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Natural Secondary Metabolites

From Nature, Through Science, to Industry

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Preface

Secondary metabolites are also known as specialized molecules; they are not involved in the essential functions of an organism. These metabolites can be found in most living organisms, such as plants, fungi, animals, and bacteria.

Their importance for human activities is vast, especially given their chemical diversity and specific roles they play in the organisms that produce them, making them unique. Research around the world has been doing a remarkable, but never never-ending effort to identify, categorize, understand, and obtain secondary metabolites from several organisms. Beyond scientific curiosity, this endeavor has been driven by potential applications of these molecules in several industries, especially the food, cosmetic, textile, and pharmaceutical industries.

Much of our work is devoted to secondary metabolites; thus, we felt the need to compile most of the knowledge on secondary metabolites into a book which includes the Plant and Fungi Kingdoms, but also known applications of these marvel metabolites. To provide context, we added a historical perspective of how the study of these molecules shaped human communities, and a final chapter focusing on the future of secondary metabolism.

Our intention is for this book to be used by students and scientists, studying the several groups of plant and fungal metabolites, but also by the industries that obtain, transform, and sell these molecules or products containing them. With this book, we expect to highlight their versatility and applicability, but also to boost their exploitation and expose the state of the art of their continuous study.

Bragança, Portugal

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Part I
History of Secondary Metabolites

Chapter 1
History of Secondary Metabolites: From Ancient Myths to Modern Scientific Validation

Mariana C. Pedrosa, Laíres Lima, José Ignacio Aloso-Esteban, Custódio Lobo Roriz, Lillian Barros, Isabel C. F. R. Ferreira, and Márcio Carocho

Abstract The study of primary plant metabolism has been the main focus of scientists for several years, seconding research related to the secondary metabolism. Nowadays, with the better understanding of the processes and products involved in primary metabolism, the focus has switched to the expansion of knowledge involving secondary metabolism. Initially, plants were the focus of secondary metabolite studies due to their ease of acquisition and availability, reasons for being a term coined by plant physiologists. Only in the twentieth century has the secondary metabolites from microorganisms began to be recognized and investigated due to the value that these metabolites could bring to science. Not by chance, it was in the twentieth century that the era of antibiotics began. In this chapter, a voyage through the centuries is proposed, focused on secondary metabolism and metabolites, highlighting how they shifted from disregarded molecules to the powerhouses of bioactivity for modern science.

Secondary metabolites (SM) are part of secondary metabolism and are molecules that do not take part in essential functions, such as growth and reproduction, but rather perform protective functions. Other names used by scientists throughout history to refer to SM are "shunt metabolite," "idiolite," and "specialized

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metabolites." These molecules can be synthesized by bacteria, fungi, plants, and animals (Bennett 1983; Kumar et al. 2021; Mohan et al. 2021).

In history, the origin of secondary metabolites is not evident, possibly these molecules arose from the need to produce chemical structures for defense, communication, and competition against other microorganisms (Brakhage 2013). Complementarily, there is the hypothesis that SM were reused residues of the primary metabolism by enzymatic actions, which provided them with bioactive characteristics, generating beneficial evolutionary properties over millions of years, for communication and protection (Vicente et al. 2022). The view that SM are literally non-essential to the organism is a misconception, as the evolution over millions of years of these components was only possible because they provided evolutionary advantages to the organism relative to its ecosystem (Cavalier-Smith 1992).

The presence of secondary metabolites in plants was one of the factors responsible for the settlement and permanence of aquatic plants on dry land. Around 480–430 million years ago when this expansion began, plants had to adapt to the new conditions of the surrounding environment and the new stresses involved, therefore new metabolic pathways were established, leading to the production of lignins, flavonoids, and plant hormones. For example, flavonoids may have played a role in protecting the plant from the direct action of ultraviolet rays outside the aquatic environment (Delaux et al. 2012; Ferrer et al. 2008). Also, from an evolutionary aspect, the great variety of secondary metabolites produced by plants is the result of immune and defense response of these substances against parasites and the surrounding environment (Bednarek et al. 2011).

Components from plants were already used for the purpose of treating diseases, even without knowing their chemical composition and why they were effective. For instance, around 1630, Jesuit missionaries returning to Europe used cinchona bark (*Cinchona ledgeriana* Moens. ex Trimen) to treat malaria (a typical disease of tropical areas). Only in the following centuries was the chemical family responsible for such activity isolated and identified, the benzylisoquinoline alkaloids (Hanson 2003). In order to discover the origin of the mustard seed's pungent taste, as early as the seventeenth century, the compounds glucosinolates and isothiocyanates had already been identified as responsible for the particular properties of mustard (Fahey et al. 2001). The vast majority of structural identification of plant secondary metabolites was done in the late nineteenth and throughout the twentieth century (Hanson 2003). Also, in the plant kingdom, morphine from opium poppy (*Papaver somniferum* L.) was one of the first secondary metabolites isolated in history, specifically in 1804 by Friedrich Wilhelm Sertürner. This event was the starting point for the search of more substances, mainly toxic compounds that were designated alkaloids (Eich 2008).

In relation to fungal secondary metabolites, the scientist Raistrick was considered the "father," by helping other research groups from England characterize several fungal metabolites during the twentieth century. Another scientist of the time, Wienberg, defined fungal SM as "chemically bizarre" due to the great distinction and variety of compounds. As with plants, one of the possible explanations for the diversity of fungal SM, both chemically and biologically, was the evolutionary adaptation of these organisms, in order to survive and expand in the environment

(Bennett 1983), for instance, the production of antibiotic compounds by fungi is an example of defense against microorganisms (Nuankeaw et al. 2020). Fungi SM have been part of human society since antiquity, either positively, for example, in food production (namely fermentation), or negatively, such as by producing mycotoxins and food contaminants. In 1039, the fungus *Claviceps purpurea*, more specifically due to the production of ergot alkaloids in rye, caused an incident of ergotism, which became notorious as Saint Anthony's fire (Mosunova et al. 2021). *Penicillin* is the main and greatest example of fungal SM that transformed the course of medicine, being the primary and most notable antibiotic produced. However, there are several other examples of medicines and drugs, such as caspofungin, taxol, cyclosporine, and lovastatin that can act as antifungal, anticancer, immunosuppressive and in lowering cholesterol, respectively (Enespa and Chandra 2019).

The bacterial kingdom can also offer a variety of secondary metabolites beneficial to society and human health. It is estimated that there are around 1 trillion species of bacteria in the global ecosystem and more than 13,000 bioactive compounds derived from them. Bacterial SM are a potent source of metabolites for medicine, pharmacology, and drug discovery, with a large proportion of secondary metabolites coming from actinomycetes. For instance, Dr. William Colley was the first to try to use natural products from bacteria (*Streptococcus pyogenes* and *Serratia marcescens*) to treat cancer (Kim et al. 2021; Mohan et al. 2021), which helped these molecules gain momentum in the commonly known "antibiotic era" (Bader et al. 2020).

In the animal kingdom, carminic acid from the scales of the cochineal insect, *Dactylopius coccus* Costa, is the oldest known animal SM used by humans. The purpose of this natural substance was to be applied as a natural dye, as well as being a commodity at the time. Like many substances, it was only years later that the performance and benefits of cochineal were revealed (Gronquist and Schroeder 2010).

The importance of secondary metabolites is evidenced in their application, such as drugs, with more than 70% of antibacterial and anticancer compounds being derived from natural products (Khan et al. 2014). Secondary metabolites are no longer just laboratory substances, but are increasingly being introduced into society, namely in medicine, pharmaceuticals, food, etc., mainly due to the advance of the understanding of the processes involved and the evolution of the methods of analysis, identification, extraction, and characterization (Demain and Fang 2000; Kumar et al. 2021).

The definition of secondary metabolites is not, nor has it been immutable over time, it has undergone changes and even today due to advances in science, which allows a deepening of knowledge, especially with regard to their functions. At the beginning, it was stipulated that the products resulting from secondary metabolism were not essential for the growth and development of the organism, it was believed that they played a role in the sense of conferring some evolutionary advantage and adaptation to the environment surrounding the natural source where we can find them (Bourgaud et al. 2001; Craney et al. 2013). These compounds are characterized by an enormous chemical diversity, where each organism has a list of secondary

metabolites very characteristic of the organism in which they are found (Makkar et al. 2007; Tiwari and Rana 2015). Several authors tried to present a definition that credibly reflected the functions of these compounds, such as Bennett and Bentley (1989), who gave the following definition:

"General metabolites (hence general metabolism): A metabolic intermediate or product, found in most living systems, essential to growth and life, and biosynthesized by a limited number of biochemical pathways. Secondary metabolites: a metabolic intermediate or product, found as a differentiation product in restricted taxonomic groups, not essential to growth and life of the producing organism, and biosynthesized from one or more general metabolites by a wider variety of pathways than is available in general metabolism." This proposed definition, despite already referring to the importance of secondary metabolism in the organisms, does not fully contemplate the importance of secondary metabolites with regard to their role. Thus, Verpoorte and Alfermann (2000) then presented his version of a definition for secondary metabolites:

"Secondary metabolites are compounds with a restricted occurrence in taxonomic groups, which are not necessary for a cell (organism) to live but play a role in the interaction of the cell (organism) with its environment, ensuring the survival of the organism in its environment, ecosystem".

In this definition, it is possible to observe the importance of secondary metabolites for the organism to survive as a species in a given ecosystem (Verpoorte and Alfermann 2000).

But how does interest in secondary metabolites arise? Since the beginnings of humanity, it is possible to observe the use of plants and natural products which were called medicines at the time (several forms of potions, oils, anointments, among others) (Cragg and Newman 2001). These folk medicines had in their composition natural products, based on bioactive properties from secondary metabolites. The use of medicinal plants was based on knowledge acquired through centuries of trial and error (Ikan 2008; Kulka 2013). From different ancient civilizations, there are records about the use of plants as a form of treatment for medicinal purposes. The oldest record was found on clay tablets in cuneiform from Mesopotamia, and date back to 2600 B.C., documenting the use of cypress (*Cupressus sempervirens*) and myrrh (*Commiphora* species) oils for the treatment of cough, colds, and inflammation. There is also an ancient pharmaceutical record from Egypt known as the Ebers Papyrus, dating back to about 1550 B.C. which documents over 700 plant-based drugs, describing for instance, the useful preparations of opium and castor oil, and the use of "rotten bread" to treat infections (Dias et al. 2012). China, considered the leading civilization in the use of natural products, with its extensive knowledge documented over thousands of years, has in its Chinese Materia Medica over 52 prescriptions. The oldest compilation of Chinese herbs, with 385 entries, is the Pen Ts'ao Ma catalogue, from the Ming Dynasty (1573–1620) which presents 1898 herbal drugs and 8160 prescriptions. Among the most famous herbs present in this compilation, *Panax ginseng* appears, used to treat various diseases, and *Ginkgo biloba* described for the treatment of mental alertness and to improve memory (Ikan 2008). Ancient Greek and Roman cultures are pointed out as the basis of knowledge

of herbal therapies in the western world. Greek philosopher and natural scientist, Theophrastus (100 B.C.) wrote a compendium about medicinal herbs. This information later served as the basis for Pliny the Elder, a Roman of the first century, Galen, a Greek working at Rome in the second century, and Dioscorides, another Greek physician from the first century (Bernardini et al. 2018). Between the V and X century, Anglo, Irish, Franco, and German monks were responsible for the preservation of the western knowledge, while Greco-Roman knowledge was preserved by the Arabs, increasing this knowledge with their own resources, together with knowledge from India and China, due to the herbs from these countries, unknown to the Greco-Roman world. Interestingly, the first pharmacy appears by the hands of the Arabs, in the VIII century, more precisely Avicenna, a Persian pharmacist, physician, philosopher, and poet was responsible for the *Canon Medicinae*, a very important medical encyclopedia (Dias et al. 2012). In the middle of the X century, in southern Italy, the Salerno school appeared, under the aegis of the emperor Frederick II, due to the Greek-Roman knowledge about medicinal plants intersecting with the Arabic culture and knowledge. Johannes Gutenberg invented the letterpress, and partially due to it the XV and XVI centuries saw the resurrection of the Greco-Roman plant-based knowledge, as it was possible to disseminate knowledge through paper in Europe, allowing for the publication of books such as The Mainz Herbal (Herbarius Moguntinus 1484) and German Herbal (1484), edited by Gutenberg's partner Peter Schoffer (Bernardini et al. 2018).

During the colonization of the "New World" in the XVII century, some members of the Society of Jesus, a Catholic order, took the bark of the cinchona to America, for the treatment of malaria. Curiously, it was from this natural source that Pelletier and Caventou, in 1820, isolated quinone, an important bioactive compound against malaria. It was also from the American Indians that the powerful hallucinogenic effects of mescaline, derived from desert cacti, became known (Ikan 2008).

Ancient cultures relied on the beneficial effects of plants and natural products, but in some cases used them to satisfy their indulgencies. Coffee and tea, for instance, which present moderate stimulatory properties have been used for centuries, becoming a long cultural tradition and in our days are some of the most important agricultural products worldwide (Dickschat 2011).

Some milestones were reached in the decades following the definition of secondary metabolites by Albrecht Kossel, mainly supported by the progress of science, which provoked even more interest for "natural products," and provided new information which helped convert empirical practice into scientific knowledge. Important milestones, such as the discovery and isolation of morphine from opium in 1804 by Friedrich Serturner, or the discovery of penicillin by Alexander Fleming in 1928a, or even the discovery and identification of two new molecules, namely compactin and mevinolin in the mid-1970s, capable to inhibit cholesterol biosynthesis, are just a few examples (Bernardini et al. 2018).

During the early XIX century, the applications of natural-based products were often subjected to self-tests or discovered by serendipity. The idea that plants could get their medicinal potential through their constituents seemed to be clear, however, the exact components that handled this potential and the mechanisms of synthesis

were, thus far, unknown. The first studies involving secondary metabolites in this century were described by Friedrich Sertürner around 1805. Although he was an apprentice in medicine and pharmacy, his rational thinking led him to believe that there must be an active ingredient in opium that, if isolated, could be administered in a safe, effective, and reliable dose for analgesic treatment. Sertürner carried out experiments with opium poppy seeds (*Papaver somniferum*), dissolving them in acid and neutralizing them with ammonia (Schmitz 1985). His first experiments produced nothing more than inert compounds. However, he later isolated morphine crystals and tested them on stray animals. These tests showed that the discovered substance had sedative properties but could accompany dangerous consequences. Sertürner delivered the first short note about his discovery, *Säure in Opium* [Acid in Opium], to the editor of the *Journal der Pharmacie* [Journal of Pharmacy] in 1805, but his work was considered unscientific and not acceptable by the medical community (Schmitz 1985; Sertürner 1817). Recognition finally came with the publication *Ueber das Morphium, eine neue salzfähige Grundlage, und die Mekonsäure, als Hauptbestandteile des Opiums* [About Morphine, a new Saline Base, and Meconic Acid, as the main constituents of Opium], published in *Annalen der Physik* [Annals of Physics] in Leipzig, 1817 (Sertürner 1817). Sertürner's discovery was a pioneering demonstration that a bioactive chemical compound could be obtained from a natural product, and it strongly influenced and directed the organic, analytical, and pharmaceutical chemistry areas (Hartmann 2007).

In the 1820s, Justus von Liebig focused on the analysis of organic substances taking part in plant physiology, including the family of nitrogenous organic bases known as alkaloids. Years later, in 1843, the scientist confirmed Sertürner's hypothesis that there were several active substances present in plants that seemed to be responsible for the medicinal effects (Liebig 1843). He published these studies in the book "Handbuch der Organischen Chemie: mit Rücksicht auf Pharmacie" [Handbook of Organic Chemistry: with Consideration for Pharmacy], being an extremely important work for the studies conducted during the XIX century on plant metabolites. Unfortunately, the knowledge of the time did not allow precise identification of how these compounds were synthesized or how they acted in the plant metabolism (Liebig 1843). These substances were simply "natural products" to most organic chemists, providing endless challenges of structure determination and synthesis mechanisms. For biologists, the various alkaloids, resins, and other unique botanicals were even more puzzling (Bennett and Bentley 1989).

In the second half of the XIX century, there were already studies indicating that some substances present in plant cells did not seem to play any discernible role in the organism's active maintenance or growth metabolism; on the other hand, they seemed to have a protective role (Kerner 1879). Despite this, there was a great interest in obtaining these constituents because of their possible medicinal and physiological properties. In his book, Physiological Plant Anatomy, published in 1884, Gottlieb Haberlandt addressed some classes of compounds (certain organic acids and saline derivatives) as metabolic waste products that, in some cases, would have evolutionarily acquired secondary ecological functions, especially those of

protective nature (Haberlandt 1914) from the Darwinian paradigm perspective, which required that organism's attributes convey adaptive value.

Similarly, Julius Sachs, one of the founders of modern plant physiology, insinuated that compounds such as alkaloids, waxes, tannins, and some pectin derivatives were waste or storage plant products, considering that they had no known physiological meaning at the time (Sachs 1873). On the other hand, in 1879, Anton Kerner von Marilaun recognized that biotic interactions, as well as historical, climatic, and geological conditions directly interfered with species characteristics (Kerner von Marilaun 1895), becoming one of the first to document convincing arguments against Jean Baptist Lamarck's hypothesis about the "heritability of characters acquired," which was strongly supported at the time. Kerner stated that plant evolution is driven by environmental selection and is directly based on heritable variation and subsequent selection of competitive genotypes, both crucial aspects of species survival and perpetuation. The German scientist supported this idea by presenting in-depth studies about a vast range of mechanical and chemical defenses (alkaloids, resins, essential oils, among others) of plants in his manuscripts (Kerner 1879).

Leo Errera, a researcher in the fields of botany, chemistry, and physiology, carried out pioneering work in histochemistry and floral biology, in which he discovered the occurrence of glycogen in fungi and plants (amylopectin) through sophisticated histochemical methods. Later, Errera applied these techniques to detect different classes of compounds in plants, namely organic acids, tannins, essential oils, glucosides, and alkaloids. His work demonstrated that plants that have these classes are avoided by animals and insects, these substances being means of protection involving interactions with other plants and against environmental adversities and predators, and that chemical defenses would therefore be as important as defenses mechanics (Errera and Durand 1886).

The published information on certain substances with secondary functions which could have medicinal properties in humans stimulated several studies and promoted the first bases of the pharmaceutical industry and drug research during the XIX century. The first publication on the synthesis of a secondary product, indigo, by Adolf von Baeyer in 1883, represented a milestone in synthetic organic chemistry, as until then, most dyes and colorants were directly obtained from extracts of natural products (Huisgen 1986).

Oswald Schmiedeberg and Richard Koppe published in their book *Das Muscarin: Das Giftige Alkaloid Des Fliegenpilzes (Agaricus muscarius L.)* [The Muscarin: The Poisonous Alkaloid of the Fly Agaric (*Agaricus muscarius L.*)] in 1869, a complete study on the structure and physicochemical properties of muscarine and its physiological and toxicological effects on the human body. The authors describe the mushroom extraction protocol to obtain alkaloid rich extracts, especially muscarine (Schmiedeberg and Koppe 1869). Although the authors obtained an extract rich in muscarine, this important compound was not isolated until the beginning of the next century, by 1922, by Harold King (King 1922). Many of the works published on metabolites from fungi during the XIX century addressed the

obtainment of extracts that seemed to contain some type of active principle, with the isolation and synthesis of active substances being achieved some years later.

Friedrich A. Flückiger and Daniel Hanbury, for example, conducted a study in 1874 on the botanical and historical origin, morphology, and chemical composition of species of lichen (*Lichen Islandicus*), fungus (*Secale cornutum*), and algae *(Chondrus crispus and Fucus amylaceus*) (Flückiger and Hanbury 1874). In addition to Flückiger and Hanbury, Planchon and Collin reported studies of simple drugs of natural origin in 1895, covering several species of lichens, fungi, and algae (Planchon and Collin 1895). In both books, the authors gathered data from several sources, transcribing in detail protocols for obtaining extracts, mostly by decoction or infusion using water or organic solvents. They reported that the species addressed were studied by physicians and pharmacists at the time, often prescribed for the treatment of illnesses because of their medicinal capabilities. It was observed that these species had chemical defenses against environmental disturbances and predators, which could be used as an extract with therapeutic activity (Flückiger and Hanbury 1874; Planchon and Collin 1895). In the work of Planchon and Collin, *Drogues Simples d'Origine Végétale* [Simple Drugs of Plant Origin], the authors commented on the first attempts to identify the chemical compounds of *Claviceps purpurea* and the isolation of its alkaloid ergotinine by Tanret, dated 1875. The extract of *C. purpurea* was widely used at the time as an anti-hemorrhagic and labor inducing agent in pregnant women (Planchon and Collin 1895; Tanret 1875). Tanret's discovery was a crucial step toward the isolation of other active principles in fungi and their subsequent drug applications.

In the late XIX century, Ernst Stahl studied the feeding behavior of slugs, snails, and other herbivores concerning various mushroom species with chemical protection against predators. The author observed that all omnivorous species had very little or no effect on fresh fungus, while the leached pieces were devoured in a short time. Stahl then followed the same line of thinking as Errera and suggested that the various means of chemical protection of fungi are modeled and optimized according to their interactions with the environment and with possible predators (Stahl 1888). Thus, it was possible to argue that not only plants had chemical defenses, but also other groups, such as fungi which could be exploited in this sense.

In studies throughout the XIX century, there was already the idea that secondary metabolites were not participants in the growth and reproduction metabolism, but instead derive mainly from defense mechanisms. However, as the identification of compounds naturally synthesized was approached considering the limited knowledge of this period, the bioactive properties mentioned were not directly attributed to a particular chemical component or group in most cases. Remarkably, knowledge about plant metabolites is greater in this period in comparison to fungi, for example, given the complexity of the studies published throughout the century.

After the term secondary metabolite was coined by Kossel (1891) at the end of the XIX century, the XX century, especially the second half, was the time of the greatest scientific breakthroughs in this field of study (Bourgaud et al. 2001). In the 1920s, the Czapek's series was published, dedicated to plant biochemistry and these

compounds were extensively discussed in one of the volumes (Bourgaud et al. 2001; Czapek 1925).

In the specific case of phenolic compounds, Boudet (2007) pointed at the beginning of the twenty-first century that research during the previous 50 years could be described in five stages. The first was the characterization of the common precursors and enzymatic steps of the phenylpropanoid pathway. The second outlined the evidence of changes in gene expression associated with the plasticity of metabolism using molecular biology techniques. The third rendered a more accurate depiction of the diversity of genes and enzymes involved in phenolic compound metabolism by the use of functional genomics. The fourth stage was related to the optimization of phenolic compound profiles via genetic engineering. Finally, the last was the boom of epidemiological studies concerning the bioactive potential of food phenolic compounds in human health. These stages could be applied to other secondary metabolites, especially to those with a long research history.

At the beginning of the XX century, the analysis of secondary metabolites continued to be difficult because of their low abundance and overall technical limitations (Bourgaud et al. 2001). The two main challenges were to obtain enough pure compounds and to use the appropriate tools to elucidate de structure (Macías et al. 2007). The first chemical structures were mainly identified by the scrupulous chemical disassembly and reassembly of the compounds under study. That was particularly usual with terpenes because their chemical structure arose by assembly from a common five carbon precursor (Eisenreich and Bacher 2007). In the early years of the XX century, Tswett introduced column adsorption chromatography for the separation of plant pigments, and it became a widely used separation method for natural products in the 1930s (Marston 2007). In the following decade, Martin and Synge developed paper chromatography and there was a very intense activity regarding separation and identification of secondary metabolites (Haslam 2007). That technique showed slow migration rates and was overtaken by thin layer chromatography. Gas chromatography (GC) was introduced in the 1950s, and it became available a decade after. GC provided excellent results, but it was restricted to volatile secondary metabolites. High-performance liquid chromatography represented the most remarkable advance in the separation of secondary metabolites because it was an appropriate technique for water-soluble, thermally labile, and non-volatile compounds (Marston 2007). The improvement of chromatographic techniques in the middle of the century enabled the development of phytochemistry as a scientific discipline (Bourgaud et al. 2001), but the development of spectroscopic techniques during those times and the widespread application during the 1970s contributed to a huge expansion of secondary metabolite chemistry (Macías et al. 2007). Some technologies that contributed to the analysis of secondary metabolites were X-ray crystallography, mass spectrometry, and nuclear magnetic resonance (NMR) spectroscopy (Eisenreich and Bacher 2007).

As with chemical techniques, sophistication of biological assays increased in the second half of the XX century. Studies on biological activities of novel secondary metabolites became a priority in pharmacognosy research during the 1980s and

different in vitro and in vivo methods were developed to be used for initial screening and bioassay-guided fractionation of active compounds. One of the objectives was the discovery of new drugs, which targeted the study on acetylcholine antagonism, antihistamine, anticancer, antihypertensive, antispasmodic, and immunostimulant activities, as well as activities against disease-causing organisms such as bacteria, fungi, protozoa, and viruses (Phillipson 2007). However, more recently, some in vitro assays, especially those of antioxidant capacity, are occasionally used as a claim, but arguably show limited relevance in terms of in vivo effects. Thus, bioavailability, metabolism, and tissue distribution of secondary metabolites in humans are important issues which need to be plainly determined in association with their biological activities (Espín et al. 2007). Computer advances allowed complicated statistical studies that showed the correlation between pharmacological information and chemical composition, as well as to relate the chemical structure of secondary metabolites to their biological activity through quantitative structure–activity relationships (QSAR) (Reynolds 2007).

First classifications of secondary metabolites took place after a considerable number of chemical structures were collected (Robins et al. 2007; Robinson 1955). The three main groups were alkaloids, terpenes, and phenolic compounds (Bourgaud et al. 2001). Phenolic compounds were one of the most studied groups, in addition to chromatographic techniques, the technical advances in mass spectrometry enabled the identification of a large number of compounds with complex structures and low stability and abundance (Boudet 2007). One of the first classifications of phenolic compounds was done by Bate-Smith and the three major groups were leucoanthocyanins, flavonol glycosides, and hydroxycinnamic acid derivatives. Leucoanthocyanins were described by Robert and Robinson in the 1930s and they were considered colorless compounds with a chemical structure similar to anthocyanins. Bate-Smith related leucoanthocyanins with condensed tannins, corresponding to proanthocyanidins. Bate-Smith also studied hydrolyzable tannins, particularly vic-trihydroxyaryl grouping, which were esters of gallic acid, gallotannins, and ellagitannins. Their synthesis, as well as leucoanthocyanin synthesis, was considered primitive. Those studies were carried out from the 1950s onward, and together with those of Swain were the most outstanding in tannin analysis. Until that time, Fisher was the main contributor to tannin knowledge because of his studies at the beginning of the XX century, and in the 1920s it was assumed that there were two kinds of tannins, non-hydrolyzable and hydrolyzable (Haslam 2007). In the 1950s, several European scientists were interested in separation and structural elucidation of phenolic compounds, as well as in their taxonomic distribution, and founded the Plant Phenolics Group, which was the forerunner to the Phytochemical Society of Europe (Robins et al. 2007).

Studies on the biosynthetic origin of secondary metabolites were initially based on predictions made according to common structural features and evidence was produced experimentally by analyzing enzyme activities (Robins et al. 2007). In the 1950s, studies using radioactive tracers were the most common due to being simple techniques. After the development of sensitive and specific detection methods, such as advanced mass spectrometry and high-resolution nuclear magnetic resonance

spectrometry, stable isotopes started to be used because they were safer and easier to work with (Eisenreich and Bacher 2007). These refined tracer techniques enabled an outline of the main biosynthetic pathways of secondary metabolites, and it was the basis for the characterization of the enzymes that played a part in the biosynthetic routes in the 1970s (Hartmann 2007). Still, in the 1980s, first genes related to secondary metabolite biosynthesis were identified and isolated (Kutchan et al. 1988; Robins et al. 2007). In the XXI century, the obtention of genome data became relatively easier and, as a result, the genetic information simplified the analysis at the level of the metabolic network. However, despite the abundance of sequence information from different species, the role of many genes is still uncertain (Fernie 2007). Only the biosynthetic pathways of major secondary metabolites have been clarified and the enzymes involved in the different steps have been characterized, but it is still unknown for most of the structurally known natural compounds, as well as those with unknown structure (Petersen 2007).

In the first half of the XX century, secondary metabolites were reckoned as metabolic waste or detoxification products (Hartmann 2007). That was the case for "faecal theory," which was formulated by Pictet in the first decade of the XX century and suggested that alkaloids were degradation products of complex nitrogen-containing compounds (Zenk and Juenger 2007). That view changed several years after, and they started to be considered dynamic components of plant metabolism rather than inert end products (Hartmann 2007). The phytoalexin theory was proposed by Mueller and Börger in the 1940s considering that the de novo synthesis of secondary metabolites was an equivalent to immunological response against microorganisms. Another function of secondary metabolites proposed at that time was allelopathy, which was a term coined by Molisch (Macías et al. 2007). Besides, entomologists realizing that there was an interaction between secondary metabolites and herbivores (Hartmann 2007). So, it was expected that the investment of resources in secondary metabolite production had a reasonable reward in terms of advantages of survival, so the aim of several studies from them on was to clear up the role that secondary metabolites played in living organisms (Macías et al. 2007). Besides, that presented an opportunity for protecting agricultural production against pests, diseases, and weeds through the induction of the biosynthetic pathways of defense secondary metabolites (Pickett et al. 2007).

Once the ecological role of secondary metabolites was generally accepted, the pharmacological potential was taken into consideration (Bourgaud et al. 2001). However, sustainability was an important point of discussion because the chemical synthesis of secondary metabolites was not considered economically viable, so the compounds had to be obtained directly from natural sources, endangering the survival of high demand species (Macías et al. 2007). Therefore, at the end of 1960s, plant cell culture techniques were applied to the study and production of secondary metabolites. In vitro studies carried out with undifferentiated cell cultures were extensively used with this purpose, although they were not the only ones. Organ cultures, especially hairy roots, were tested to meet the requirements for the production of secondary metabolites in bioreactors, but little commercial success was achieved after 30 years of efforts (Bourgaud et al. 2001).

The metabolic engineering techniques opened a new possibility because they allowed the modification of the genetics of secondary metabolite biosynthesis. Besides the own biosynthetic capacities of each species, these techniques were the base of "molecular pharming," which consisted of the use of plants as host organisms to produce secondary metabolites naturally synthesized by other species. However, due to environmental concerns about transgenic plants, it did not gain public acceptance (Bourgaud et al. 2001).

Plant classification based on chemical composition was not a new practice and experienced a boom in the 1960s and 1970s (Reynolds 2007; Waterman 2007). At that time, the rise of secondary metabolites brought with it discussions of their occurrence in different species, so chemosystematics took account of these compounds for botanical classification (Larsson 2007). Later, sequence comparisons allowed the study the evolutionary origin of secondary metabolites and to establish phylogenetic relationships. The next step was to recognize the causal connection between gene diversity and plasticity of secondary metabolism, since secondary metabolites play a crucial ecological role in the changing interactions of the organisms that produce them and the environment in which they live (Hartmann 2007). However, the progress has been slow and the most outstanding observations into phylogeny offered by secondary metabolites took place at the end of 1960s. Nevertheless, modern taxonomic descriptions now include information about secondary metabolites, corroborating their relevance in plant identification (Waterman 2007).

Although technical advances in the field of study of secondary metabolites have been remarkable, there continue to be challenges to overcome, such as an improvement of sensitivity, selectivity, and speed in analysis, which would simplify the solution of structural problems that some compounds present, as well as the establishment of general rules of structure/function relationships. Given the importance of secondary metabolites as raw materials, especially for pharma-food industries, future research is likely to be driven by these sectors (Robins et al. 2007). Secondary metabolite studies have experienced an enormous evolution over time and the point of view changed from a descriptive approach to comprehensive knowledge. Complexity has been increasing, which includes different scientific disciplines, such as chemistry, pharmacy, pharmacognosy, botany, plant and fungal physiology, ecology, and biochemistry (Macías et al. 2007). A future goal, and a realistic one according to Hartmann (2007), is to trace back the genes which are responsible of the biosynthesis of secondary metabolites to their origin in order to identify the ancestors and, at best, to reconstruct the evolution of whole plant secondary pathways. A very similar future is predicted for fungi secondary metabolites, with faster and cheaper methods for genetic analysis of soil and marine species and a better understanding of biosynthetic pathways (Keller et al. 2011). Therefore, all advances in analytical instrumentation, the refinement of separation techniques, and metabolomic knowledge culminated in the chemistry of natural products study, also focusing on secondary metabolism, expressing great interest, which continues to this day in secondary metabolites, their functions and bioactivities, as potential candidates to explore in the development of natural medicinal compounds. Thus, the improvement of the analytical techniques will surely be closely related and

correlated with the discovery of new secondary metabolites and new functions for them, both in plants and fungi.

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Part II
Plant Secondary Metabolites

Chapter 2
Biochemistry of Secondary Metabolism in Plants

Nemanja Rajčević, Danka Bukvički, Dejan Stojković, and Marina Soković

Abstract Through secondary or specialised metabolites plants adapt to their environment. A myriad of different compounds plays a significant role in the plant–environment interaction. These compounds help plants in attracting pollinators or deterring herbivores, in their protection from pathogen microorganisms, or in protection from various abiotic stresses. The vast diversity of these compounds is based not only on the ability to rapidly evolve biochemical pathways but also on differential expression of already existing genes in different tissues, and the substrate-binding plasticity of the existing enzymes. The general diversity of biochemical pathways and their evolution is discussed in this chapter.

2.1 Introduction

Plants cannot adapt to their environment through behaviour like animals. Instead, they rely heavily on the biosynthesis of a myriad of different compounds. These compounds are called secondary or specialised metabolites (Tissier et al. 2014). Secondary metabolites are oftentimes described in relation to the primary or general metabolites. The primary metabolites are necessary to carry out the fundamental biochemical processes responsible for maintaining life, they are few in numbers and their biosynthesis is more or less conserved across taxa. So, by definition, secondary metabolites would be all the other metabolites synthesised by the organism. While the term "secondary metabolites" is more common, it can be misleading, implying they are of secondary significance to the organism, and sometimes even a "waste product" of the primary metabolism (Bennett and Wallsgrove 1994). So, recently

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another term was proposed for this group of metabolites, that would be more accurate—specialised metabolites (Davies 2013; Tissier et al. 2014). Verpoorte defined these metabolites as compounds with a restricted occurrence in taxonomic groups, that are significant for the interaction of the cell (organism) with its environment, thus ensuring the survival of the organism in its ecosystem (Verpoorte 2000).

The secondary metabolites have an enormous chemical diversity, owing to their specialised roles in every organism. While every organism has its own set of specialised metabolites, they can be shared between different taxa, even those not closely related (Verpoorte 2000; Pichersky and Gang 2000; Arimura and Maffei 2016). While over 50,000 different compounds have been elucidated thus far, no one knows the exact number of these compounds produced by plants (Verpoorte 2000; Pichersky and Lewinsohn 2011; Tissier et al. 2014). However, considering the number of plant taxa, their massive genomes (especially in seed plants), as well as the ability of some enzymes to produce multiple products, some authors argue that there might be over 200,000 different specialised metabolites (Pichersky and Gang 2000; Kampranis et al. 2007; Tissier et al. 2014; Rajčević et al. 2015).

Based on their chemical nature and, sometimes, their role in the plant organism, specialised metabolites can be divided into several groups. The three most numerous groups are terpenoids, alkaloids, and phenolics, encompassing over 60,000 known structures. Of course, this classification is not complete since there are many others. Some, like very-long-chain aliphatic lipids that are an important part of (epi)cuticular waxes present in all plant taxa, while others are present only in certain families, e.g. glucosinolates (Brassicaceae, Capparaceae, and some other families), organic disulfides in the Amaryllidaceae family, unusual fatty acids in certain gymnosperms and angiosperms, cyanogenic glucosides in particular members of the Rosaceae family, bis-bibenzyls present in Marchantiophyta (liverworts), some Primulaceae and Orchidaceae (Buchanan et al. 2009; Tissier et al. 2014; Arimura and Maffei 2016; Osei-Safo et al. 2017; Asakawa et al. 2021; Bukvicki et al. 2021).

Specialised plant metabolites play an important role in the interaction between plants and their environment. Long-chain aliphatic hydrocarbons (alkanes, aldehydes, ketones, and other long-chain-fatty acid derivatives) are part of (epi)cuticular waxes that protect plants from uncontrollable water loss, but also likely function in light reflectance, provide a surface for a variety of biotic and abiotic relationships, repel fungal pathogens, and provide water-repellency (Barthlott and Neinhuis 1997; Rajčević et al. 2014a, b, 2020a; Dodoš et al. 2015, 2017, 2019a; Jocković et al. 2020). Terpenoids play an important role in attracting pollinators, deterring herbivores, or protecting plants from infections caused by microorganisms (Buchanan et al. 2009; Ludwiczuk et al. 2017; Rajčević et al. 2018, 2019, 2020b; Novaković et al. 2019; Dodoš et al. 2019b, 2021). Some terpenoids (e.g. iridoids, sesquiterpene lactones), alkaloids, cyanogenic glucosides, tannins, and coumarins are bitter tasting and oftentimes toxic to mammals and other animals (Bennett and Wallsgrove 1994; Buchanan et al. 2009; Tissier et al. 2014; Gupta and Birdi 2017). Flavonoids also play a significant role in plant–pollinator interaction (i.e. colouring compounds), but they also are fundamental in photoprotection and antimicrobial protection (Grotewold 2006; Buchanan et al. 2009; Tissier et al. 2014; Gupta and Birdi

Table 2.1 Origin of specialised metabolites from glycolysis, TCA (Krebs) cycle, and Shikimate

Glycolysis	TCA cycle	Shikimate
Glycosides	Alkaloids	Phenylpropanoids
Terpenoids	Purines	Coumarins
Saponins	NPAAs	Alkaloids
Cucurbitacins		Quinones
Terpenoid alkaloids		Flavonoids
Waxes		Stilbenes
Fatty acids		Anthocyanins
Polyketides		Monoterpenes
Phenolics		
Flavonoids		

2017). Many of the specialised metabolites from plants have been shown to possess antibacterial and antifungal properties, as well as a potential to influence mammal metabolism (Tyagi et al. 2013; Bukvicki et al. 2013, 2015, 2016; Stojković et al. 2020a, b, 2021, 2022; Velickovic et al. 2020; Ivković et al. 2021a; Ivanov et al. 2022).

Despite the huge diversity of specialised metabolites, the number of corresponding basic biosynthetic pathways is restricted and distinct. Precursors for their synthesis usually come from basic metabolic pathways, such as glycolysis, the TCA (Krebs) cycle, or the shikimate pathway (Wink 2010). The diversity of specialised metabolites comes from (1) evolution of new genes and new biosynthetic pathways, (2) flexibility of biosynthetic pathways, (3) differential expression of genes in tissues (Muzac et al. 2000; Verpoorte 2000; Pichersky and Gang 2000; Yazaki 2006; Smetanska 2008; Pichersky and Lewinsohn 2011; Kabera et al. 2014; Tissier et al. 2014; Takahashi and Asakawa 2017). Table 2.1 shows the origin of most secondary metabolites based on their pathways.

2.2 Shared Pathways: An Example from Waxes

Some of the groups of specialised metabolites are almost universally present, e.g. phenolics, flavonoids, and aliphatic hydrocarbons, which indicates an ancient origin of these pathways. Probably the only universally present group of specialised metabolites is the ones forming the cuticle. The cuticle represents one of the most important adaptations to life on land. Terrestrial plants must face dehydration, fluctuations in temperature, and high solar radiation (Dodd and Poveda 2003; Kunst and Samuels 2003; Knight et al. 2004; Xue et al. 2017). The cuticle consists of two major parts—cuticular matrix (insoluble polymer of ω-hydroxy fatty acids and cutan) and cuticular wax—the outermost hydrophobic layer (Dodd and Afzal-Rafii 2000; Neinhuis et al. 2001; Rajčević et al. 2014a, b). While the cuticle itself is present in algae, fungi, and all plants, its composition varies between taxa and presents the evolutionary response to the specific environment. Hence, it is no surprise that cuticular wax biosynthesis is under strong genetic control. Biosynthesis

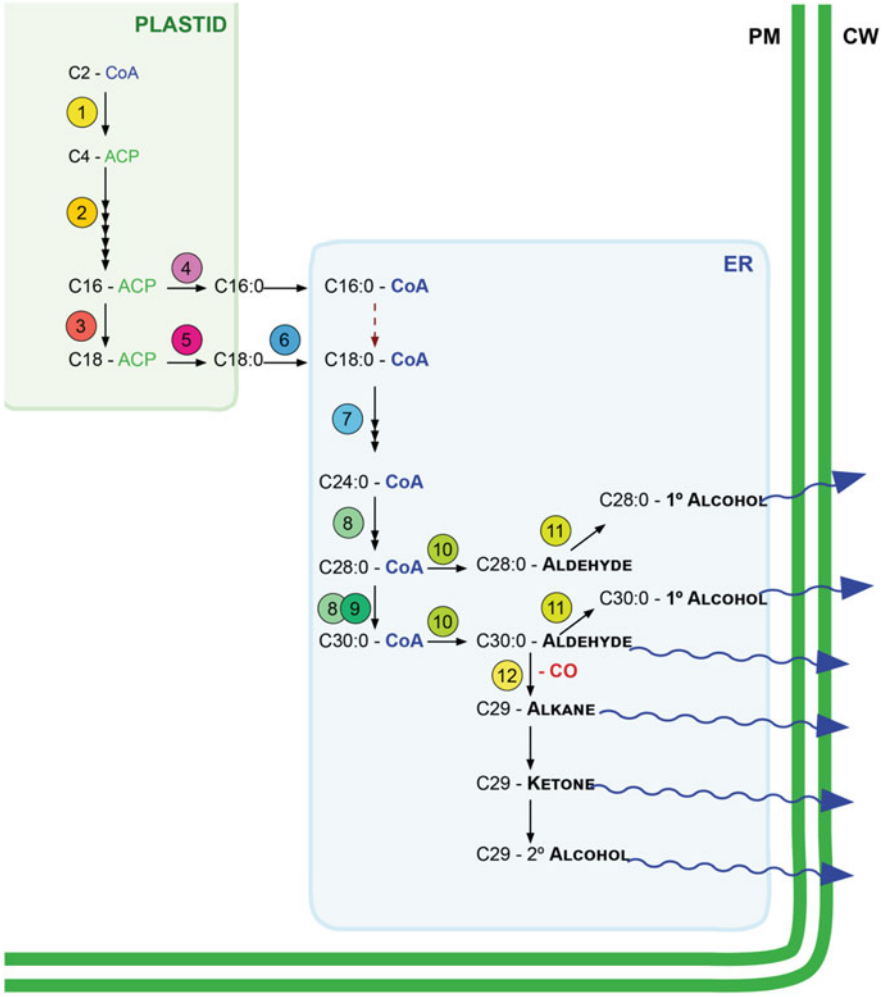

Fig. 2.1 Depiction of wax formation: ER—endoplasmatic reticulum, PM—plasma-membrane, CW—cell wall, 1–3 FAS complex enzymes differ in a single enzyme 1—KASIII, 2—KAS I, 3-KASII. 4—palmitoyl-ACP-thioesterase, 5—stearoyl-ACP-thioesterase, 6—acyl-CoA synthetase; 7- KCS, 8—CER6; 9—CER2, 10—CER10, 11—CER4, 12—CER13

of the (epi)cuticular waxes can be separated into three phases: (1) de novo biosynthesis of fatty acids in plastids, (2) elongation of these acids into very-long-chain fatty acids (VLCFA) in the endoplasmatic reticulum (ER), and (3) transformation of VLCFA into different components through several parallel biosynthetic pathways (Fig. 2.1).

Precursors for the biosynthesis of VCLFAs are fatty acids that are synthesised de novo in plastids (usually leucoplasts). Indication of the synthesis is the condensation of malonyl-ACP (Acetyl Carrier Protein) with Coenzyme A (Ohlrogge et al. 1993).

After this step, next following reactions repeat cyclically, adding two carbon atoms at the end of each cycle. The reactions in the cycle are reduction of β-ketoacyl-ACP complex, dehydration of β-hydroxy acyl-ACP, and the reduction of *trans*-δ-2-enoyl-ACP. The product of the first cycle remains esterified to ACP, and C_2 moiety is added to that part of the molecule in the following cycles. The donor of the two carbon atoms is malonyl-ACP, and NAD(P)H donors two hydrogen atoms for two reduction reactions. For a C_{18} fatty acid to be synthesised, several condensing enzymes are necessary. They can be differentiated through strict association to a specific acyl-chain length. KASIII (C_2 to C_4), KASI (C_4 to C_{16}), and KASII (C_{16} to C_{18}) (Shimakata and Stumpf 1982; Clough et al. 1992). Two reductases and a dehydrogenase do not have a specific acyl-chain length preference. These C_{16} and C_{18} fatty acids are precursors for various lipid components (Post-Beittenmiller 1996). While this first step of wax formation is shared with other lipid biosynthetic processes, the latter phases are not. In most plant tissues, products of de novo synthesis of fatty acids—$C_{16:0}$ and $C_{18:1}$ are precursors for the synthesis of structural biomolecules (glycolipids of the cell membrane, cutin or suberin of the cell wall) (Samuels et al. 2008; Kunst and Samuels 2009).

The second phase is the elongation of these C_{16} and C_{18} fatty acids to VLCFA. Once the fatty acids are esterified to CoA, they are translocated to the ER, where additional acyl-chain elongation and modification of VLCFAs to diverse aliphatic wax components occur (Samuels et al. 2008). These reactions are catalysed by the extra-plastid membrane-bound multienzyme complexes also known as fatty acid elongases (von Wettstein-Knowles 1982). Similarly, to the previous step, a series of four enzymatic reactions result in the extension of the molecule by two carbon atoms: condensation, followed by a β-keto-reduction, dehydration, and, finally, enoyl reduction (Fehling and Mukherjee 1991). Multiple cycles of elongation are needed for the chain to grow to a certain length. VLCFAs are usually between 20 and 60 carbon atoms long (Post-Beittenmiller 1996). NAD(P)H is a donor of hydrogen for the reduction reactions. However, there are significant differences in this enzymatic reaction: (1) the carbon atom donor is malonyl-CoA (not malonyl-ACP), (2) elongases are extra-plastid and bound to the cell membrane, and (3) elongases need ATP (Post-Beittenmiller 1996).

The third step is the biosynthesis of wax lipid components. Precursors for this step are VLCFAs. There are two main biosynthetic pathways in most plants: the acyl-reduction pathway which leads to the production of primary alcohols and esters (components with a predominantly even number of carbon atoms), and the decarboxylation pathway which leads to the formation of aldehydes, alkanes, secondary alcohols, and ketones (components with a predominantly odd number of carbon atoms). In some taxa, both pathways can have some or almost all of the reactions catalysed by the same enzyme and differ only in the last step. For example, *Arabidopsis cer4* mutants are considered defective in aldehyde reduction (Jenks et al. 1995). In these mutants, the amount of aldehyde is high and primary alcohols are low, whereas there are no changes in the decarboxylation pathway products, while *Cer13* mutants have higher production of C_{30} alcohol and C_{31} alkane (Rashotte et al. 2001). The decarboxylation pathway begins with the production of

aldehyde from VLCFAs with the help of acetyl-CoA reductase, a membrane-bound protein. This enzyme is quite different in size and sequence from those involved in the production of primary alcohols (Vioque and Kolattukudy 1997). Synthesised aldehydes are henceforth decarboxylase to the odd-number-chain alkane with the release of carbon monoxide. As can be seen from this example, there is a different set of enzymes for each step of the biosynthetic pathway, so different expressions of the respective genes can be achieved in different plant tissues or at different stages of development. Alternatively, different ratios of the products can be easily achieved the same way, thus significantly influencing the properties of the cuticular wax in response to different environmental influences.

2.3 Patchy Distribution

While only a few specialised metabolites are universally present, most have a patchy distribution. In other words, an ability to synthesise a specific metabolite evolved multiple times across plant lineages (Pichersky and Gang 2000; Pichersky and Lewinsohn 2011). Convergent evolution, defined as the independent occurrence of similar or identical features in more or less distant lineages, has been shown multiple times in specialised metabolism (Bennett and Wallsgrove 1994; Verpoorte 2000; Pichersky and Lewinsohn 2011; Kabera et al. 2014; Tissier et al. 2014; Buchanan et al. 2015; Xue et al. 2017; Bukvicki et al. 2021).

One example for patchy distribution is a small group of specialised metabolites called macrocyclic bis-bibenzyls (MBBs). MBBs are the most characteristic compounds in the Marchantiophyta (liverworts) (Asakawa et al. 2021; Novaković et al. 2021). There are only a few literature reports about the presence of MBBs in vascular plants—Primulaceae (*Primula veris* subsp. *macrocalyx*) (Kosenkova et al. 2009; Bukvicki et al. 2021), Orchidaceae (*Dendrobium*), and Dichapetalaceae (*Dichapetalum heudelotii*) (Osei-Safo et al. 2017; Li et al. 2020). Bis-bibenzyls possess interesting biological activities (Asakawa and Matsuda 1982; Bukvicki et al. 2012, 2013, 2016; Anchang et al. 2016; Novakovic et al. 2019; Asakawa et al. 2021; Ivković et al. 2021a, b, c; Novaković et al. 2021).

Professor Yoshinori Asakawa discovered in 1979 a new class of specialised metabolites, the cyclic bis-bibenzyl, marchantin A (interesting structural scaffolds that belong to the class of stilbenoids), a major specialised metabolite in the cosmopolitan distributed liverwort *Marchantia polymorpha*. From that time, more than 150 related compounds, riccardins, perrottetins, and plagiochins have been isolated from liverworts and their structures established and characterised till now (Asakawa 1982; Asakawa et al. 1987, 2021; Novaković et al. 2021). Marchantin A and Riccardin C were originally isolated from liverworts by Asakawa et al. (Asakawa et al. 1979; Asakawa and Matsuda 1982).

There are three structure types of naturally occurring bis-bibenzyls based on how connected benzene rings are (C-C type, C-O type, and O-O type) (Fig. 2.2). Those

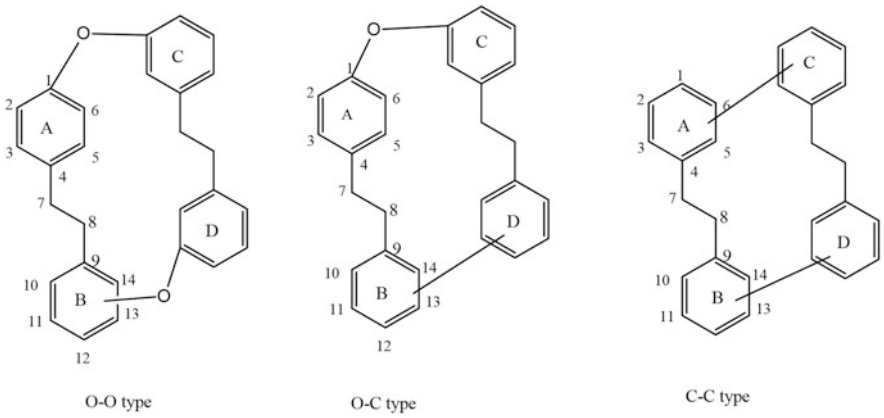

Fig. 2.2 General structures of three types of macrocyclic bis-bibenzyls (MBBs)

types are made of macrocyclic rings connected via two biphenyl ether C–O bonds or one biphenyl ether C–O or one biaryl C–C bond, and two biphenyl bonds.

Bis-bibenzyl skeleton comprises four aromatic rings (A-D), biosynthesised from dimerization of lunularic acid through dihydro-*p*-coumaric acid and prelunularin (labile aromatic compound) (Fig. 2.3). Riccardin C, cyclic bis-bibenzyl has been isolated from the liverwort *Reboulia hemisphaerica* for the first time in 1982, by Asakawa and Matsuda and authors proposed that cyclic bis-bibenzyls may be synthesised from two coupled molecules of lunularin.

Asakawa and Matsuda (1982) proposed that bis-bibenzyls might be synthesised from bibenzyl that chemically correspond to dihydro-stilbenes. Friederich et al. (1999a) confirmed this hypothesis while investigating the biosynthesis of the marchantin A, using thallus tissue of the liverwort *Marchantia polymorpha*. Feeding experiments with radioactively ^{13}C-labelled precursors indicate that A and C rings from the molecule of Marchantin A originated from the benzene ring of L-phenylalanine through *trans*-cinnamic acid and *p*-coumaric acid. In this paper, it is confirmed that dihydro-*p*-coumaric acid is an intermediate in marchantin A biosynthesis through ^{13}C NMR spectroscopy. Bibenzyl monomers were confirmed to be the building blocks of the marchantin molecules through the phenylpropane/polymalonate pathway using dihydro-*p*-coumaric acid and acetate/malonate (Fig. 2.3). Coupled bibenzyls form the bis-bibenzyl structure (Friederich et al. 1999a). Lunularin and lunularic acid are biosynthesised by the shikimate-malonate pathway. The co-occurrence of and the presence of a lunularic acid decarboxylase in *Lunularia cruciata* and *Conocephalum conicum* indicated that a decarboxylation step was involved in forming naturally occurring C-14 stilbenes and their dihydro products (Asakawa 1982). The crucial step in the biosynthesis of marchantins is the intermolecular phenolic coupling reaction of the bibenzyl monomers, lunularic acid, to form the macrocyclic marchantin (Friederich et al. 1999a, b), two specific cytochrome P-450 enzymes catalyse the formation of marchantins A and C. The first enzyme ("marchantin C synthase") catalyses the coupling of two molecules of

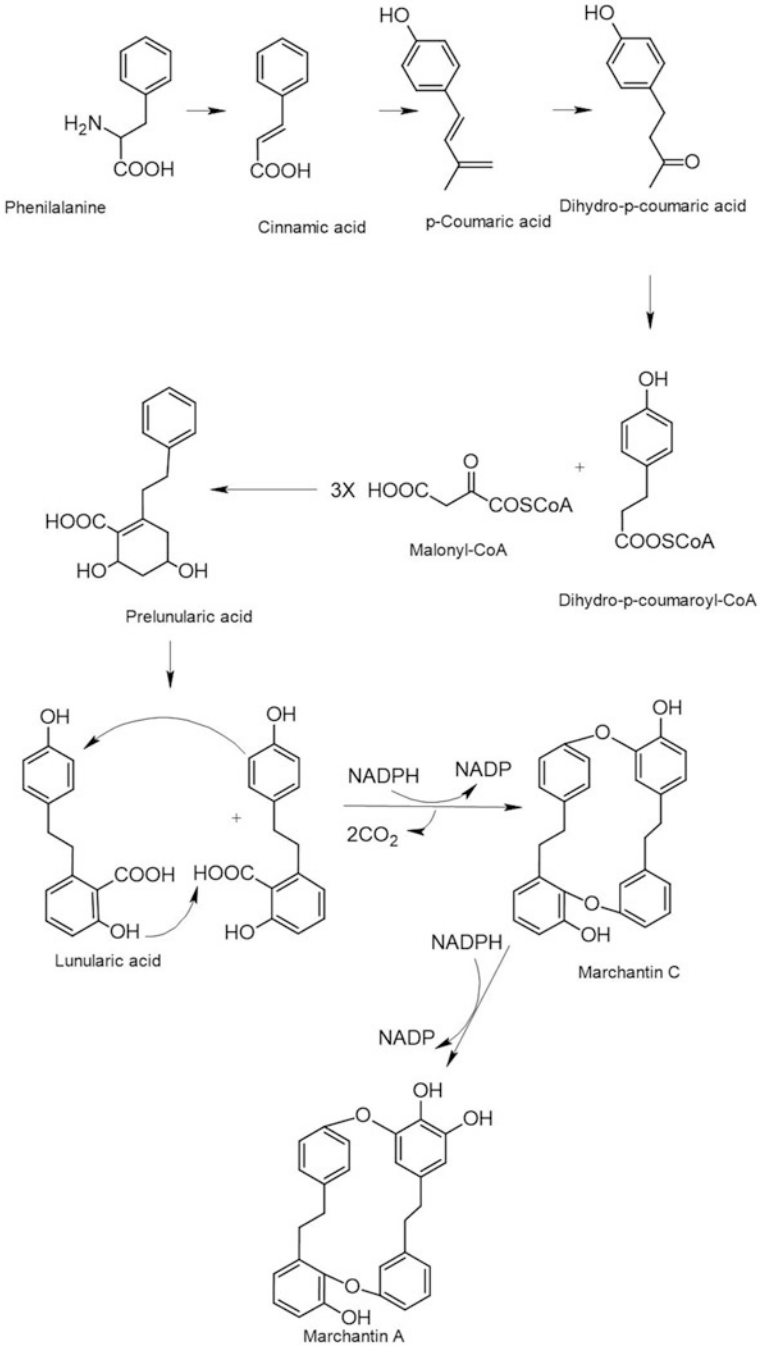

Fig. 2.3 Proposed pathway for the biosynthesis of bis-bibenzyls Marchantin A and Marchantin C

lunularic acid to form marchantin C and CO_2 (Fig. 2.3), while the second P450 enzyme ("marchantin C hydroxylase") catalyses the hydroxylation of marchantin C to marchantin A. As can be seen from this example, enzymes that catalyse these reactions are, in essence, similar to other enzymes of secondary metabolism, but have different products due to their different substrate-binding preference. It would be interesting to identify and sequence these genes and compare them across taxa that also produce bis-bibenzyls.

2.4 The Flexibility of Biosynthetic Pathways

Most of the pathways to produce specialised metabolites have not yet been elucidated. Based on what it is known, there are possibly hundreds of thousands of different enzymes involved in specialised metabolism in plants (Pichersky and Gang 2000). So, how did such a diverse group of metabolites evolve? Gene duplication usually plays a role in the emergence of new protein functions since the presence of two or more copies of the same gene permits mutations in the copies without a negative influence on the organism's fitness (Tissier et al. 2014). Duplication can occur in a number of ways, e.g. through polyploidization, hybridisation, local duplications of chromosome regions with the participation of transposons. Interestingly, recent research has shown that unlike all other genes in eukaryotes that are typically dispersed throughout the genome, those responsible for specialised metabolite pathways are clustered together. Though molecular mechanisms of these clusterings in a single locus are still not known, the formation of these operon-like loci certainly is an interesting one. Molecular evidence for proving such an event requires the analysis of the presence of the gene in related taxa and the comparison of its sequences. We can find this, for example, in a gene from *Clarkia breweri* (Onagraceae) that encodes the enzyme responsible for methylation of eugenol to methyl-eugenol. This enzyme evolved from the enzyme which methylates caffeic acid to ferulic acid (Wang and Pichersky 1999).

Duplication of genes is not the only process of obtaining new qualities. Mutations can occur in one of the alleles of the given locus. This mutation would not have a negative impact on the plant's fitness. Through evolution, the changed function of the gene can be fine-tuned, and heterozygous individuals for this locus could, in fact, have better fitness in comparison to other individuals. Alternatively, the obtained new characteristic could in fact enable expansion of the distribution range.

The diversity of chemical structures can also be achieved through the flexibility of the metabolic pathways. The flexibility of the specialised metabolites biochemical pathways offers a possibility to produce various sets of compounds in different tissues with specific biological and ecological functions. In other words, many specialised metabolites arrive through a network of metabolites connected with a limited number of enzyme activities. These enzymes show a somewhat loose specificity towards substrates, allowing them to modify in the same way a myriad of related metabolites. The amount of each of the metabolites will be determined by

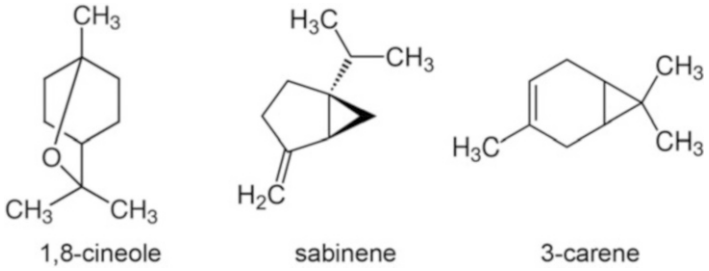

Fig. 2.4 Three monoterpenes with similar terpene synthase sequences

the expression level of different enzymes in the given plant tissue (Pichersky and Gang 2000; Tissier et al. 2014).

This redundancy can be seen especially in the biosynthesis of different terpenoids. For example, precursors for terpenoid biosynthesis can be created through two independent biosynthetic pathways which are also separated in different cell compartments: cytosol mevalonate (MVA) pathway and plastid Methyl erythritol 4-phosphate (MEP) pathway. Both pathways end in isoprenyl diphosphate (IDP), which can be exchanged between cytosol and plastids (Adam and Zapp 1998; Bick and Lange 2003; Bartram et al. 2006). Further down the line, alternative splicing combined with the low-substrate-specificity can lead to the production of different compounds even though there is only a single gene involved. This is especially true for terpenoid synthases that can have different N-terminus, which is responsible for the transport of the enzyme into different cell compartments. Sesquiterpene synthases differ from monoterpene synthases in the N-terminus of the polypeptide. By removing this sequence, the enzyme is not able to enter the plastid, so it is active in the cytosol. Even though the primary substrate of these enzymes is GPP, monoterpene synthases can also bind FPP, and thus the different products will be synthesised (Tissier et al. 2014). Phylogenetic analyses of terpene synthases (TPS) sequences discovered that many sesquiterpene synthases originated by changes in the N-terminus region of the protein from genes encoding monoterpene and diterpene synthases (Martin 2004).

The vast diversity of specialised metabolites can be achieved relatively easily. Enzymes prenyl diphosphatase and terpene synthases share similar characteristics and contain conserved sequences, especially in the substrate-binding regions (Buchanan et al. 2009). In fact, small changes in the gene sequence oftentimes lead to completely different products in specialised metabolism. For example, just based on the sequence of the terpene synthase genes, it is very difficult, if not completely impossible, to conclude the product of the enzyme. A change of a single amino acid (Arg instead of Ile) will change the product from 1,8-cineole to sabinene, while a further change of three amino acids would shift production to 3-carene (Fig. 2.4). As little as 9% difference in the coding region is detected between many terpene synthases, e.g. limonene/α-pinene synthase (Katoh et al. 2004; Kampranis et al. 2007; Roach et al. 2014). A small change in sequence can lead to different

preferences to substrate. In a similar way, concluding the role of an enzyme based on its sequence or similarity of a sequence can be misleading. For example, the methyltransferase gene from *Arabidopsis* is similar to other Catechol-O-methyltransferases (COMTs), but instead of caffeic acid, it methylates quercetin—a flavonol (Muzac et al. 2000).

While terpene synthases share a basic metabolic pathway, however, in sesquiterpene synthases, many different intermediary carbocations exist (e.g. farnesyl, nerolidyl, germacredienyl, humulyl, bisabolyl, cyclopentenyl, eudesmyl, etc.). This, in turn, leads to multiple pathways for the synthesis of the same product (Degenhardt et al. 2009). Additionally, in conifers, up to 52 different products were detected from a single enzyme (Phillips and Croteau 1999; Buchanan et al. 2009). Additional diversity in the terpene composition can be achieved through the modifications of the synthesised terpenes, e.g. oxidations, reductions, isomerization, and conjugations. Some of these modifications are achieved through the activity of cytochrome P450 oxidase (Buchanan et al. 2009). The number of cytochrome oxidases is huge, and only a fragment of these have been analysed thus far.

2.5 Conclusion

Specialised metabolites play an important role in the life of plants. They enable quick adaptation to ever-changing environments. Unlike primary metabolism, the relationship between different metabolic pathways, the ability of plants to utilise alternative metabolic routes in biosynthesis, and rapidly evolving enzymes make the field of study of these metabolites both challenging and interesting. Different evolution of specialised metabolites across plant lineages prevents "quick solution" research and simple extrapolation of function or biosynthetic pathway from one plant lineage to another. Small changes in the amino acid sequences in these enzymes can completely shift the production, not just from within one subclass of compounds (e.g. monoterpene) to another, but between subclasses (e.g. from monoterpene to a sesquiterpene), or even from one class of compounds to another (e.g. from phenolic acid to flavonoid). Thus, extensive phytochemical surveys on composition, variability, biosynthetic pathway, and biological role of specialised metabolites in different taxa are paramount in understanding plants' complex metabolism, especially taking into consideration the rapidly changing climate and the many benefits humans can get from the plants' specialised metabolites.

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Chapter 3
Phenolic Acids and Derivatives: Description, Sources, Properties, and Applications

Celestino Santos-Buelga, Ana M. González-Paramás, and Susana González-Manzano

Abstract Phenolic acids are one of the major classes of phenolic compounds occurring as secondary metabolites in plants; among them, hydroxybenzoic and hydroxycinnamic acids are outstanding. These compounds are widespread in the human diet through plant-based foods, where they contribute to sensory and functional properties. Their consumption has also been associated with positive effects in human health, owing to their recognized biological activities (e.g., antioxidant, anti-inflammatory, antimicrobial, antidiabetic, or anticarcinogenic). Technological and functional properties of phenolic acids have made them interesting compounds for food, pharmaceutical, and cosmetic companies, so that suitable preparation processes are required to meet their increasing research and industrial demand. To fulfill these needs, an efficient production of pure compounds is required that cannot be fully satisfied by their isolation from natural sources or chemical synthesis, which suffer limitations such as low yield, time-consuming, or non-environmentally friendly processes. Biotechnological approaches including the construction of heterologous plant or microbial systems can be an alternative for enhancing phenolic acid production or addressing pathways toward the biosynthesis of particular target compounds. This chapter offers an overview on phenolic acids occurrence in food and natural sources, biosynthesis and advances in their biotechnological production.

3.1 Introduction

Phenolic acids are one of the major classes of phenolic compounds (also commonly referred to as polyphenols) that occur as secondary metabolites in plants, where they play essential physiological and metabolic functions for plant life. They have structural roles, regulate processes related with plant development and growth, cell

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division, seed germination, and plant pigmentation, and are also involved in the mechanisms of natural plant resistance against biotic and abiotic stresses (Lattanzio et al. 2008, 2012). They are also widespread in plant-based foods, in which they contribute to sensory (flavor, astringency, bitterness, or color) and functional properties, and may also serve as food preservatives helping to prevent processes of enzymatic browning. As for other phenolic compounds, the phenolic acids possess a series of biological activities that have been related with positive effects in human health. Among others, different compounds of this family have been reported to act as antioxidant, anti-inflammatory, antimicrobial, antidiabetic, or anticarcinogenic agents (Kiokias and Oreopoulou 2021; Kumar and Goel 2019; Ruwizhi and Aderibigbe 2020; Sova and Saso 2020).

Antioxidant activity is one of their more recognized properties. Indeed, phenolic acids behave as efficient antioxidants helping to counteract cell oxidative damage, owing to their ability to scavenge oxidizing species, e.g., reactive oxygen and nitrogen species, through mechanisms that involve the transfer of an H atom or of a single electron to the radical. They may also act as indirect antioxidants by modulating cell oxidative stress via the induction of endogenous protective pathways, such as the antioxidant response element (ARE) regulatory system (Santos-Buelga et al. 2019). Anti-inflammatory activity of phenolic acids and derivatives has been related to their ability to suppress the activity of the nuclear transcription factor NF-κB, and downregulation of pro-inflammatory cytokines, such as IL-6, IL-8, or TNF-α, as well as to their antioxidant properties (Ali et al. 2020; Nagasaka et al. 2007). Similarly, anticancer effects of different phenolic acids have been associated to their antioxidant and anti-inflammatory activity and the regulation of diverse transcription factors and signaling pathways related to cell proliferation, apoptosis, or angiogenesis, such as NF-κB, cyclin-dependent kinases (CDKs), vascular endothelial growth factor (VEGF), phosphoinositide 3-kinase (PI3K), or protein kinase B (Akt), as mostly demonstrated in in vitro assays and animal studies (De et al. 2011; Abotaleb et al. 2020).

Antimicrobial properties of phenolic acids seem to depend on their lipophilicity. As weak organic acids, they partially exist in undissociated lipophilic form in biological systems, which allow them to diffuse across cell membranes, disturbing the membrane structure and acidifying the cytoplasm causing protein denaturation (Campos et al. 2009). The lipophilicity of hydroxybenzoic acids basically correlates with the type and number of functional groups, decreasing with the number of hydroxyl groups and increasing with that of methoxy substituents; in hydroxycinnamic acids it is much lesser dependent on the substitutions of the aromatic ring, but strongly dependent on the double bond of the side chain (Sanchez-Maldonado et al. 2011). The pH is also determining in the antimicrobial activity of phenolic acids, which increases at lower pH values, making cell membrane more permeable and affecting the sodium-potassium ATPase pump implicated in ATP synthesis (Cueva et al. 2010).

The antidiabetic potential of phenolic acids has been associated to their ability to interfere with glucose metabolism. Several mechanisms have been described, including inhibition of enzymes involved in carbohydrate digestion and intestinal glucose

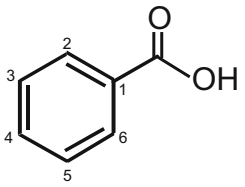
uptake, stimulation of insulin secretion from the pancreatic β-cells, modulation of glucose transporters GLUT2 and GLUT4, or activation of insulin receptor and glucose uptake in insulin-sensitive tissues (Vinayagam et al. 2016). Detailed information on biological activities and health benefits of phenolic acids can be found in recent reviews (Kiokias and Oreopoulou 2021; Kumar and Goel 2019; Ruwizhi and Aderibigbe 2020; Sova and Saso 2020).

Technological and functional properties of phenolic acids make them highly interesting for food, pharmaceutical, and cosmetic industries, so that suitable preparation processes are required to meet their increasing industrial demands. Just as an example, phenolic acids like ferulic, gallic, vanillic, or salicylic acids are widely utilized as ingredients or raw materials for the preparation of preservatives, flavors and fragrances, skin protective agents, or edible films (Valanciene et al. 2020).

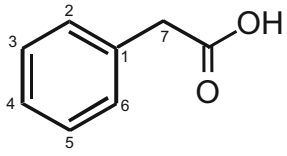
This chapter offers an overview on phenolic acids occurrence in food and natural sources, biosynthesis and advances in their biotechnological production.

3.2 Description

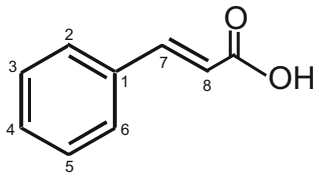
Structure of phenolic acids consists of a phenolic ring with an attached carbon chain bearing a carboxylic group (Fig. 3.1). The most important compounds of this family are hydroxybenzoic (C6-C1) and hydroxycinnamic acids (C6-C3); hydroxyphenylacetic acids (C6-C2) also occur, usually as minor components, in



C3	C4	C5	
H	OH	H	*p*-hydroxybenzoic acid
OH	OH	H	protocatechuic acid
OCH_3	OH	H	vanillic acid
OH	OH	OH	gallic acid



C3	C4	C5	
H	OH	H	4-hydroxyphenylacetic acid
OCH_3	OH	H	homovanillic acid



C3	C4	C5	
H	OH	H	*p*-coumaric acid
OH	OH	H	caffeic acid
OCH_3	OH	H	ferulic acid
OCH_3	OH	OCH_3	sinapic acid

Fig. 3.1 Structures of main phenolic acids

plants and food, being 4-hydroxyphenylacetic acid and 3-methoxy-4-hydroxyphenylacetic (homovanillic) acid and the most representative compounds of this group.

In their natural sources, phenolic acids can be found in free form, but most frequently linked to other compounds such as carbohydrates, organic acids, flavonoids, terpenes, or sterols, as well as bounded to plant matrix components (cellulose, proteins, lignin) through ester, ether, or acetal bonds. Free forms may also appear in processed foods resulting from chemical or enzymatic cleavage during fruit and vegetable processing.

3.2.1 Hydroxybenzoic Acids

Figure 3.1 shows the structures of the most common hydroxybenzoic acids (HBAs), differing in the number of hydroxyl and methoxyl substituents on their aromatic ring. In general, they are present in plants as conjugates, usually glucosides. Four HBAs, namely *p*-hydroxybenzoic, vanillic, syringic, and protocatechuic acid, are constituents of lignin; as an approximation, it can be said that plants which do not contain lignin do not contain these acids either (Tomás-Barberán and Clifford 2000). Other common hydroxybenzoates are salicylic and gallic acids.

Gallic acid esterified to glucose and less usually to other polyols, such as hamamelose, quinic acid, shikimic acid, or other cyclitols, giving rise to the so-called hydrolyzable tannins (HTs). Successive substitution of the hydroxyl groups of glucose by gallic acid leads to a series of simpler galloyl esters from monogalloylglucose (1-*O*-galloyl-β-d-glucose; β-glucogallin) to 1,2,3,4,6-penta-*O*-galloyl-β-d-glucose (Fig. 3.2). This latter is considered the biosynthetic precursor of both gallotannins (GTs) and ellagitannins (ETs). Complex GTs are formed by substitution of pentagalloylglucose with additional galloyl residues affording

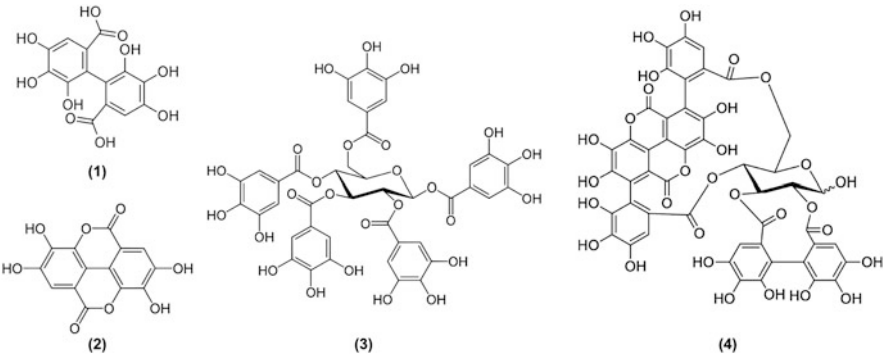


Fig. 3.2 Structures of hexahydroxydiphenic (1) and ellagic acids (2), and two representative hydrolyzable tannins: a gallotannin,1,2,3,4,6-penta-*O*-galloyl-β-d-glucose (3) and an ellagitannin, punicalagin (4)

polygalloylglucoses. Tergallic and gallagic acids (the gallic acid trimer and tetramer, respectively) may also be found in some HTs. Tannic acid is a generic name given to variable mixtures of GTs that are present in several plant sources. GTs are less distributed in plants than ETs, which also display a much more pronounced structural variability; their strong tendency to form dimeric and oligomeric derivatives contributes significantly to the vast number of described structures (Gross 2008). Gallic acid also makes part of the structure of condensed tannins, in this case esterified to flavan-3-ols (i.e., catechins and proanthocyanidins). ETs derive from the oxidative formation of secondary C-C bonds between adjacent galloyl residues to produce hexahydroxydiphenoyl residues (Gross 2008). Acid or alkaline hydrolysis of ETs releases hexahydroxydiphenic acid that spontaneously rearranges into the dilactone ellagic acid (Fig. 3.2), which explains their denomination.

3.2.2 Hydroxycinnamic Acids

Hydroxycinnamic acids (HCAs) are more widespread in nature than hydroxybenzoic acids. The main HCAs are *p*-coumaric, caffeic, ferulic, and sinapic acids (Fig. 3.1). These compounds predominantly occur in plants and food as *trans* (*E*)-isomers, although low amounts of *cis* (*Z*)-isomers may also be found, mostly originating from isomerization by exposure to UV-irradiation or produced enzymatically. HCAs can be present in plants and food in free form, but more usually they occur in conjugated forms esterified with hydroxyl acids, such as quinic, shikimic, and tartaric acid, sugars or flavonoids. The largest group of HCAs are chlorogenic acids (CGAs), a term that includes a series of compounds resulting from the esterification of HCAs with 1L-(-)-quinic acid. Caffeoylquinic acids (CQAs) are the main group of CGAs. 5-*O*-Caffeoylquinic acid (5-CQA) is the most abundant CGA in nature and commonly referred to as chlorogenic acid. Nevertheless, in the literature, there is some confusion between 5-CQA and 3-CQA, which are often mistaken, a discrepancy that derives from either using or not the IUPAC numbering for 1L-(-)-quinic acid (Fig. 3.3). Indeed, if the spatial arrangement of the substituents for representing chlorogenic acid is not used, it is not possible to differentiate both enantiomers, as also depicted in Fig. 3.3. Using IUPAC rules, the trivial name "chlorogenic acid" should be retained for 5-CQA, while 3-CQA would correspond to the so-called neochlorogenic acid (Kremr et al. 2016). Unfortunately, as indicated by Clifford et al. (2017), many publications and online sources (including Wikipedia) are unaware of this situation and 3-CQA (non-IUPAC) is treated as the same compound as 3-CQA (IUPAC). Coffee beverage rather than fruits and vegetables is probably the main dietary source of CQAs for many people (Clifford et al. 2017).

HCAs can also occur in polymerized forms, as present in the polyaromatic domain of suberin and other cell wall matrix components like cutin or lignin (Strack 1997), as well as conjugated with amino acids and amines giving rise to phenolamides (Fig. 3.4). These latter can be classified into different subgroups according to the type of amine moiety, namely anthranilic acids (avenanthramides,

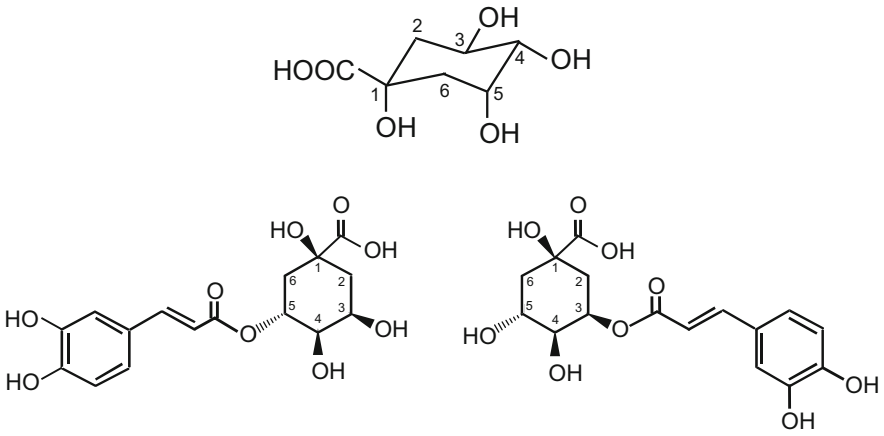


Fig. 3.3 Numbering of 1L-(-)-quinic acid according to IUPAC 1976 and structures of 5-O-caffeoylquinic acid (5-CQA; chlorogenic acid) and 3-*O*-caffeoylquinic acid (3-CQA; neochlorogenic acid)

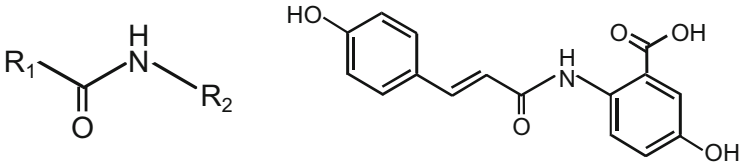


Fig. 3.4 General structure of phenolamides, where R1 corresponds to phenolic acid and R2 to amine residues, and example of an avenanthramide (avenanthramide A, consisting of *p*-coumaric acid and 4-hydroxyanthranilic acid)

AVAs), aromatic monoamines (e.g., tryptamine, tyramine, serotonin, dopamine, octopamine), aliphatic di- and polyamines (putrescine, cadaverine, spermine, spermidine), and agmatine. AVAs are unique compounds that are found exclusively in oats, while phenolamides with aromatic monoamines are more widely distributed, being reported in a variety of foods, including tomatoes, paprika, lettuce, garlic, or potatoes. HCAs conjugated to aliphatic di- and polyamines and agmatine have been mainly described in the Poaceae and Solanaceae families, among edible plants (Wang et al. 2020).

3.3 Biosynthesis

Phenolic acids are synthesized by the shikimate pathway, a route of the primary metabolism for the formation of aromatic amino acids that is present in plants, bacteria, and fungi. Aromatic amino acids, i.e., L-phenylalanine and L-tyrosine, further enter the secondary metabolism through the phenylpropanoid pathway to

yield the different polyphenol classes. Main steps of the shikimate and general phenylpropanoid pathways are depicted in Fig. 3.5.

The shikimate pathway consists of a sequence of seven enzymatic steps leading to chorismate, which is the common precursor to aromatic amino acids. Firstly, erythrose-4-phosphate (E4P), derived from the non-oxidative branch of the pentose phosphate pathway, and phosphoenolpyruvate (PEP), produced by the glycolytic pathway, are condensed into 3-deoxy-D-arabinoheptulosonate 7-phosphate (DAHP) by DAHP synthase (DAHPS). Following reactions lead successively to the formation of 3-dehydroquinate (DHQ), 3-dehydroshikimate, shikimate, shikimate 3-phosphate (S3P), and 5-enolpyruvylshikimate 3-phosphate (EPSP), to finally produce chorismate catalyzed by chorismate synthase (CS) (Marchiosi et al. 2020).

From chorismate onward the carbon flow is channeled to different branches of secondary metabolism through the phenylpropanoid pathway. Hydroxycinnamic acids (HCAs) result from the deamination of L-phenylalanine involving the enzyme phenylalanine ammonia lyase (PAL) to produce trans-cinnamic acid, as core structure for phenylpropanoids. Further hydroxylation by the action of cinnamate 4-hydroxylase (C4H) yields *p*-coumaric acid that through a series of sequential hydroxylation and methylation reactions lead to the formation of the rest of common HCAs, caffeic, ferulic, and sinapic acids (Strack 1997). C4H is a cytochrome P450-dependent monooxygenase and composes a multi-enzyme complex with the enzyme *p*-coumaroyl 3-hydroxylase (C3H), which hydroxylates the *p*-coumaric acid to caffeic acid. In some plants, tyrosine can also be directly converted into *p*-coumaric acid by tyrosine ammonia lyase (TAL), thus bypassing C4H. Nevertheless, in most plants phenolic compounds are biosynthesized from phenylalanine, so that PAL reaction represents the usual branching point between primary and secondary metabolism, being an important regulatory step in polyphenol formation (Marchiosi et al. 2020).

HCAs can be then converted to their CoA derivatives catalyzed by 4-hydroxycinnamoyl-CoA ligase (4CL), an enzyme that acts on different HCA substrates, to be subsequently reduced to *p*-hydroxycinnamoyl alcohols, such as *p*-coumaryl, sinapyl, and coniferyl alcohols, also termed monolignols, which are precursors of lignin and lignans (Umezawa 2010). Condensation of *p*-coumaroyl-CoA with three molecules of malonyl-CoA, derived from citrate produced by the tricarboxylic acid (TCA) cycle, catalyzed by chalcone synthase (CHS) leads to the formation of naringenin chalcone, branching the phenylpropanoid pathway toward the formation of flavonoids (Davies and Schwinn 2006).

Hydroxycinnamates are often accumulated in conjugated forms basically with carboxylic acids or carbohydrates, but also with proteins, lipids, amino acids, amines, terpenoids, alkaloids, or flavonoids. Furthermore, insoluble forms of hydroxycinnamates occur in plant cell walls bound to polymers such as cutins and suberins, lignin or polysaccharides (Strack 1997). The widespread chlorogenic acids are formed by esterification of quinic acid with hydroxycinnamoyl-CoA through hydroxycinnamoyl-CoA quinate:hydroxycinnamoyl transferase (HQT). Quinic acid results from the reduction of 3-dehydroquinate (DHQ) or dehydration of shikimate,

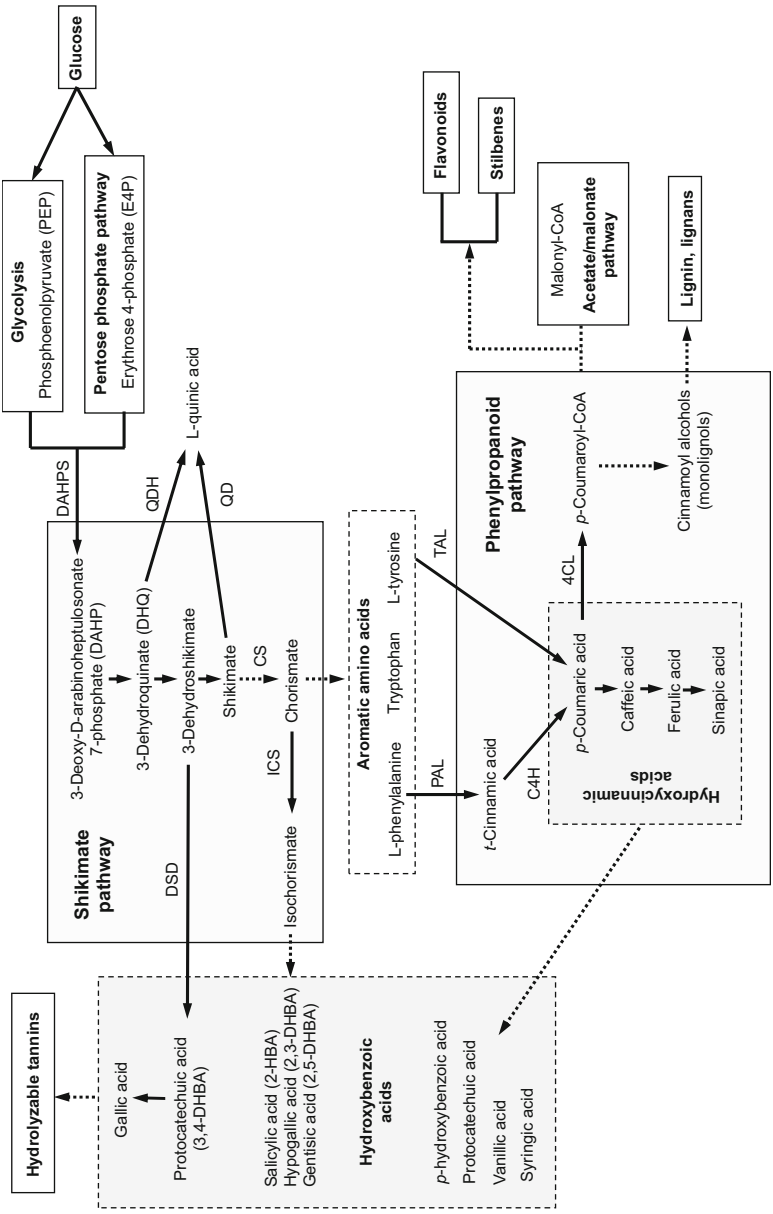


Fig. 3.5 Scheme of main steps of the shikimate and general phenylpropanoid pathways. Enzyme abbreviations: DAHPS, D-arabinoheptulosonate 7-phosphate synthase; DSD, 3-dehydroshikimate dehydratase; QDH, quinate dehydrogenase; QD, quinate dehydratase; CS, chorismate synthase; ICS, PAL, phenylalanine ammonia lyase; TAL, tyrosine ammonia lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-hydroxycinnamoyl-CoA ligase

catalyzed by quinate dehydrogenase (QDH) and quinate dehydratase (QD), respectively (Marchiosi et al. 2020).

The biosynthesis of hydroxybenzoic acids can occur from intermediates of the shikimate or phenylpropanoid pathways. In the first case, 3-dehydroquinate, 3-dehydroshikimate, shikimate, and chorismate act as precursors. Chorismate is a precursor of salicylic, 2,3-dihydroxybenzoic (hypogallic), and 2,5-dihydroxybenzoic (gentisic) acids through isochorismate formed by the action of isochorismate synthase (ICS). Gallic acid can be formed by oxidation of 3-dehydroshikimate, while dehydration of this latter would lead to 3,4-dihydroxybenzoic (protocatechuic) acid that can be further hydroxylated to gallic acid (Marchiosi et al. 2020).

Hydroxybenzoic acids can also derive from the phenylpropanoid pathway from structurally analogous hydroxycinnamic acids by cleavage of an acetate fragment from their side chain. The reaction sequence involves coenzyme A (β-oxidative), but it can also be CoA-independent (non-β-oxidative) or a combination of both pathways (Valanciene et al. 2020). The substitution patterns of hydroxybenzoates may be determined by the hydroxycinnamate precursor, although hydroxylations and methylations may also occur in an analogous way to the hydroxycinnamate pathway (Strack 1997).

Hydrolysable tannins, i.e., gallo- and ellagitannins, are synthesized from gallate and UDP-glucose through UDP-glucose:gallate *O*-glucosyltransferase yielding 1-*O*-galloyl-β-d-glucose; sequential incorporation of galloyl residues takes place catalyzed by galloyltransferases up to 1,2,3,4,6-pentagalloylglucose. Further galloylations (depside formation) leading to the gallotannins are also catalyzed by such 1-galloylglucose-dependent acyltransferases. Ellagitannins arise from secondary C-C linkages between adjacent galloyl groups and may further combine to yield oligomeric derivatives coupled through C-O-C linkages (Strack 1997).

The accumulation of phenolic acids, both hydroxybenzoic and hydroxycinnamic acids, varies according to the part of the plant (leaves, flowers, stalks, roots) and is highly dependent on factors like cultivar, seasonality, and agricultural and edafoclimatic conditions, being boosted by biotic (e.g., fungal or bacterial pathogens) or abiotic stresses (e.g., salinity, UV light, water deficit, wounding, metal ions, or treatment with elicitors like ethylene or methyl jasmonate) (Valanciene et al. 2020).

3.4 Phenolic Acids in Food

3.4.1 Occurrence

Phenolic acids are widespread in the human diet through plant-based foods, with hydroxycinnamic acids being better represented than hydroxybenzoic acids.

HCAs commonly occur in plants and foods esterified with sugars, organic acids (especially quinic or tartaric acids), or flavonoids, as well as bound to plant structural

elements (i.e., cellulose, lignin, and proteins), while they are less usually found in free form. Caffeic and ferulic acids, either free or esterified, are the most abundant HCAs in foodstuffs. Caffeic acid is more usual in fruits and vegetables, being reported that it may account for 75%–100% of the total HCA content in most fruits, while ferulic acid predominates in cereal grains, which represent its major dietary source (Manach et al. 2004).

Chlorogenic acids (CGA), including caffeoyl-, feruloyl-, dicaffeoyl-, and coumaroylquinic acids, are the most ubiquitous phenolic acids, especially the caffeoylquinic acid isomers (CQA), with 5-caffeoylquinic acid as the most common isomer in food products, except in stone fruits and brassicas where the 3-isomer seems to dominate (Clifford 2000). Green coffee beans are among the richest plant sources of CGA, with contents that may range 3.5–7.5% of bean dry matter (Jeszka-Skowron et al. 2016), and 5-CQA representing about 76–84% of total CGAs (Tajik et al. 2017). Nevertheless, the roasting process has a profound effect on CQA contents and isomer distribution. Clifford (2000) estimated a decrease of 8–10% of CGA for every 1% loss of dry matter during roasting, while Crozier et al. (2012) found that in this process 5-CQA was destroyed faster than 3-CQA and 4-CQA, although it still would be the main isomer in coffee with percentages ranging 43–58%. On the other hand, Moeenfard et al. (2014) reported 3-CQA as the major isomer in coffee brews, accounting for about 50% of the total CQAs, followed by 5-CQA and 4-CQA, with around 25–26% each, which was explained not only by their relative rates of destruction of the isomers during roasting but also by the fact that 3-CQA would be more water-soluble. These latter authors determined total CGA concentrations within a large range between 46 and 1662 mg/L in coffee brews, depending on their preparation, with greater levels in boiled and filtered coffees and lowest in instant coffees (Moeenfard et al. 2014). For their part, Crozier et al. (2012) reported an average amount of 145 mg of total CGA (range of 24–422 mg) per espresso serving (43 ml), while Clifford (1999) estimated that a 200 ml-cup of coffee may contain 70–350 mg CGA.

In addition to coffee, tea (*Camellia sinensis*) and mate (*Ilex paraguariensis*) are also relevant sources of CGA. Contents of 10–50 g/kg have been reported for green and black tea leaf, while a brew of green mate (approx. 200 ml) may contain 107–133 mg of CGA (Clifford 1999). Other rich dietary sources of CQAs are fruits and vegetables like some berries, apples, stone fruits, potatoes, or aubergines, as well as some species from the Asteraceae family, such as lettuce, endive, and artichoke. Fruits with the highest contents (blueberries, kiwis, plums, cherries, apples) may contain 0.5–2 g/kg fresh weight (Manach et al. 2004), while concentrations of 500–1200 mg/kg dry weight have been described in potato tubers and around 600 mg/kg in aubergines (Clifford 1999). Artichoke (*Cynara scolymus*) may provide some 450 mg/kg, and levels of total cinnamates of 50–120 mg/kg and 200–500 mg/kg were cited lettuce and endive, respectively (Clifford 1999).

Ferulic acid is particularly abundant in cereal grains, in which it may constitute up to 90% of total phenolic acid content (Manach et al. 2004). It mostly accumulates in the outer parts of the fruit (pericarp and aleurone layers) bound to cell wall polysaccharides. Concentrations as high as 30 g/kg have been reported in maize bran

(Clifford 1999), while wheat bran, and especially aleurone, may present more than 8 g/kg (Barron et al. 2007); contents of 0.8–2 g/kg dry weight have been described in whole wheat grain (Manach et al. 2004). Cinnamoyl-tartaric acid esters, namely caftaric, fertaric, and *p*-coutaric acids, are especially abundant in grapes, being caftaric acid the predominant one; average concentrations about 170 mg/kg have been indicated for *Vitis vinifera* grapes, although large variations exist depending on the grape variety and geographical and agricultural factors (Waterhouse 2002).

The concentrations of hydroxybenzoic acids in food are usually low, with the exception of certain berries (e.g., blackberry, black and redcurrant, raspberry, or strawberry), green and black tea, or pomegranate. Gallic and ellagic acids are usually the commonest phenolic acids, although they occur in good extent as a part of the hydrolyzable tannins. Contents of gallic acid up to 3.5 g/kg have been reported in black tea leaves, and concentrations of 20–50 mg/l of brew have been estimated for a typically prepared tea infusion (Tomás-Barberán and Clifford 2000). The contents of gallotannins in foods are not well characterized; mango fruits and dried sumac (*Rhus coriaria*), used as a condiment, could be dietary sources (Clifford and Scalbert 2000). The highest levels of ellagic acid (EA) are found in berries, although a relevant part of it is present as ellagitannins (ETs). Contents of ETs from 1 to 400 mg/100 g fresh weight have been reported in berries, as reviewed by Landete (2011). Greater concentrations of ETs have been found in berries of the genus *Rubus*, such as raspberries and cloudberries, while they represent the second largest phenolic group in genus *Fragaria* (strawberry) after anthocyanins (Kähkönen et al. 2001; Koponen et al. 2007). Other relevant sources of EA and ETs are pomegranate and walnut. Contents of ETs from 68 to more than 1800 mg/L have been determined in pomegranate juice, depending on the way of preparation (Gil et al. 2000), while 59 mg of total EA/100 g dry weight have been reported in walnut (Daniel et al. 1989). Berry fruits can also be significant dietary sources of salicylates, with a range from 0.76 mg/100 g for mulberries to 4.4 mg/100 g for raspberries (Swain et al. 1985). Some condiments are particularly rich in salicylates. Contents around 200 mg/100 g were determined in curry powder, thyme, or hot paprika, and between 50 and 100 mg/100 g in dry thyme, dill, oregano, rosemary, or turmeric (Swain et al. 1985). Nevertheless, given the small amounts used in food, they cannot be considered as significant contributors to the dietary intake. Besides plants, some edible mushroom species can also contain significant amounts of phenolic acids, mostly benzoic acids, examples are *Ramaria botrytis*, which is rich in protocatechuic acid (343 μg/g DW), *Agaricus silvicola* that mostly contains *p*-hydroxybenzoic acid (343 μg/g DW) (Barros et al. 2009), or *Agaricus brasiliensis* (syn. *A. blazei*), with high amounts of *p*-hydroxybenzoic (333 μg/g DW), gallic (492 μg/g DW), and ferulic acids (753 μg/g DW) (Bach et al. 2019).

3.4.2 Dietary Intake

Several studies over different countries have estimated the dietary intake of phenolic acids, obtaining results that vary within a wide range from some tens mg/day to more

than 1 g/day depending on the target population and the methodological approach, i.e., the type of dietary assessment and composition databases used. Indeed, the existing databases for phenolic compounds have limitations, lacking data for some compounds or foods, which, on the other hand, can show variable polyphenol contents as influenced by factors like plant origin, cultivation, processing, or storage.

Radtke et al. (1998) in a study on a Bavarian cohort calculated an average intake of total phenolic acids of 222 mg/day, from which 211 mg corresponded to hydroxycinnamic acids and 11 mg to hydroxybenzoic acids. Caffeic acid (206 mg/day) was by far the most important contributor to that intake, while the mean intake of ellagic acid represented up to 5.2 mg/day, *p*-coumaric acid up to 3.8 mg/day, and ferulic acid up to 1.9 mg/day. Coffee was the major phenolic acid contributor, providing around 92% of the caffeic acid. Very large differences in the individual dietary intakes of phenolic acids were observed, ranging between 6 and 987 mg/day of total cinnamates, mostly depending on coffee consumption. Clifford (1999), in a rough estimation based on composition data collected from the literature and the probable burden of food intake, proposed a similar range of values for total cinnamates consumption by the UK population, comprised between less than 25 mg/day, in people that do not take coffee and have little intake of fresh fruit or vegetables, and 800–1000 mg/day, in great consumers of coffee, bran, and citrus fruits. Again, coffee was the main source of phenolic acids, namely caffeoyl-derived hydroxycinnamates.

In a large study across ten European countries in the frame of the European Prospective Investigation into Cancer and Nutrition (EPIC), Zamora-Ros et al. (2013) calculated a mean phenolic acid intake of 512 mg/day, with hydroxycinnamic acids as the predominant class (around 85% of total phenolic acids), followed by hydroxybenzoic acids (some 14%) and hydroxyphenylacetic acids (below 1%). Significant differences were observed among individuals and regions. Total phenolic acid intake was greater in northern than in southern European countries, although coffee was the principal source in all cases. Caffeic acid was again the most consumed phenolic acid, followed by ferulic acid, with cereals, bran, and whole-grain products as main dietary sources, while intake of *p*-coumaric acid was low and primarily provided by fruits, nuts, and spices. Tea, wine, and rosaceous fruits were the principal sources of hydroxybenzoic acids, whose intake was higher in southern countries associated to their greater consumption of nuts, seeds, and fruit and vegetables. Among hydroxyphenylacetic acids, homovanillic acid was the most important compound (77.8%), being primarily found in olives and olive oils more frequently consumed in southern European diets.

In a highly cited paper, Scalbert and Williamson (2000) proposed that flavonoids contributed around two-thirds of the dietary intake of total polyphenols, while phenolic acids accounted for one-third, an assertion that was assumed and spread by many other authors. However, several prospective studies over different populations seem not support it as a general rule, but similar consumption of both classes of polyphenols, or even higher of phenolic acids, are usually found in most studies, although notable variations obviously exist, reflecting the distinct dietary behaviors, e.g., coffee, fruits, or cereal consumption.

In a survey over a random sample of Finnish people using a 48-h dietary recall and data on phenolic composition collected in the Finnish National Food Composition database, Ovaskainen et al. (2008) calculated a daily polyphenol intake of 863 mg, from which 75% corresponded to phenolic acids. Coffee was the primary food item contributing to phenolic acid intake and also to the total intake of polyphenols; other relevant contributors were cereals (especially rye bread), tea, and fruits. Average intakes of 639 ± 273 mg/day and 506 ± 219 were calculated for phenolic acids and flavonoids, respectively, in a French cohort, being hydroxycinnamates the most largely consumed polyphenols, mostly originating from coffee (83%), followed by potatoes (4%), apples (2%), and green chicory (2%) (Pérez-Jiménez et al. 2011). Similar intakes of flavonoids (897 ± 423 mg/day) and phenolic acids (800 ± 345 mg/day) were found by Grosso et al. (2014) in a prospective study over a Polish cohort. Hydroxycinnamic acids were far more abundant (around 88% of total phenolic acids) than hydroxybenzoic acids. Newly, coffee was the primary food item contributing to phenolic acid intake (66%) and caffeoylquinic acids the most consumed polyphenols.

In a study within the frame of the EPIC study, Zamora-Ros et al. (2016) calculated mean total polyphenol intakes in the range 584–744 mg/day in Greece (the lowest consumption) to 1626–1786 mg/day in Denmark (the highest). In general, phenolic acids were the best represented phenolic class (52.5–56.9%). Higher or similar intakes of phenolic acids and flavonoids were also calculated in other cohorts from Italy (Godos et al. 2017; Vitale et al. 2018), Spain (Tresserra-Rimbau et al. 2014), or Brazil (Miranda et al. 2016; Nascimento-Souza et al. 2018). By contrast, in a large survey on the UK population, Ziauddeen et al. (2019) determined significant higher intakes of flavonoids than phenolic acids in all age subgroups from children of 1.5–3 years to people aged more than 65 years. The group with greater consumption of polyphenols was that of 50–64 years with an average intake of flavonoids of 714.5 ± 415.2 mg/day for 336.7 ± 292.0 mg/day of phenolic acids, from which 231.8 ± 289.9 mg/day hydroxycinnamic acids and 104.6 ± 82.5 mg/day hydroxybenzoic acids.

All in all, the overall intake of polyphenols is highly variable. Phenolic acid derivatives seem to represent the more prevalent class of phenolic compounds in most individuals, although flavonoids can predominate over phenolic acids in some populations. Hydroxycinnamoyl derivatives, and especially caffeoylquinic acids, are the main phenolic compounds in usual diets, with coffee as the most important contributor to their intake, followed by tea and cereals, with fruits and vegetables in the next level. Nevertheless, it is necessary to consider that the observations from prospective studies are determined by their estimative nature, owing to the limitations of the dietary assessments and composition databases, incomplete and lacking data on bound phenolics and complex tannins, including gallo- and ellagitannins, which are difficult to quantify and can represent a relevant part of food polyphenols that are, thus, overlooked in most studies. Actually, the non-extractable polyphenols fraction was estimated by Saura-Calixto et al. (2007) that may contribute up to double amount than extractable phenolic compounds to the dietary phenolic intake.

3.4.3 *Influence on Sensory Properties*

Phenolic acids are considered important contributors to the sensory properties of food due to their inherent taste but also because they can prevent rancid flavors by acting as antioxidants influencing lipid oxidation (Duizer and Langfried 2016). Classically they have been classified as possessing sensory properties described as being sour, astringent, and bitter. Nevertheless, studies carried out with isolated phenolic acids have shown the ability of these compounds to elicit a complex mixture of taste and oral sensations (Maga 1978; Peleg and Noble 1995).

Physiologically, to detect taste, compounds must first be dissolved in saliva and transported to taste cells, located at the base of the tongue, where they can either interact with taste receptors on the surfaces of these cells, leading to sweet and bitterness detection, or with ion channels, leading to salty and sour detection (Rawson and Li 2004). Taste thresholds of individual phenolic acids range from 5 ppm for the *m*-anisic acid to 240 ppm for syringic acid (Maga 1978), although combinations of them, as occurring in foods, may decrease the taste threshold due to synergistic effects (Ferrer-Gallego et al. 2014). For example, a recent study has shown that phenolic acids and quercetin rutinoside interact synergistically in tea infusion enhancing bitterness and astringency of tea infusion (Chen et al. 2022). On the other hand, it is known that sensory properties of phenolic acids can be influenced by their structure. Thus, it has been reported that functional groups in the meta position increase sensitivity, whereas the same functional groups in the ortho position decrease sensitivity. For benzoic acid derivatives, a methoxy group results in a more tasting compound than a hydroxy group (Maga 1978).

Astringency is not a taste, but a tactile sensation described as drying, roughening, and constricting within the oral cavity. In general, astringency has been thought to be the result of the interactions of astringent compounds, such as phenolic compounds, with salivary proteins. Interestingly, the precipitation of salivary proteins was not found to be required for the development of astringency of ferulic, vanillic, or gallic acid, suggesting that salivary protein binding activity may not be an accurate measurement of the astringency of all phenolic compounds. Indeed, it looks like that the perceived astringency of organic acids is a function of pH, suggesting that the inverse relationship between pH and astringency may be explained by reduced salivary lubricity due to denaturation of salivary proteins under conditions of reduced pH (Lawless et al. 1996).

Studies carried out with individual phenolic acids have established that among benzoic acid derivatives, gentisic acid had the highest sourness, astringency, and bitterness intensity, while salicylic had similar astringency than gentisic acid and *m*-hydroxybenzoic acid was the sweetest sample, and the prickling feeling was especially contributed by benzoic acid (Peleg and Noble 1995). However, based on the amounts found in food and their respective taste threshold, vanillic, *p*- and *o*-coumaric and ferulic acids are the principal phenolic acids to be organoleptically detectable (Maga 1978). *p*-Coumaric acid is perceived as bitter, astringent, and unpleasant at 48 ppm while ferulic acid is perceived as a sour at 90 ppm. However,

the combination of *p*-coumaric and ferulic acids is felt as bitter and sour at 20 ppm (Huang and Zayas 1991). Vanillic acid taste is significantly sourer than ferulic acid, while ferulic acid is considered to be significantly more bitter than vanillic acid.

Chlorogenic acid isomers are precursors of caffeic acid and quinic acid that contribute to the formation of sensory attributes and are related to the sensory characteristics of coffee beverage, mainly to astringent, acid, and bitter ones (Aree 2019; Moon and Shibamoto 2010). In the same way, it has been described that the sensory properties of virgin olive oil may be differently affected by its phenolic acid content depending on the type of cultivar (Rivas et al. 2013).

In general, the native phenolic compounds in plant-based foods have been considered to negatively influence food selection due to imparting negative bitter flavor attributes to foods (Drewnowski et al. 1997; Heiniö et al. 2008). On the other hand, it is also well known that some hydroxycinnamic acids undergo decarboxylation and oxidative reactions during thermal processing leading to volatile products that can impact on both aroma and taste attributes of foods as well as food palatability (Jiang and Peterson 2010). For example, under thermal processing, ferulic acid generates aroma compounds such as 4-vinylguaiacol, guaiacol, and vanillin (Fiddler et al. 1967), while catechol and 4-ethylcatechol can derive from thermal degradation of caffeic acid and 5-caffeoylquinic acid (Frank et al. 2006). Decarboxylation processes can also occur during fermentation by lactic acid bacteria (LAB) (Couto et al. 2006) or yeast (Shinohara et al. 2000), resulting in the production of 4-vinylplenols, or their reduced form 4-ethylphenols, with great impact on the sensorial characteristics of foods. In fermentation by LAB, *p*-coumaric acid had the highest conversion efficiency, followed by caffeic acid and lastly ferulic acid (Miyagusuku-Cruzado et al. 2020), while yeast strains are very unselective and decarboxylate cinnamic, *p*-coumaric, and ferulic acids with similar conversion rates (Goodey and Tubb 1982). In fermented products such as wine, HCA degradation by yeast results in the formation of 4-vinylplenols that are responsible for undesirable phenolic off-flavors in the final product (Shinohara et al. 2000). On the other hand, phenolic acids remarkably inhibit terpene glycosides hydrolysis and free terpene volatilization and affect the profile and amounts of free terpenes, thus influencing the tropical, sweet, small berry, and floral aromas of wine (Wang et al. 2021). However, these compounds may be desirable, for example, in wheat beer, where the reduction of HCA in 4-vinylphenol and 4-vinylguaiacol results in a less pronounced wheat beer aroma (Kalb et al. 2020; Langos and Granvogl 2016), or in bread where decarboxylation of ferulic acid generates 4-vinylguaiacol contributing to its flavor profile (Wang et al. 2012).

3.4.4 Effect of Processing and Storage on Phenolic Acids

Most food processing techniques involve a sequence of operations bringing about changes in the raw material, with each operation having impact on the food constituents. In general, food processing operations, such as thermal processing,

fermentation, or freezing lead to a release of matrix-bound phenolic acids increasing the contents of free forms (Dewanto et al. 2002). The changes in free and bound forms of phenolic acids induced by processing depend on the type of matrix and the processing technique employed (Nayak et al. 2015). Detrimental effects can also be produced, being temperature, oxygen, and enzymes activity major factors affecting the stability of phenolic acids (Dewanto et al. 2002).

A majority fraction of phenolic compounds present in cereal and cereal-based products are bound to cellulose and hemicellulose structures, which are partially broken down during *thermal processing*. Thus, after thermal processing of wheat bran, increases in the content of ferulic, vanillic, and *p*-coumaric acids of 70% have been reported, while in oat bran dihydroxybenzoic acids increased almost 40% (Călinoiu and Vodnar 2019). Likewise, extrusion processes (120–200 °C) enhanced by two to three times the levels of free forms of vanillic, syringic, and ferulic acids (Zielinski et al. 2001). Bryngelsson et al. (2002) found that autoclaving of oats increased the content of *p*-coumaric, vanillin, and ferulic acids, while it had a negative impact on caffeic acid. Szwajgier et al. (2014) reported detrimental effects after heating of different fruits, with a significant reduction of total phenolic acids in compotes and jams compared with the corresponding fresh-frozen (thawed) fruits; the most dramatic loss of phenolic acids was predominantly observed in the case of jams. In the same way, a significant decrease in the levels of *p*-hydroxybenzoic, *p*-coumaric, caffeic, chlorogenic, and syringic acids was observed in dried fruit homogenates in comparison with fresh fruits. Thermal processing of orange juice resulted in the hydrolysis of ferulic acid esters with subsequent release of the free acids, which later undergo decarboxylation, leading to the formation of 4-vinyl guaiacol, a potent off-flavor compound imparting an unpleasant odor to the final product (Lee and Nagy 1990). Changes in free and bound phenolic acids were found in artichokes during cooking, being the decrease in total caffeoylquinic acids higher in boiling than in frying and griddling, although the formation of new isomers due to transesterification phenomenon partially compensated the loss of total mono- and di-caffeoylquinic acids (Domínguez-Fernández et al. 2021). As for coffee roasting, the higher the roasting degree the lower the content of chlorogenic acids, however, as they are decomposed a rise in caffeic and quinic acids levels is produced (Awwad et al. 2021; Fuller and Rao 2017).

Increases in the contents of some phenolic acids in thermally processed fruits have been reported by several authors. Thus, the concentration of ellagic acid was found to raise in preserves of strawberry in relation to fresh fruit due to partial degradation of ellagitannins, while it was reduced during processing of blueberries into jams (Häkkinen et al. 2000), highlighting the importance of the matrix effect. Similarly, an increase in the level of caffeic acid was observed in pear juice in relation to fresh fruit, which was attributed to the thermal hydrolysis of chlorogenic acids (Spanos and Wrolstad 1990). Pressure cooking of legumes also resulted in improved bioaccessibility of phenolic acids, released from plant matrix (Chen et al. 2015). Significant enhancement in phenolic acids contents was also found in black currant juices submitted to pasteurization, and further increase by a factor of two to four was produced in the levels of hydroxycinnamic acids during one-year storage,

which could be possibly explained by acid hydrolysis of conjugated forms (Mäkilä et al. 2017). However, no changes were observed in the concentration of total phenolic acids (the sum of *p*-hydroxybenzoic, vanillic, chlorogenic, caffeic, syringic, ferulic, and *o*-coumaric acids) in blueberry juices, either pressed, clarified, pasteurized, or concentrate, submitted to heat and SO_2 treatments (Lee et al. 2002).

Other technological processes, such as *pulsed electric field* (Agcam et al. 2014; Morales-De la Pena et al. 2011), *gamma radiation* (Breitfellner et al. 2002), or *high pressure processing* (Marszałek et al. 2017; Pérez-Lamela et al. 2021) have been reported to increase the content of individual phenolic acids when applied on juices or fruits, also attributable to the release of bound compounds through hydrolysis reactions.

Fermentation processes on matrices rich in phenolic acids lead to similar events as thermal processing, due to the activity of hydrolytic enzymes. Thus, an increase in the levels of free phenolic acids initially bound, mainly but not exclusively ferulic acid, is produced during fermentation of cereal doughs (Amaya Villalva et al. 2018; Bhanja et al. 2009; Katina et al. 2007). Different lactic acid bacteria (LAB) have shown capacity to release caffeic acid from chlorogenic acids from fruit and vegetable substrates as a result of their cinnamoyl esterases activity (Filannino et al. 2015; Fritsch et al., 2016). Biotransformation of phenolic acids by LAB and yeast decarboxylases or reductases has also been documented in numerous investigations. Yeasts present variable hydroxycinnamate decarboxylase activity according to the strain, which is responsible for the transformation of hydroxycinnamic acids into vinylphenols during wine fermentation, a process with significant influence on red wine color, while their further conversion to ethylphenols have a negative impact on wine flavor (Morata et al. 2016). Ripari et al. (2019) observed that co-fermentation of wheat and rye malt sourdoughs by *L. plantarum* and *L. hammesii* released bound ferulic acid, which was further converted to dihydroferulic acid and volatile compounds (e.g., vinylguaiacol and ethylguaiacol) with an impact on bread flavor; however, no conversion of other phenolic acids, including coumaric, sinapic, and vanillic acids was noticed. Svensson et al. (2010) found that ferulic acid was reduced into dihydroferulic acid by *L. plantarum* and *L. fermentum* but not decarboxylated during fermentation of sorghum doughs. However, while *L. plantarum* decarboxylated caffeic acid to vinylcatechol and ethylcatechol, *L. fermentum* reduced and decarboxylated it to dihydrocaffeic acid and vinylcatechol, respectively, without production of ethylcatechol. Filannino et al. (2015) showed that degradation of caffeic acid would depend on the *L. plantarum* strain, whereas some strains mostly degraded it into vinylcatechol, others released dihydrocaffeic acid or ethylcatechol as end products. Leonard et al. (2021) also reported that *p*-coumaric acid was generally degraded into *p*-vinylphenol and phloretic acid by *L. plantarum*, but the proportion of these two metabolites and the percentage of degraded *p*-coumaric acid substantially differed among strains. Hydroxybenzoic acids can also be transformed by LAB. Catechol was identified as main end product from protocatechuic acid conversion by *L. plantarum* in cherry juice, while no formation of catechol was observed when inoculated with *L. reuteri* (Filannino et al. 2015). The ability of different strains of *L. plantarum*, *Enterococcus mundtii*, and

Pediococcus pentosaceus to decarboxylate caffeic, ferulic, and *p*-coumaric acids into 4-vinylcatechol, 4-vinylguaiacol, and 4-vinylphenol, respectively, in model in vitro systems was demonstrated by Miyagusuku-Cruzado et al. (2020). All these studies reveal that different LAB strains can show distinct metabolic pathways and substrate specificities leading to different products when degrading phenolic acids.

Changes in phenolic acids content and composition have also been observed during *storage*. Ellagic acid was found to be released from the hydrolysis of ellagitannins during refrigerated storage of fruits such as strawberry and juices (Häkkinen et al. 2000), as well as during their storage under modified atmospheres (Gil et al., 1997). By contrast, Klaiber et al. (2005) observed that the level of phenolic acids strongly diminished in fresh vegetables during storage in the absence of oxygen or at carbon dioxide levels >30%, which was explained by inhibition of PAL activity; however, high accumulation was seen when they were stored in air. An increase in the content of hydroxycinnamic acids was found in cherries during postharvest storage at 15 ± 5 °C for 6 to 30 days, although a tendency to decline was observed at 1–2 °C (Goncalves et al. 2004). Several phenolic acids (protocatechuic, *p*-hydroxybenzoic, syringic, salicylic, *p*-coumaric, chlorogenic, ferulic, and β-resorcylic acids) were analyzed in soybean flour stored under different conditions of time and temperature by Prabakaran et al. (2019), concluding that whenever temperature and time increased an enhancement was also produced in total contents of phenolic acids.

Light is another important factor that influences phenolic acids evolution during storage of fruits and vegetables. Continuous light irradiation (30 μmol m^2 s^{-1}) was shown to produce positive effects in the accumulation of phenolic acids in minimally processed spinachs, which was explained by the activation of PAL activity (Zhan et al. 2020). Friedman (1997) and Griffiths et al. (1995) also reported significant greater increases in chlorogenic acid levels in light-stored potatoes than in those stored in darkness. By contrast, Slimestad and Verheul (2005) found that the content of chlorogenic acid felt in cherry tomatoes from 0.51 to 0.06 mg/100 g after 3 weeks of postharvest storage at 20 °C in the absence of light.

Overall, processing can have both positive and detrimental effects on phenolic acid content and composition depending on the plant matrix and the processing method. Therefore, it is critical to determine not only optimal processing conditions to extend the shelf life of products, but also to reduce the degradation of bioactive compounds (Ifie and Marshall 2018).

3.5 Biotechnological Production

3.5.1 Preparation from Natural Sources

In view to their use by food, cosmetic, or pharmaceutical industries, large production of phenolic acids is required. Although plant extracts or compounds mixtures can be appropriate for some purposes (e.g., dietary supplements, cosmeceuticals), the

preparation of pure compounds is necessary for their use as food ingredients or additives, as well as for the adequate assessment of their technological, healthy, therapeutic, or safety characteristics.

Concentrations of phenolic acids in plants are usually low and strongly variable depending on plant species and tissues, as well as geographical, seasonal, and edafoclimatic factors. Despite some plant materials are very rich in some particular compounds, such as coffee beans (chlorogenic acids) or cereal brans (ferulic acid derivatives), their extraction on a large-scale is normally unaffordable. Extraction is usually carried out with organic solvents and only allows obtaining free extractable phenolic acids; furthermore, the process may involve the use of non-environmentally friendly solvents and, in general, extraction yields are low and lead to complex mixtures of compounds, so that further purification is required, making the process tedious and expensive. Although chemical synthesis might be an alternative for the preparation of some compounds, the existing approaches are often inefficient, especially in the case of complex products, and suffer from similar limitations of low yields, formation of side-products, challenging reactions, use of non-green or toxic solvents and reagents, laborious purification, waste production, and heavy pollution.

Hydrolysis or fermentation processes allow releasing phenolic acids from complex structures (e.g., hydrolyzable tannins) or bound to matrix components, thus increasing the extraction yields. Acid and alkaline hydrolyses can provide an effective cleavage of bound phenolics, although these treatments are environmentally unsuitable; while enzymatic hydrolysis is more friendly it can be expensive. In this respect, processes taking advantage of the enzymatical machinery of microorganisms would be more appropriate, such as extracellular enzymes produced during microbial growth like pectinases, cellulases, amylases, xylanases, or esterases able to degrade cell walls. Residues from agro-food industries can be particularly suitable sources, as they are rich in lignin, from which phenolic acids like ferulic, *p*-coumaric, syringic, vanillic, or *p*-hydroxybenzoic acids can be released. In this respect, biotechnological processes using different microorganisms and substrates have been explored, either in liquid media containing essential nutrients for the growth of the microorganisms (submerged fermentation processes) or in the absence of free-flowing water on a moist solid substrate that acts both as physical support and source of nutrients for microorganisms (i.e., solid-state fermentation) (Šelo et al. 2021). Solid-state fermentation (SSF) offers particular advantages such as low energy requirements and not requiring complex machinery and control systems, thus providing a low-cost process. Nevertheless, no high yields are usually obtained, and process scaling-up and compound purification are challenging. Yeasts and fungi are the microorganisms most commonly used due to their lower water activity requirements compared to bacteria (Thomas et al. 2013). Some processes developed for phenolic acids production are below given as examples.

Saccharomyces cerevisiae was employed by Santos da Silveira et al. (2019) in an SSF process to obtain chlorogenic acid from coffee pulp; optimization of the process at a pilot scale allowed the preparation of extracts 400% richer in chlorogenic acids (600 mg/kg of coffee pulp) and with lower sugar amounts, which facilitated further

purification. Solid-state fermentation with the fungus *Rhizopus oryzae* was found to strongly enhance the content of free phenolic acids in rice bran, especially gallic and ferulic acids, reaching levels 170 and 765 mg/g in the fermented bran, respectively (Schmidt et al. 2014). Significant increases in the contents of ferulic, sinapic, vanillic, caffeic, syringic, and 4-hydroxybenzoic acids were also found in rice bran by Abd Razak et al. (2015), following SSF with single and mixed cultures of two fungi, *Rhizopus oligosporus* and *Monascus purpureus*.

A range of bacteria, including strains of genera such as *Streptomyces, Rhodococcus, Pseudomonas, Bacillus, Alcaligenes, Arthrobacter,* or *Nocardia*, have also been reported to be capable of degrading lignin to low molecular weight compounds (Bugg et al. 2011; Lee et al. 2019). An anaerobic strain from the *Acetoanaerobium* genus, isolated from the sludge of a pulp and paper mill, was used by Duan et al. (2015) for the production of ferulic and syringic acids as final metabolites from the biodegradation of kraft lignin derived from pulp and paper industries, using it as the sole carbon source.

Mushrooms have also been used to recover phenolic acids from different substrates. The food grade fungus *Lentinus edodes* (shiitake mushroom) has been shown to be able to produce high amounts of extracellular β-glucosidase, an enzyme capable of hydrolyzing phenolic glycosides to release free phenolic acids, during solid-state growth. SSF processing of cranberry pomace with *L. edodes* was employed for effective production of free gallic, *p*-hydroxybenzoic and *p*-coumaric acids (Zheng and Shetty 2000), and ellagic acid (Vattem and Shetty 2003). The ability of the white-rot fungus *Trametes versicolor* to produce lignolytic enzymes was explored by Bucic-Kojic et al. (2017) to release phenolic acids from corn silage, reaching 10.4, 3.4, 3.0, and 1.8-fold increases in the yield of free syringic, vanillic, *p*-hydroxybenzoic, and caffeic acids, respectively, after 20 days of treatment with the fungus.

Nevertheless, these and other studies were intended to optimize fermentation conditions in order to increase the extraction yields of phenolics. However, to obtain a ready-to-market product, downstream concentration and purification of the compounds using sustainable techniques is still required and the processes have to be scaled-up, so that their cost-effectiveness and environmental impact can be evaluated, which is yet a long way to go. To overcome some of the constraints posed by the extraction of phenolics acids from natural sources, genetic and molecular biology techniques have been explored for engineering plants or microorganisms, some of them aimed at channeling the synthesis toward target compounds, thus requiring minimum purification.

3.5.2 Plant Genome Engineering

As above discussed, polyphenols are produced through the shikimate-phenylpropanoid pathway, a route that is involved in the biosynthesis of both

phenolic acids and flavonoids, as well as other phenolic compounds, like stilbenes or lignans.

Plants present several advantages over microorganisms for the construction of heterologous systems in view to produce phenolic compounds. Firstly, plants already contain genes of the phenylpropanoid pathway, so that there is no need to reconstitute the entire pathway. Actually, producing the array of enzymes needed to reproduce all the phenylpropanoid diversity seems unaffordable in microbial hosts; besides, plant enzymes may not behave as efficiently in microbes as in plants, especially in prokaryotes lacking post-translational modifications and organelles for enzyme compartmentalization. While phenolic compounds can be accumulated in large amounts in plant vacuoles, their accumulation in microorganisms would be more limited; further, some phenylpropanoids may also have antimicrobial activity, thus killing hosts. On the other hand, plants can be readily edible or require minimal processing, which is advantageous for their delivery, reducing costs associated with the production chain (Ferreira and Antunes 2021). By contrast, plant growth is slow and strongly influenced by external factors (soil, climate, biotic, and abiotic stresses) (Rainha et al. 2020). Another difficulty for phenolic acid production in plants is that specialized enzymes involved in the biosynthesis of phenylpropanoids are able to utilize multiple substrates and, therefore, pathways are not straightforward, but rather structured as complex grids, with coexistence of multiple and sometimes competitive pathways, so that it is difficult to foresee the metabolic consequences of gene engineering. Furthermore, plants are more difficult to manipulate genetically than microorganisms.

Strategies to engineer plants in order to increase the biosynthesis of secondary metabolites can target enzymes or transcription factors. A key enzyme in phenylpropanoid biosynthesis is phenylalanine ammonia lyase (PAL), whose activity represents the branching point between primary and secondary metabolism, so that PAL gene expression is a relevant target for the activation of the phenylpropanoid pathway and increasing phenolic production (Marchiosi et al. 2020). Other relevant enzymes are cinnamate 4-hydroxylase (C4H), 4-coumaroyl CoA ligase (4CL), responsible for the formation *p*-coumaroyl CoA, the activated intermediate for the various branches of phenylpropanoid metabolism, and cinnamoyl-CoA reductase (CCR) that catalyzes the reduction of cinnamoyl-CoA esters to their corresponding cinnamaldehydes, a key step in the biosynthesis of lignin (Umezawa 2010). Pathway perturbations modifying the activity of these enzymes alter the synthesis of phenylpropanoids. Accumulation of phenolic acids, such as *p*-coumaric, caffeic, ferulic, 5-hydroxyferulic, and sinapic acids and derivatives has been demonstrated to occur in low producing lignin mutants, e.g., c4h, 4cl1, or ccr1 mutants (Vanholme et al. 2012). Production of high levels of ferulic acid was shown by Xue et al. (2015) in *Arabidopsis* ccr-1 mutants, which also presented multiple developmental defects including increased cell proliferation, explained by the cell proliferative effect of ferulic acid.

Compound accumulation resulting from enzyme activation may trigger a feedback inhibition in the activities of pathway enzymes with subsequent arrest in metabolite production. A strategy to overcome this shortcoming is the introduction

into plants of genes encoding feedback-insensitive enzymes from microbes or plants with unique phenotypes (Yuan and Grotewold 2015). The introduction in *Arabidopsis* of a bacterial AroG gene encoding a feedback-insensitive 3-deoxy-d-arabino-heptulosonate 7-phosphate synthase (DAHPS), a key enzyme of the primary metabolism regulating the carbon flow through the shikimate pathway, resulted in the enhancement in the plant of the levels of aromatic amino acids, i.e., phenylalanine and tryptophan, but also of derived secondary metabolites, including phenylpropanoids (Tzin et al. 2012).

Transcription factors (TFs) usually control genes of multiple enzymes within a metabolic pathway; thus, their use can be an effective strategy to engineer plants in order to increase the biosynthesis of secondary metabolites. Metabolic engineering can be focused on early TF genes so as to push the flux downstream, or on late pathway genes to address substrates toward a particular final product (Yuan and Grotewold 2015). Regulatory genes encoding the MYB family of transcription factors are particular good candidates, as they are present in all eukaryotes and regulate a variety of plant-specific processes, including phenylpropanoid biosynthesis (Ambawat et al. 2013). Several MYB transcription factors involved in phenylpropanoid biosynthesis have been characterized and proposed to engineer plants in order to promote phenolic acid production. The expression of the R2R3-MYB transcription factor ZmMyb-IF35, with high identity with the P regulator of 3-deoxy flavonoid biosynthesis in maize, does not induce the accumulation of flavonoids, but that of ferulic and chlorogenic acids not found in Black Mexican Sweet maize cells (Dias and Grotewold 2003). Tang et al. (2021) observed that overexpression of another R2R3 MYB transcription factor, LmMYB15 from *Lonicera macranthoides*, led to increased accumulation of chlorogenic acid (CGA) in tobacco leaves, underlying new breeding strategies to enhance CGA content in *L. macranthoides*, the main biologically active compound in this plant used in traditional Chinese medicine.

Methyl jasmonate is an elicitor that has been used to activate the transcripts of PAL and C4H enzymes from the phenylpropanoid pathway, as well as tyrosine aminotransferase (TAT) and 4-hydroxyphenylpyruvate reductase (HPPR) from the tyrosine-derived pathway (Xiao et al. 2009, 2011). Overexpression of the methyl jasmonate-responsive R2R3-MYB transcription factors SmMYB1 (Zhou et al. 2021) and SmMYB2 (Deng et al. 2020) was shown to upregulate the expression of genes encoding key enzymes in the phenolic acid biosynthesis leading to increased accumulation of phenolic acids and anthocyanins in *Salvia miltiorrhiza*, especially salvianolic acids such as rosmarinic acid and salvianolic acid B, which are important bioactive components in this medicinal herb. By contrast, the content of *p*-coumaric acid, rosmarinic acid, salvianolic acid B, salvianolic acid A, and total phenolics was dramatically decreased in *S. miltiorrhiza* following overexpression of SmMYB39, suggesting that this TF acts as a repressor through suppressing transcripts of key enzyme genes (Zhang et al. 2013).

Attention has also been paid to the basic helix loop-helix (bHLH) MYC family of transcription factors, such as MYC2, the up-regulation of which enhances the production of phenolic acids in *S. miltiorrhiza*, namely rosmarinic acid and

lithospermic acid B, whose concentrations increased by 2.46-fold and 1.88-fold, respectively (Yang et al. 2017). Previously, Bovy et al. (2002) also showed that ectopical expression in tomatoes of genes of two transcription factors from maize, i.e., MYB-type C1 and MYC-type LC, upregulated the phenylpropanoid pathway resulting in a strong accumulation of phenolic compounds, and especially flavonols, in tomato flesh, a tissue that normally does not produce flavonoids. However, the own authors indicated that the results are difficult to predict, and the type and content of metabolites can vary between plant species and varieties, owing to the induced expression of structural genes and the substrate specificities of the enzymes involved may differ from plant to plant. Similarly, expression in transgenic tomato of AtMYB12, a TF regulating flavonol biosynthesis in *Arabidopsis thaliana*, led to the accumulation of high levels of chlorogenic acids and flavonols in the fruit, which might represent a way for the effective production of these phenylpropanoids in tomato (Zhang et al. 2015).

In order to minimize possible adverse effects derived from pathway modification, the use of autoregulatory synthetic genetic circuits controlled by biosensors may help the transgenic organism address the metabolic output and trigger an on or off response as needed. Several phenylpropanoid-related TF sensors have been characterized in prokaryotes, although at present their use has been mostly limited to microbial systems, such as bacterial or yeast strains engineered for flavonoid production (Ferreira and Antunes 2021). A promising phenylpropanoid-related biosensor is the Q-system from the fungus *Neurospora crassa*. This is a gene cluster involved in the catabolism of quinic acid for its use as a carbon source. The cluster contains a transcriptional activator (QF) and its repressor (QS); the binding of QF to a minimal promoter presenting the QUAS regulatory sequence triggers downstream gene expression, while binding of QS to QF inhibits the activity of QF. It has been shown that quinic acid can regulate this system by restoring QF activity through inhibition of QS. The functionality of the Q-system in plants has been demonstrated through expression in *Glycine max* protoplasts and *Nicotiana benthamiana* leaves (Persad et al. 2020), envisaging its use as a tool for precise control of gene expression in plant metabolic engineering. As quinic acid is an essential metabolite for redirecting phenylpropanoids toward chlorogenic acids, the Q-system could be a suitable biosensor to regulate this pathway. Nevertheless, de-repression of the system by quinic acid has not been yet tested in plants, although the fact that it functions in other heterologous systems, such as *Caenorhabditis elegans* (Wei et al. 2012) or drosophila (Riabinina et al. 2015), allows thinking that quinic acid-dependent de-repression might also be possible in plants (Ferreira and Antunes 2021).

Biosensors are only able to detect intracellular ligand concentration, which represents a limitation when cells secrete the desired product (Ferreira and Antunes 2021). A biosensor allowing detection of extracellular *p*-coumaric acid in yeasts was developed by Siedler et al. (2017). The system was based on a *Bacillus subtilis* transcriptional repressor (PadR), which was introduced in *Escherichia coli* to generate *E. coli*-biosensing cells that were further encapsulated into p-coumaric acid-producing yeasts, which could, thus, be sorted using the fluorescent *E. coli* biosensor

signal. Other phenylpropanoid-related TF sensor proteins have been characterized in prokaryotes, as reviewed by Ferreira and Antunes (2021), a few of which have been engineered for high-throughput screening of metabolites in bacterial or yeast strains, especially for flavonoids. As biosensors become available, they are expected to find applications for detecting metabolites not only in microbial systems but also in plants.

Despite relevant progresses have been made, up to now, plant engineering developments have mostly been carried out at lab scale and not upgraded to industrial production, where outcomes are subject to cultivation factors and difficult to predict and standardize.

3.5.3 *Engineered Microorganisms for Phenolic Acid Production*

As above indicated, microorganisms do not produce polyphenols naturally, so that the phenylpropanoid pathway has to be reconstructed. However, primary metabolism is similar to plants and, therefore, they are able to provide the aromatic amino acid precursors for the phenolic biosynthesis, representing an attractive platform when the plant pathways can be functionally introduced (Milke et al. 2018). Indeed, microorganisms may have advantages over plants for the production of secondary metabolites. They can grow in inexpensive substrates and have a rapid growth rate, so that the process is faster, more economical, and easier to upgrade to a large-scale production. Furthermore, the diversion of intermediate products to competing pathways often present in the natural host can be reduced, and the process addressed to the compounds of interest, avoiding simultaneous formation of related molecules, which facilitates further purification (Krivoruchko and Nielsen 2015). *Saccharomyces cerevisiae* and *Escherichia coli* have been most commonly used microorganisms for metabolic engineering purposes, having the advantage of being well-characterized and genetically tractable, with multiple tools available for their genetic manipulation; also, other bacteria such as *Lactococcus lactis, Corynebacterium glutamicum*, or *Pseudomonas* spp. have been explored (Dudnik et al. 2018; Valanciene et al. 2020).

Although successful developments have been achieved using *E. coli* as a platform, *S. cerevisiae* presents advantages, due to its robustness and tolerance toward harsh fermentation conditions, as well as its superior capability of expressing membrane-bound cytochrome P450 oxidases (e.g., C4H), which are key catalysts in most relevant plant-based biosynthetic pathways (Liu et al. 2019b). As a eukaryote, *S. cerevisiae* is able to perform post-translational modifications such as glycosylation and it possesses intracellular organelles similar to plant cells, which is crucial to functionally express membrane-bound cytochrome P450 enzymes. Besides, unlike *E. coli*, it has a GRAS status, that is important to produce both nutraceutical and pharmaceutical compounds (Gomes et al. 2022). Nevertheless, it is

to say that most attempts of using *S. cerevisiae* as a chassis have been addressed to the production of flavonoids, prenylflavonoids, or stilbenes, while less developments have been focused on phenolic acids (Gomes et al. 2022; Rainha et al. 2020).

Aromatic amino acids (AAA) are the primary substrates for the biosynthesis of phenolic compounds and their availability represents a major bottleneck and a metabolic engineering challenge for microbial polyphenol production. AAA can be directly added into the media as precursors, although this makes the process more expensive for industrial applications. An alternative is the use of microbial strains capable of producing phenolic compounds directly from glucose (de novo production), which can be achieved by engineering the pentose phosphate and the shikimate pathways to provide sufficient amounts of L-phen or L-tyr.

Aromatic amino acid biosynthesis is subject to feedback inhibition by the product (i.e., L-phen or L-tyr). To overcome it, the most successful strategies have dealt with overexpression of the feedback-insensitive mutant enzymes 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase (DAHPS) and chorismate mutase (CM), which catalyze the first steps in the AAA biosynthesis and are the branching point toward the production of tyrosine and phenylalanine, respectively (Liu et al. 2019b). A tyrosine-insensitive ARO4 allele in conjunction with deletion of the ARO3 allele of the DAHP synthase was introduced by Koopman et al. (2012) in *S. cerevisiae* engineered with *Arabidopsis thaliana* genes for phenylpropanoid synthesis. This allowed accumulation of aromatic amino acids that, together with elimination of phenylpyruvate decarboxylase to reduce pathway diversion to phenylethanol formation, led to a notable increase in the extracellular accumulation of the target polyphenols (in this case, the flavanone naringenin), using glucose as sole carbon source. Liu et al. (2019b) constructed a *S. cerevisiae* platform able to produce high levels of *p*-coumaric acid. The AAA biosynthesis pathway was modified by introducing a feedback-insensitive DAHPS and CM, and a heterologous phosphoketalose-based pathway to divert glycolytic flux toward formation of the shikimate precursor erythrose 4-phosphate; distribution of the carbon flux from glycolysis toward the AAA biosynthesis was optimized by replacing the promoters of key node genes between these two pathways. To increase production of *p*-coumaric acid two different strategies were explored consisting of introducing (i) PAL, C4H and a cytochrome P450 reductase from *A. thaliana*, together with the native cytochrome (CYB5) from *S. cerevisiae*, or (ii) a highly specific TAL from *Flavobacterium johnsoniae*. The authors concluded that the PAL branch was far more efficient than the TAL branch.

Efficient production of caffeic acid in *S. cerevisiae* from L-tyrosine was achieved by Liu et al. (2019a) by recruiting a heterologous tyrosine ammonia lyase (TAL) from *Rhodosporidium toruloides* and two enzymes, HpaB from *P. aeruginosa* and HpaC from *E. coli*, to take on the role of the plant-specific cytochrome P450-dependent monooxygenase, *p*-coumarate 3-hydroxylase (C3H) to hydroxylate *p*-coumaric acid to caffeic acid.

The expression of heterologous genes in *S. cerevisiae* can lead to translation errors, resulting in the expression of non-functional enzymes; thus, codon optimization can be required for an efficient production of phenolic compounds (Rainha

et al. 2020). For instance, expression of the TAL gene from *Rhodobacter sphaeroides* into *S. cerevisiae* failed to produce *p*-coumaric acid when feeding with tyrosine substrate, despite bacterial TAL activity had been confirmed in vitro and the transcripts of TAL were present at high levels in the transgenic yeast. This was attributed to differences in codon usage, with many of the Rhodobacter codons having very low usage frequency in the yeast. Using multi rounds of PCR-based mutagenesis, Wang et al. (2011) succeeded to replace inefficient bacterial codons by yeast favorable codons, which strongly increased *p*-coumaric acid and resveratrol biosynthesis.

Escherichia coli has also been extensively explored for the heterologous production of phenolic compounds. Comparing with *S. cerevisiae*, it has the advantage of having higher growth rates and shorter time of doubling, which is relevant to develop competitive processes of production. Besides, the expression levels of heterologous genes in *E. coli* are usually higher than those in *S. cerevisiae*. However, it does not perform post-translational modifications and does not possess intracellular organelles, which can be unfavorable for the functional expression of plant-derived enzymes (Gomes et al. 2022).

De novo biosynthesis of *p*-coumaric acid from glucose as the sole carbon source in *E. coli* was achieved by Li et al. (2018), using a plant-specific cytochrome P450 enzyme (C4H) encoding the gene *Lau*C4H gene isolated from *Lycoris aurea* fused with the CYP450 redox partner from *A. thaliana*, as well as PAL from *A. thaliana* into the recombinant *E. coli* cells. Synthesis of four cinnamates (cinnamic, *p*-coumaric, caffeic, and ferulic acids) was obtained by An et al. (2016) in engineered *E. coli* harboring different combination of four genes: PAL from *A. thaliana*, TAL and a monooxygenase (Sam5) from *Saccharothrix espanaensis*, and an *O*-methyltransferase (ROMT9) from *Oryza sativa*. Rodrigues et al. (2015) engineered *E. coli* for production of caffeic acid adding tyrosine as a precursor in a two-step conversion using TAL from *Rhodotorula glutinis* (RgTAL) and 4-coumarate 3-hydroxylase from *S. espanaensis* or cytochrome P450 CYP199A2 from *R. palustris*. A limitation of the system was the accumulation of *p*-coumaric acid, owing to its cytotoxicity and feedback inhibition of TAL activity. Improved caffeic acid production could be obtained by changing genes arrangement through codon optimization and different plasmid constructions, together with recurrent addition of the substrate (L-tyr). The same authors further demonstrated that this biosynthetic pathway could be triggered using heat shock promoters, suggesting its potential for the future production of these compounds at industrial scales without chemical induction (Rodrigues et al. 2017).

Jendresen et al. (2015) screened a series of PAL and TAL enzymes from different origins, finding that TAL from *Herpetosiphon aurantiacus* and *Flavobacterium johnsoniae* showed higher substrate specificity than other characterized enzymes, resulting in enhanced production of *p*-coumaric acid in bacteria like *E. coli* and *Lactococcus lactis*. A system for caffeic acid production was developed by Haslinger and Prather (2020) by expressing TAL from *F. johnsoniae*, which performed better at low L-tyr concentrations, and CYP199A2 from *R. palustris* altering the redox partners to increase the efficiency of the cytochrome P450.

Using this strategy, the pathway performance was enhanced under low L-tyr conditions, which allowed de novo production of caffeic acid with glucose as the only carbon source in an otherwise wild type *E. coli* without supplementing L-tyr. For their part, Lin and Yan (2012) used a dual pathway to convert tyrosine to caffeic acid consisting of the enzymes TAL from *Rhodobacter capsulatus* and an *E. coli* native hydroxylase complex (i.e., 4-hydroxyphenylacetate 3-hydroxylase, 4HPA3H). Through this heterologous pathway, they avoided the use of the cytochrome P450-dependent monooxygenases C4H and C3H difficult to be functionally expressed in prokaryotic systems.

Bacterial platforms have also been used for the biosynthesis of hydroxybenzoic acids. A *Corynebacterium glutamicum* strain was constructed by Kallscheuer and Marienhagen (2018) for the production of hydroxybenzoates derived from chorismate. In this microorganism, the existence of a catabolic network for aromatic molecules prevents the microbial production of aromatic compounds other than aromatic amino acids. To allow formation of hydroxybenzoates, the genes involved in the catabolism of aromatic compounds were deleted. Further, the carbon flux into the shikimate pathway was increased by manipulation of the glucose transport and key enzymatic activities of the central carbon metabolism. Allosteric inhibition of DAHP synthase by L-tyr was avoided by introduction of tyrosine-insensitive DAHP synthases from *E. coli* (aroH or aroF*) in combination with a second gene (ubiC) coding for an enzyme capable of converting shikimate pathway intermediates to the desired hydroxybenzoate. The obtained strain (*C. glutamicum* DelAro^5) allowed efficient production of 2-hydroxybenzoic (salicylic), 3-hydroxybenzoic, 4-hydroxybenzoic, and protocatechuic acids. Microbial production of protocatechuic acid was also achieved by Okai et al. (2017) from ferulic acid by expressing the vanillate *O*-demethylase gene (vanAB) from *Corynebacterium efficiens* in *C. glutamicum*.

Further detailed information about phenolic acids production by heterologous microorganisms can be found in several recent reviews (Hernández-Chávez et al. 2019; Gomes et al. 2022; Rainha et al. 2020; Valanciene et al. 2020; Vargas-Tah and Gosset 2015).

3.6 Concluding Remarks and Prospects

Plant phenolic acids are recognized to possess a range of noteworthy biological activities that are thought to contribute to the health-promoting effects associated to the consumption of plant-derived foods. Their antioxidant, anti-inflammatory, or antimicrobial properties have had them interesting for food, cosmetic, and pharmaceutical companies, so that there is an increasing research and industrial demand for these compounds. In order to fulfill these needs, an efficient production of pure compounds is required, so that they can be available for adequately assessing their biological activity and effects, and sufficient amounts can be delivered to the

industry for their applications as nutraceutical, cosmeceutical, or functional food ingredients.

Over the last decades, relevant advances have been made in the knowledge of the biosynthetic pathways involved in the formation of the distinct phenolic classes including phenolic acids, and many approaches have been developed to enhance their production or address it toward particular compounds, such as processes of plant selection, tissue culture, fermentation, or construction of heterologous plant or microbial platforms. However, despite improvements in plant optimization and breeding, plants are subject to cultivation factors and production outcomes are difficult to predict and standardize. Although plant tissue cultures allow more control and may overcome some of these shortcomings, they also show problems associated with low and variable product yields, largely influenced by the type of culture, e.g., differentiated (shoots or roots) or non-differentiated cells (callus or suspensions), their instability over time, susceptibility to several stresses, or formation of aggregates limiting oxygen and nutrient diffusion (Wilson and Roberts 2012). Critical aspects for obtaining high yields in bioreactors are the application of adequate selection of process parameters, medium optimization, precursor feeding, or elicitation (Marchev et al. 2020).

Plant engineering constitutes an attractive alternative to increase phenolic acids production. However, plants are difficult to manipulate genetically, and challenges are even higher when the interest is focused on particular compounds and not in the production of a phenolic pool. In this respect, heterologous microorganisms incorporating specialized genes from plants or other microorganisms represent a promising approach. Genetic manipulation is much easier in microorganisms than in plants and, as the polyphenol pathway has to be reconstructed, more straightforward routes to target compounds can be designed avoiding diversion of substrates through competing pathways of secondary metabolism. Microbes also possess the advantages of a rapid growth and the possibility of growing in inexpensive carbon sources. Nonetheless, pathways for phenolic biosynthesis are not always easy to reproduce in microorganisms. Plant enzymes may not work efficiently in microorganisms and gene introduction may entail errors and sequence mismatch at DNA assembly, such as long repeat sequence and hairpin loop structure, which hinder the precise construction of biosynthetic pathways (Chen et al. 2020). Novel technologies have become available as powerful tools for site-specific genome modification, such as the Clustered Regularly Interspaced Short Palindromic Repeats associated caspase 9 endonuclease system (CRISPR/Cas9), which can be expected to break ground for large-scale genome editing and thus provide more rapid developments in plants and microbes genome engineering in the near future (Yuan and Grotewold 2015). Actually, CRISPR/Cas9 approaches for genome engineering of plant secondary metabolism have already been applied in plant in vitro systems (Marchev et al. 2020). The use of these innovative genomic tools together with the advances in computational omics also enables the construction of microbial chassis increasingly adapted to the synthesis of phenolic compounds and other groups of secondary metabolites. Nevertheless, there are still many challenges to overcome before

profitable industrial processes for phenolic acid production using microbial cell factories become a reality (Rainha et al. 2020).

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Chapter 4
Flavonoids

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Abstract Flavonoids are secondary metabolites found in fruit, vegetables, grains, beverages (tea and wine), and also in food by-products. Food by-products have been recognised as significant sources of flavonoids that have been undervalued. Several studies identified that by-products flavonoids content is frequently higher than in the raw source material. Flavonoids stand out by their diversity in chemical structure, activities, and potential application in industry. Flavonoids have been reported as antioxidant, antibacterial, antiviral, anti-inflammatory, antihypertensive, and antihyperglycemic agents in few clinical trials but in several in vitro and in vivo tests. The cardioprotective and antidiabetic activities of flavonoids are well-documented, being valuable beneficial effects in disease prevention and treatment with applicability in the pharmaceutical and nutraceutical industry. Antioxidant and antimicrobial activity of flavonoids were also applied in food preservation, and the food industry has also been exploring flavonoids as potential natural colouring agents. Cosmetic is another industry where flavonoids have been gaining importance due to their antioxidant, anti-inflammatory, and antimicrobial activity, but also to their UV-protecting and even wound healing properties.

4.1 Introduction

Plants synthesise a wide variety of secondary metabolites. These compounds are not essential for plant survival, but they have vital functions related to the plant–environment interaction acting as pigments or protective compounds. Among these secondary metabolites, the phenolic compounds are the major class in diversity and bioactivity (Kaleem and Ahmad 2018). Phenolic compounds have at least one phenol group in their structure and are generated in plants' pentose phosphate, shikimate, and phenylpropanoid pathways. According to the number of phenol

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rings and the structural elements that attach these rings, phenolic compounds can be divided into five principal classes: flavonoids, phenolic acids, tannins, stilbenes, and lignans (Carvalho et al. 2021). Between phenolic compounds, flavonoids stand out as one of the largest groups of secondary plant metabolites, present almost in all parts of the plants. Many flavonoid compounds have been already identified (about 8000) (Mutha et al. 2021). The structure of flavonoids is also vastly variable, and consequently, these compounds exhibit various functions and roles in plant metabolism. Flavonoids are primarily responsible for plants' colour, fragrance, and taste (including fruits, flowers, and seeds). Besides that, flavonoids have a fundamental role in protecting plants against insects and herbivores by altering the palatability of the plants, reducing their nutritive value, decreasing digestibility, or even acting as toxins (Mutha et al. 2021; Mierziak et al. 2014). Furthermore, many studies attributed to flavonoids a wide range of biological activities and health-promoting effects explored in human nutrition and tested in other applications, namely as food ingredients, nutraceuticals, pharmaceutical and cosmetic formulations, with promising results (Mutha et al. 2021; Mierziak et al. 2014; Ruiz-Cruz et al. 2017a).

In conclusion, flavonoids have attracted much attention due to their wide range of biological and health-promoting activities. Consequently, many studies have described the flavonoids' activities and mechanisms of action concerning human health, extraction, and quantification in plants and foods. The increasing importance of accomplishing sustainable agro-food economy systems has led to the intensified exploration of agro-food by-products to extract flavonoids using conventional and emergent technologies. These flavonoids extracted from by-products have been incorporated in several products to increase their biological and health benefits, and consequently, new applications have been rising. This chapter focused on recording the primary plant and agro-food by-products sources of flavonoids, enumerating and explaining flavonoids' principal biological and health-promoting activities with application in various industries.

4.2 Flavonoids Sources: From Plants to Foods and Their By-Products

Flavonoids are an important class of secondary metabolites with more than 10,000 compounds identified, widely found in fruit, vegetables, grains, and certain beverages, such as tea and wine (Panche et al. 2016; Hossain et al. 2016). They are reported as the most abundant plant pigments and carotenoids, and chlorophylls, providing fragrance and taste, making them attractants for other organisms (Ruiz-Cruz et al. 2017b). Besides their relevance in plants defence systems, flavonoids are important for human health because of their broad spectrum of health-promoting activities reported in several clinical and research studies (Ullah et al. 2020).

Overall, flavonoids are composed of a 15-carbon (C6-C3-C6) skeleton and two benzene rings joined by a linear 3-carbon chain (Hossain et al. 2016). They can be

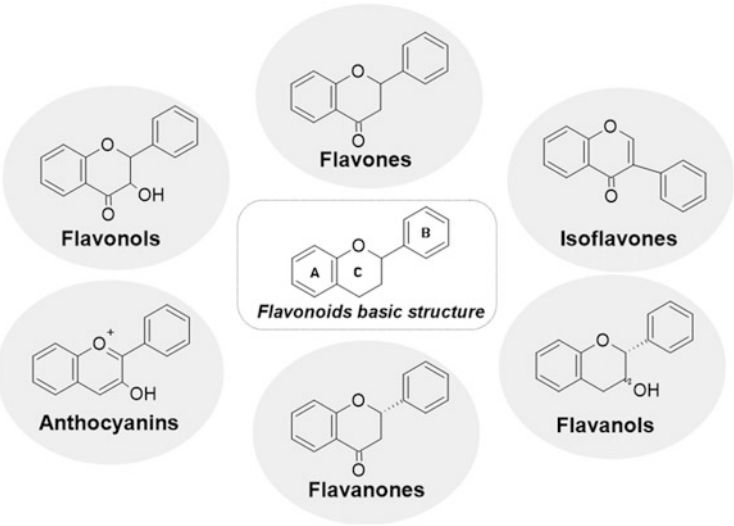

Fig. 4.1 Flavanoid basic structure and general structures of the main flavonoids (Structures and drawing developed in ACD/ChemSketch)

divided into six classes depending on the carbon of the C ring on which the B ring is attached and the degree of unsaturation and oxidation of the C ring (Fig. 4.1). These groups include flavonols (quercetin, kaempferol, and rutin), flavanols (catechin and epicatechin), flavones (apigenin and luteolin), isoflavones (daidzein and genistein), flavanones (hesperidin and naringenin), and anthocyanidins (malvidin, petunidin, and pelargonidin) (Skarpalezos and Detsi 2019). They diverge in the structure around the heterocyclic oxygen ring, but all have the characteristic C6-C3-C6 skeleton. Nevertheless, their functions depend on their structural class, degree of hydroxylation, conjugation, and degree of polymerisation.

The flavonoids content in food depends on the source (vegetable, fruit, cereal, seed), as well as the process used and the type of matrix (e.g. juice, milk, meat, etc.). The content of some important flavonoids in spinach, carrots, peas, cabbage, peaches, strawberries, orange juice, white wine, and brewed coffee is less than 10 mg/kg or mg/L. However, in other vegetables and fruits, such as beans, red pepper, tomato, lettuce, cherries, tea, and red wine, their content is about 50 mg/Kg or mg/L. On the other hand, the flavonoid content in broccoli, kale, French beans, celery, and cranberries is higher than 50 mg/kg or mg/L (Ruiz-Cruz et al. 2017b) (Table 4.1). Nevertheless, the main dietary sources of flavonoids in eastern and western societies include tea, citrus fruit, citrus juices, berries, red wine, apples, soybeans, onions, berries, and leafy vegetables. For instance, apple contains flavonoids such as quercetin (92 mg/100 g) and epicatechin (90 mg/100 g) (Bondonno et al. 2012). Oranges contain as main phenolic compound hesperidin, and orange juice contains 28.9 mg/100 mL juice of this molecule (Bellavite and Donzelli 2020). Berry fruits contain high levels of anthocyanins in their flesh and skin; strawberries

Table 4.1 The major food sources of flavonoids selected by classes

Flavonoid group	Example compounds	Main food sources
Flavonols	Myricetin	Tomatoes, orange, red wine
	Fisetin	Strawberry
	Quercetin	Onions, asparagus, green peppers, green tea, tomatoes
	Kaempferol	Spinach, kale
	Isorhamnetin	Parsley
Flavanols	Catechins	Red wine, broad beans, black grapes, apricots, strawberries
	Epigallocatechin gallate	Green, black, and white tea
	Epicatechin	Apples, blackberries, cherries, pears, raspberries, chocolate
	Procyanidin	Berries (blueberries, cranberries, and black currant), plums, avocado seeds
Flavones	Apigenin	Cereals, fruits, parsley, thyme, bee pollen, passiflora plant
	Luteolin	Celery, parsley, broccoli, cabbages, apple skins
	Chrysin	Honey, propolis
Isoflavones	Genistein	Soy-based foods
	Daidzein	Soy-based foods, peas, lentils
Flavanones	Hesperidin	Citrus fruits, curcumin, oranges, grapefruits, peppermint, lemon
	Eriodictyol	Lemon, orange
	Naringenin	Citrus fruits, tomatoes
	Narirutin	Orange, onions, broccoli, and blueberries
Anthocyanins	Cyanidin, delphinidin, malvidin, pelargonidin, petunidin, peonidin	Blueberries, strawberries, red wine, cranberries, blackcurrants

contain 33.63 mg/100 g, whereas blueberries contain 13.52 mg/100 g (Basu et al. 2010). Onions have up to 22 mg of quercetin/100 g (Nishimura et al. 2019). Tea was identified as the most important source of flavanols and flavonols, contributing 157 mg of daily flavonoid intake (Song and Chun 2008). In addition, kombucha, a fermented tea beverage prepared with bacterial cultures and yeast, is highly consumed nowadays and shows a total content of flavonoids between 111 and 180, depending on the tea type used (white, green, or black) (Jakubczyk et al. 2020).

The human food intake of flavonoids varies significantly by geographical region due to different dietary patterns (Escobar-Cévoli et al. 2017). According to several epidemiological studies, the worldwide mean intake of flavonoids ranges between 150 and 600 mg/day (expressed as aglycones form) (Johannot and Somerset 2006; Zamora-Ros et al. 2013; Chun et al. 2007; Zamora-Ros et al. 2018; Zhang et al. 2014; Jun et al. 2016; Zamora-Ros et al. 2016). However, Europe has the most number of studies assessing the intake of total flavonoids. Therefore, it is feasible to describe their intake rather accurately. In non-Mediterranean countries, the total

intake of flavonoids (350–600 mg/day) is higher than in Mediterranean countries (250–400 mg/day), even though their high intake of fruits, vegetables, and red wine. This is due to the much higher tea consumption in the last ones. The United Kingdom showed the highest flavonoid intake in Europe countries (1000 mg/day) due to the traditional tea culture consumption (Zamora-Ros et al. 2016). Countries from Eastern Europe, such as Poland, also showed a high intake of flavonoids (about 800 mg/day) related to their extraordinary amount of tea consumption (Grosso et al. 2014). In Scandinavian countries, such as Finland, the consumption of tea and fruit is deficient; therefore, the intake of flavonoids is lower than in Mediterranean countries (~200 mg/day); however, they have a higher intake of phenolic acids (Ovaskainen et al. 2008). In the USA, the total mean intake of flavonoids ranges from 250 to 400 mg/day. Although tea consumption is low, it is still the main food source of flavonoids because the consumption of fruits and vegetables is even lower. Data on other countries in the American continent is very limited. There are only information about Brazil and Mexico, where the total intake of flavonoids is approximately 150 and 50 mg/day, respectively. These are the lowest intake of flavonoids worldwide and are highly associated with the fact that society does not consume tea. The main food source of flavonoids is citrus juices, followed by fresh fruit consumption in Mexico and beans in brazil. In China, the total flavonoid intake range in total 225 mg/day, associated with the consumption of green tea. In other East Asian countries, soy and soy products (rich in isoflavones) are one of the most significant source of flavonoids. While, a recent study in Iran (Middle East) has shown a mean intake of 1650 mg of flavonoids per day. This is the highest mean worldwide, associated with the eminent consumption of black tea in these populations.

It is well-known that flavonoids consumption enhances human health; therefore, the food, nutraceutical, and pharmaceutical industry have been increasing the search for new sources of flavonoids to provide supplements rich in flavonoids, mainly for the countries where the dietary patterns do not provide a higher intake of these bioactive compounds (Ayala-Fuentes and Chavez-Santoscoy 2021). Flavonoid compounds are found in different parts of the plants. Usually, the societies use just the edible piece of the fruit or vegetables; therefore, stay large amounts of flavonoid content in the non-edible parts (Panche et al. 2016; Vilas-Boas et al. 2021).

In fruit and vegetable production, the food losses and waste reach almost 50% of the total production and, in most cases, have a higher content of nutritional and functional compounds than the fresh or final product. Tomatoes, onion, cucumbers, cabbage, and carrot are the most produced and consumed vegetables. Bananas, watermelons, apples, mangos, oranges, and grapes are the fruit with more worldwide production; therefore, they generate more by-products. Likewise, the growing popularity of tea, coffee, fruit juice, frozen fruit/vegetables, and minimally processed products has also increased the production of by-products and wastes in recent years (Fernandes et al. 2019). Apple is one of the most consumed fruit worldwide, and a huge amount of the production is used for the juice and cider process. This industrial processing generates about 4 million tons per year of apple pomace (Fernandes et al. 2019). This by-product represents one of the best sources of flavanols (catechin and

epicatechin) and flavonols (quercetin) (Rana et al. 2021). Recent studies of Fernandes et al. (Fernandes et al. 2019) showed a total amount of 31.37 ± 0.32 mg/g dry weight (DW) of quercetin-3-O-galactoside and 26.05 ± 0.27 mg/g DW of quercetin-3-O-rhamnoside in apple pomace. Orange peels, a by-product from orange juice production, represent a rich source of flavonols (rutin and quercetin), flavones (diosmin and tangeretin) and also flavanones (hesperidin, narirutin, and naringenin) (Singh et al. 2020). Similarly to fruit and other vegetables, cauliflower and broccoli production has increased substantially over the past decades, as well as its use in the processed products, producing huge quantities of by-products (leaves and stem). For instance, cauliflower has the highest waste index of non-edible to edible portions and represents an important source of flavonols, mainly kaempferol and quercetin (Soengas et al. 2012).

Hence, in the last years, the research papers focusing on evaluating the amount of flavonoids present in these kinds of by-products increased to find other natural sources of these compounds. However, part of them is usually not accessible in the plant tissues and is embedded within the plant cellular matrix, so some extractive methodologies are required for their recovery. Besides extracting the added-value molecules of interest, extraction aims to maximise the yields while minimising the extraction of undesirable compounds.

4.2.1 Flavonols

Flavonols are the largest among all flavonoid classes, with more than 6000 identified compounds (Dias et al. 2021). They occur abundantly in various fruits and vegetables, and the most studied are kaempferol, quercetin, myricetin, and fisetin. Kaempferol is a major flavonol found in beans, broccoli, cabbage, garlic, fennel, and chia seeds (Imran et al. 2019). However, quercetin is contained mainly in blueberries, onion, red grapes, cherries, green leafy vegetables, citrus fruits, apples, and honey (Guven et al. 2019).

Kale, a leafy green vegetable belonging to the Brassicaceae family, showed thirty-two phenolic compounds, including quercetin and kaempferol glycosides. The total flavonol content in kale (determined as rutin equivalents) was 646 mg of RE/100 g FW (fresh weight). The contents of quercetin and kaempferol range from 44 and 58 mg/100 g FW, respectively (Olsen et al. 2009). Grape is one of the world's most important fruit crops, mainly due to a wide range of dietary products deriving from it, such as fresh fruit and wine. During winemaking, only a small part of phytochemicals is transported to wine; therefore, large quantities remain in pomace. This by-product contains pressed grape wastes (e.g. seeds, skin, stems). Depending on the grape variety the quantities of flavonols content could vary: 9.39–78.9 mg/kg FW of quercetin; 6.42–72.9 mg/kg FW of isorhamnetin; 0.11–8.19 mg/kg FW of rutin; 0.99–3.99 mg/kg FW of quercitrin; 2.45–53.1 mg/kg FW of kaempferol, and 73.9–143 mg/kg FW of myricetin (Pintać et al. 2018). Furthermore, onion and garlic contain high amounts of flavonols, mainly quercetin, which contribute to the high

intake of flavonoids in the diet. However, onion processing generates copious waste, mainly skin. For example, the European Union (mainly Spain, the Netherlands, and UK) discard 500,000 tons of onion waste per year, which has created an environmental issue. Depending on the cultivar, the total flavonoid content ranged from 1.31 to168.77 mg QE/g DW. Main onion flavonoids are quercetin, quercetin derivatives, and kaempferol (Sagar et al. 2020). Apart from fruit and vegetables, cocoa, green tea, and red wine are also great sources of flavonols. Green tea presents 20.09 mg/g of myricetin, 45.93 mg/g of quercetin, and 40.62 mg/g of kaempferol. Whereas, in red wine, the amount of myricetin and quercetin ranges from 0 to14.6 mg/L and 1.2 to 19.4 mg L, respectively.

4.2.2 Flavanols

Flavanols are the most consumed flavonoid class worldwide, contributing to more than 80% of the total flavonoids consumption (Escobar-Cévoli et al. 2017). The most common flavonols are (+)-catechin, (−)-epicatechin, (−)-epigallocatechin, and procyanidins (B2, B1 and C1). The major food sources of flavanols include apples, bananas, tea, blueberries, peach, beer, and pears.

The edible part of the banana represents only 12% of the total banana weight, generating a large amount of waste such as peel. The flavanols were the largest group of phenolics found in the banana peel. Depending on the variety, these by-products showed about 1.34 (+)-catechin, 9.94 (−)-epicatechin, and gallocatechin 8.59 mg/100 g (Vu et al. 2018).

Tea is the only beverage that contains gallocatechin, epigallocatechin, epicatechin gallate, and epigallocatechin gallate, in addition to catechin and epicatechin (Yao et al. 2004).

Avocado consumption (fresh or processed products) has become increasingly famous due to its high nutritious value with health benefits (Araújo et al. 2018). Therefore, large amounts of by-products (peel and seed) are generated, mainly disposed of as non-valuable waste. The most representative flavonoids in avocado peels and seeds are procyanidin B2, (−)-epicatechin, and (+)-catechin.

4.2.3 Flavones

Flavones are structurally similar to flavonols; however, they have an additional hydroxyl group at the carbon 3-position (Guven et al. 2019). These groups of flavonoids are widely present in leaves, flowers, and fruits as glucosides and are the primary pigment in cream-coloured flowers and co-pigments in blue flowers and are also commonly found in the leaves and fruiting bodies of plants (Panche et al. 2016). Green and black olives, chamomile, *Ginkgo biloba*, parsley, red peppers, celery, and thyme are among the major sources of flavones. The most known

flavones include luteolin and apigenin (Guven et al. 2019). Moreover, orange peels are rich in a particular type of flavones, polymethoxylated flavones, tangeretin, nobiletin, and sinensetin (Singh et al. 2020). In addition, this by-product presents also significant amounts of diosmin (47.78 mg/g DW).

Tea also represents an excellent source of flavones intake. For instance, chamomile tea provides about 106 mg of apigenin/serving and 1 mg of luteolin (Carnat et al. 2004), while green tea provides between 1 and 5 mg of apigenin/serving and less than 1 mg of luteolin (Engelhardt et al. 1993). On the other hand, orange juice provides 20 mg of apigenin, 1 mg of luteolin, and 1 mg of diosmetin per 250 mL (Mullen et al. 2007). Regarding vegetables, parsley undoubtedly has the highest values of luteolin and apigenin, 1484 and 22 mg/100 g FW (Hostetler et al. 2017), respectively. Black olives showed between 3.2 up to 17.5 mg of luteolin/100 g FW, while green olives showed about 0.2–1.2 mg of luteolin/100 g Fw and olive oil less than 1 mg/100 g FW (Hostetler et al. 2017). The olive oil industry is one of the most polluting agro-food industries, producing the commonly known olive pomace (Ribeiro et al. 2021). Nevertheless, this by-product contains a substantial amount of phenolic compounds, particularly flavones. Ribeiro et al. (2020) identified luteolin-7-O-glucoside, luteolin, and apigenin in crude olive pomace from two different olive mills in Portugal's Inner Centre Region; however, only luteolin was quantified (18.40 ± 0.51 mg/100 g DW). After that, the same authors developed powdered ingredients without any extraction, which revealed a more viable and low-environmental effect approach than the traditional and emerging technologies involving using organic solvents or higher operational costs. Other interesting food sources of flavones are millet, wheat grain, and rice. The cereal with more luteolin and apigenin are millet, 35 and 15 mg/100 g DW, respectively. However, celery, parsley, and chicory showed higher levels than cereals (Hostetler et al. 2017).

4.2.4 Flavanones

Flavanones are another important class widely distributed in about 42 higher plant families, especially in Compositae, Leguminosae, and Rutaceae (Khan and Dangles 2014). The highest concentration of flavanones is found in the peel of citrus fruit, such as orange, lemon, grapefruit, and bergamot and is responsible for the bitter taste of the juice and peel of citrus fruits (Singh et al. 2020).

Among flavanones, naringenin and hesperidin are the most important and common because of their high prevalence in foods (Guven et al. 2019). Naringenin is mainly present in grapefruit, sour orange, and tomatoes, which can be present in both aglycone and glycoside form. Also, sweet orange, lemon, and lime have naringenin but lower concentrations. Hesperidin is the principal flavanone in sweet oranges, lemons, limes, and tangerine. For instance, the by-products from these raw materials also showed high amounts of hesperidin (del Carmen Razola-Díaz et al. 2021). Generally, the daily intake of flavanones in the Europe population was found to be,

on average, 25.7 ± 27.1 mg, which is most often consumed in fruits (72.0%), juices (17.2%), wine (5.4%), and soft drinks (1.7%) (Stevens et al. 2019).

4.2.5 *Isoflavones*

Isoflavonoids have a limited spreading in the plant kingdom and are mostly present in legumes, particularly soybean. In addition, green split peas, chickpeas, black beans, lima beans, clover sprouts, and sunflower seeds also contain isoflavones (Guven et al. 2019). The most important dietary isoflavones are genistein and daidzein (Panche et al. 2016). These compounds are the flavonoid class with the lowest intake (Escobar-Cévoli et al. 2017). According to the extraction method used, a massive amount of soybean is used for vegetable oil production or soya milk, which generates by-products called flour (or expeller soybean meal) and okara, respectively. Standing out is its isoflavones concentration; recent studies demonstrate that applying a hydroalcoholic extraction under subcritical conditions made it possible to obtain extracts with a high flavonoid content (7.34 mg QCE/g by-product) with a good antioxidant capacity (Rodríguez-Ruiz et al. 2022).

4.2.6 *Anthocyanins*

Anthocyanins are pigments responsible for the colours red, purple, and blue in plants, flowers, and fruits. In the USA, the average consumption of anthocyanins is 9 mg/day, and the primary sources of anthocyanins include berries (39%), red wine (18%), banana (12%), vegetables (9%), and other kinds of fruits (9%). Although in Europe, the average anthocyanins consumption is higher, corresponding to 19 mg/day, whereas the main sources of anthocyanins are almost the same: berries (43%), red wine (22%), and pome fruits (19%) (Khoo et al. 2017; Wallace and Giusti 2015).

The most commonly studied anthocyanins are malvidin, delphinidin, cyanidin, pelargonidin, and peonidin. Among the plants especially rich in anthocyanins are: acai berries, black beans, red grapes, blackberries, raspberries, black rice, blueberries, pomegranate, red cabbage, and red onions, cherries, tomatoes. In addition, there are several non-edible fruits and/or vegetable parts rich in anthocyanins, such as the skin of the apple, eggplant, and plums. And also, the red grape pomace is the major by-product generated in the winemaking process, with 23 million tons discarded every year only in Europe (Coelho et al. 2020). For instance, Coelho et al. (2021) applied ohmic heating (OH) for the first time and combined water with citric acid to recover anthocyanins from grape pomace, namely. The authors obtained an extract rich in malvidin-3-O-glucoside (125.94 ± 1.25 μg/g DW), delphinidin3-O-glucoside (36.77 ± 0.81 μg/g DW), and petunidin-3-O-glucoside (27.46 ± 0.92 μg/g DW). On the other hand, a syrah grape pomace extract obtained

by enzymatic extraction showed that its encapsulation protected anthocyanins throughout the gastrointestinal tract, principally the petunidin-3-O-glucoside (Costa et al. 2021). Still, in the context of the by-products valorisation, works from Condurache et al. (2021) intended to use eggplant peels as a natural source of anthocyanins with biological activities. Therefore, the authors applied an ultrasound-assisted extraction method and obtained an extract rich in five different anthocyanins: delphinidin 3-O-rutinoside-5-glucoside, delphinidin 3-O-glucoside, delphinidin 3-O-rutinoside, cyanidin 3-O-rutinoside, and petunidin 3-O-rutinoside. The anthocyanin with the highest concentration from each extract was delphinidin 3-O-rutinoside. On the other hand, the red/purple onion bulb presents about 63% (dry weight) of anthocyanins, which means that only 27% of the total anthocyanins will be consumed because the bulb is peeling (Rodrigues et al. 2017).

4.3 Biological Functions and Health Benefits of Flavonoids Through Science to Industry Application

Health consumers' consciousness has been increasing, driving the attention of researchers and the food industry towards natural and bioactive compounds and their incorporation into foods and nutraceutical formulations. Flavonoids are among the most important secondary metabolites of plants that could be extracted from several sources, as reported before in this chapter. Food by-products have been highlighted among flavonoids sources, owing to their richness in flavonoids. Besides that, food by-products are available in large amounts due to their overproduction by the food industry. The valorisation of by-products into functional ingredients to increase the nutritional content of food or its health-promoting benefits or even its shelf-life has been explored as a suitable strategy for food by-products management (Gómez-García et al. 2021; Coderoni and Perito 2020). Flavonoids are one of the compounds most used to attain these new value-added products due to their wide range of biological and health-promoting effects has been intensively explored (Kaleem and Ahmad 2018; Ballard and Maróstica 2019). Furthermore, flavonoids have been studied to adjust food products' organoleptic, sensory, and conservation attributes (Carpena et al. 2021). And so forth, flavonoids have been employed in the formulations of several functional products, mainly food products and nutraceuticals, and drug and even feed/pet food formulations (Ruiz-Cruz et al. 2017a).

Flavonoids have complex and unknown biologic functions based on their chemical and structural characteristics. Among these specific activities of flavonoids, a considerable emphasis is given to their (Kaleem and Ahmad 2018; Ballard and Maróstica 2019):

1. Antioxidant activity by different mechanisms.
2. Affinity to estrogenic receptors.
3. Anti-inflammatory activity.

4. Impact on the cardiovascular system.
5. Influence on tissue signal transmission and regulatory systems.
6. Microorganism growth inhibition.
7. Interaction with enzymes.
8. Interaction with transcription factors.
9. Interaction with receptors.

All these specific activities are ascribed to flavonoid action and have attracted attention due to their action as food preservatives and the capacity to decrease the incidence of many diseases. In the next section, some of the more relevant biological and health-promoting activities of flavonoids reported in the literature will be overviewed.

4.3.1 Antioxidant Activity

Flavonoids have been named as natural antioxidants with higher activity based on their ability to scavenge free radicals and active oxygen species (Tiwari and Husain 2017; D'Amelia et al. 2018; Karak 2019; Ciumărnean et al. 2020). The antioxidant activity of flavonoids depends on their structure, mainly in the arrangement of functional groups. They contain conjugated ring structures and hydroxyl groups that have the antioxidant potential in vitro or cell-free systems by scavenging singlet oxygen, superoxide anion, lipid peroxy radicals, and stabilising free radicals involved in oxidative processes through hydrogenation or complexing with oxidising species (D'Amelia et al. 2018). The number of hydroxyl groups, configuration, and substitution greatly influence several antioxidant mechanisms such as radical scavenging and metal ion chelation ability (Pandey et al. 2012; Catapano et al. 2017). Some of the effects mediated by flavonoids may be the combined result of radical scavenging activity and the interaction with enzyme functions. Flavonoids inhibit the enzymes involved in ROS generation: glutathione S-transferase, microsomal monooxygenase, NADH oxidase, mitochondrial succinoxidase, xanthine oxidase, lipoxygenase, and so forth, avoiding thus the formation of reactive oxygen species and organic hydroperoxides (Kumar and Pandey 2013; Nile et al. 2016).

Flavonoids are quite effective against lipid peroxidation, which is a common consequence of the oxidative process. They act by protecting lipids against oxidative damage by several mechanisms (Kumar and Pandey 2013). Quercetin, in particular, is known for its iron-chelating and iron-stabilising properties (D'Amelia et al. 2018; Terao 2017).

Many studies have already proven the antioxidant activity of flavonoids. A study evaluating the inhibitory activity of linoleic acid peroxidation, xanthine oxidase and scavenging capacity of DPPH with some flavonoids showed that for linoleic acid peroxidation, quercetin showed the highest inhibition (82%), followed by catechin (71%). For xanthine oxidase, morin, catechin, quercetin, and naringenin showed 100% of inhibition, and for DPPH, catechin, morin and quercetin obtained activity

100% when tested at a concentration of 100 μg/ml (Choi et al. 2002). The flavonoids such as quercetin, luteolin, isorhamnetin, rhamnetin, and apigenin, e.g., were studied, and their antioxidant activity was compared with DPPH radical. Overall, quercetin showed the highest antioxidant activity at a lower concentration (0.1–5 μg/sample) (Majewska et al. 2011).

In recent years, several restrictions have been imposed on using synthetic antioxidants, such as the case of propyl gallate, butylated hydroxyanisole (BHA), and butylated hydroxytoluene (BHT); in consequence, natural flavonoids have gained a lot of prominence. Nevertheless, they have been extracted and used in the food industry instead of synthetic compounds due to their capacity to retard lipid oxidation, improving the nutrition and quality of food while reducing toxicity (Ruiz-Cruz et al. 2017a; Pateiro et al. 2018; Mbah et al. 2019; Leyva-Porras et al. 2021).

The protective effect on meat quality and other products by these antioxidant compounds was indicated by several studies (Kumar et al. 2015; Papuc et al. 2017; Reddy et al. 2018; Mitterer-Daltoé et al. 2021). The most studied applications of flavonoids in foods are in red meats and poultry to inhibit lipid oxidation, retard spoilage microorganism growth in meats, and use as functional ingredients (Kaleem and Ahmad 2018).

Maqsood et al. (2015) used some natural antioxidant compounds to evaluate the antioxidant activity during 9 d of refrigeration after applying 200 ppm of catechin in ground camel meat (Maqsood et al. 2015). They found that lipid peroxidation was retarded, and catechin inhibited the oxidative degradation of proteins. A study evaluated the antioxidant capacity of catechins present in tea and grape seeds extracts, compared to BHA, carnosine, and vitamin E in raw beef patties during 8 days of storage at 4 °C and found that the antioxidant treatment significantly inhibited discolouration and lipid oxidation. The lower redness value was found in BHA, and the highest reducing activity was reported in the patties treated with grape seed extract (Liu et al. 2015).

In addition, the quercetin and quercetin + α-tocopherol antioxidant properties were studied in chicken meat. Sohaib et al. (2017) found that the addition of quercetin dihydrate alone and combined with α-tocopherol improved oxidative stability, total carbonyls, and volatile off-flavour compounds in treated chicken patties (Sohaib et al. 2017). The compounds such as catechins, epicatechins, epigallocatechin, epigallocatechin gallate, and epicatechin gallate presented great affinity for lipid bilayers of muscle and the radical scavenging activity, which prevent lipid oxidation, and also improve the antibacterial action (Goto et al. 1999; Higdon and Frei 2010). The incorporation of quercetin at 100 mg/kg meat with α-tocopherol at 100 and 200 mg/kg meat delayed the protein and oxidation of cooked meat products. Furthermore, the supplementation with quercetin also reduced the aldehydes volatiles, particularly hexanal and pentanal, considered a significant index to judge the shelf-life of cooked meat products. The oxidation of lipids in chicken pate was evaluated in the presence of quercetin and BHT, and quercetin was eight times more efficient in preventing lipid oxidative reactions than BHT (de Carli et al. 2018). Hernández-Hernández et al. (2019), in a study with cocoa bean husk, showed that this by-product is an important source of flavonoids,

including epicatechin and catechin, suitable for applications in the food, cosmetic, and nutraceutical industries (Hernández-Hernández et al. 2019).

4.3.2 Antimicrobial Activity

Several research studies attribute antimicrobial capacity to flavonoids, and much research is aimed to isolate and recognise the structures of flavonoids with antiviral, antibacterial, and antifungal capacity.

4.3.2.1 Antibacterial Activity

Several flavonoids act both as bactericidal and bacteriostatic agents by damaging the cytoplasmic membrane and inhibiting the nucleic acid synthesis and energy metabolism of microorganisms (Ahmad et al. 2015). In particular, the antibacterial action mechanism of the flavonoids might have various cellular targets rather than one specific site of action that may be related to their ability to inactivate microbial adhesins, enzymes, cell envelope transport proteins, and so forth (Kumar and Pandey 2013; Tagousop et al. 2018). Lipophilic flavonoids may also disrupt bacterial membranes, reducing the fluidity of outer and inner layers and inactivating cells (Mickymaray et al. 2020).

Different studies have reported that plant extracts rich in flavonoids from different origins have antibacterial activity against pathogenic and food spoilage bacteria (Papuc et al. 2017; Li and Xu 2008; Mishra et al. 2013; Satish et al. 2018). Flavonoids such as galangin, flavone and flavonol glycosides, apigenin, chalcones, isoflavones, flavanones, and catechin have been shown a powerful antibacterial activity (Tiwari and Husain 2017; Karak 2019).

A study using broth microdilution assay of some flavonoids, e.g., kaempferol, kaempferol-3-O-(2′′,3′′-di-O-acetyl)-α-L-rhamnopyranoside, and kaempferol-3-O-(3′′,4′′-di-O-acetyl)-α-L-rhamnopyranoside, exhibited remarkable antibacterial activities with MIC values between 62.50 μg/mL and 500 μg/mL against *Klebsiella pneumoniae, Escherichia coli, Bacillus cereus*, and *S. aureus* (Sivasothy et al. 2013). Another study using catechin, kaempferol, genistein, and naringenin isolated from Brassica *oleracea* var. Capitata L. showed that compounds possessed antibacterial activity against *E. coli* and *S. aureus* (Satish et al. 2018). The epicatechin-3-gallate flavonoid from *Euphorbia hirta* has activity against *Pseudomonas aeruginosa* (31.2 μg/mL), and its action mode targeted both the cell wall and cytoplasmic membrane (Perumal et al. 2017). Furthermore, previous studies reported that flavonoids such as kaempferol, quercetin, apigenin, chrysin, naringenin, daidzein, and genistein interfere with bacterial biofilm formation, while the luteolin, myricetin, baicalein, and quercetin inhibit bacterial DNA replication (Ginwala et al. 2019; Jucá et al. 2018; Matilla-Cuenca et al. 2020).

Flavonoid-rich grape seed extracts and green tea containing flavanols such as epicatechin, catechin, epicatechin gallate, procyanidin oligomers, and epigallocatechin were described to control the growth of pathogenic bacteria in meat products. Cooked beef samples treated with 1% grape seed extracts (GSE) significantly reduced *E. coli* O157:H7 and *Salmonella typhimurium* counts and controlled the growth of *Listeria monocytogenes* and *Aeromonas hydrophila* (Ahn et al. 2007). Yoda et al. (2004) determined the antibacterial activity of epigallocatechin-3-gallate on various strains of *Staphylococcus* and Gram-negative bacteria, including *E. coli*, *K. pneumoniae*, and *S. typhi* (Yoda et al. 2004). Catechin also effectively retarded microbial growth of psychrophilic bacterial and mesophilic bacterial in ground camel meat after 9 days of storage (Maqsood et al. 2015).

Flavonoids extracted from agro-industrial by-products have been highlighted for their antimicrobial properties (Kumar et al. 2017; Shirahigue and Ceccato-Antonini 2020; Reguengo et al. 2022). Methanolic extract of acerola bagasse flour rich in compounds such as quercetin, epigallocatechin gallate, catechin, syringic acid, and epicatechin presented bactericidal activity for *L. monocytogenes* ATCC 19117, *E. coli* ATCC 11229, *P. aeruginosa* ATCC 15442, and *S. choleraesuis* ATCC 6539, presenting the potential to be used in the food and cosmetic industry (Marques et al. 2017). Peanut skin ethanolic extract also showed bacteriostatic activity against *L. monocytogenes* and bactericidal activity against *S. aureus* (do Valle Calomeni et al. 2017).

4.3.2.2 Antifungal Activity

The flavonoids exert antifungal activity by several mechanisms, such as induction of several mitochondrial dysfunctions, inhibition of cell wall formation, cell division, RNA and protein synthesis, disruption of the plasma membrane, and the efflux mediates pumping systems (Al Aboody and Mickymaray 2020). Several studies reported the antifungal activity of flavonoids. Serpa et al. (2012) evaluated the in vitro anti-candidal activity of baicalein, a flavone of *Scutellaria baicalensis* found anti-candidal activity against *Candida tropicalis* 170.06, *C. albicans* ATCC 64550, and *C. parapsilosis* 153.07 with the MIC50 of 2.6, 26, and 13 μg/mL, respectively, using a microdilution test (Serpa et al. 2012). Quercetin-3-O-rutinosides had beneficial effects on *C. albicans* and *C. krusei* with MICs of 16 and 32 μg/mL, respectively (Orhan et al. 2010).

Medicinal plants contain several fractions of flavonoids that show antifungal properties. Propolis and its high flavonoid content have antifungal activity against dermatophytes and *Candida* spp. Exclusively, propolis contains a flavonol, galangin, which has been demonstrated to have antifungal activities against *Penicillium digitatum, A. flavus, A. tamarii, Cladosporium sphaerospermum*, and *P. italicum* (Cushnie and Lamb 2005). Sanguiin H-6 and lambertianin C isolated from raspberry (*Rubus idaeus* L.) fruit were reported as antifungal agents against *Geotrichum candidum* (Klewicka et al. 2016). Natural prenyl flavonoid isolated

from *Dalea elegans* was reported to show antifungal effects against *C. albicans* biofilms (Peralta et al. 2015).

Flavonoids extracted from by-products have been strongly reported for their antifungal effects. The extract obtained from grape pomace rich in flavonoids has been reported to inhibit the growth of *Zygosaccharomyces bailii, Zygosaccharomyces rouxii,* and *Botrytis* cinerea (Mendoza et al. 2013; Han 2007). Citrus peel ethanolic extract was found to inhibit A. flavus growth and aflatoxin production effectively in rice during two months of storage (Naseer et al. 2014). The naringin, neohesperidin, and quercetin flavonoids from citrus residues severely affected *A. parasiticus* ultrastructure and aflatoxin reduction. These flavonoids are an excellent alternative strategy to replace chemical additives for stored maize, demonstrating its potential to be applied in the food industry (Pok et al. 2020).

New antifungal agents have been developed based on flavonoids, as the available antifungal agents have not been entirely effective due to the development of undesirable side effects and resistance (Campoy and Adrio 2017). Furthermore, several studies have reported that flavonoids, especially in combination with luconazole, have been shown to display remarkable synergistic antifungal effects, and it has led flavonoids to become an important lead compound in synergistic antifungal drug research and development (Jin 2019).

4.3.2.3 Antiviral Activity

Flavonoids have also been recognised for their antiviral activity. Since the 1940s, many reports have shown that naturally occurring flavonoids exhibit a remarkable antiviral activity. Many researchers have isolated and identified the structures of flavonoids with this property (Alzaabi et al. 2022). The flavonoids work through several mechanisms against viruses. They can act by blocking the attachment and entry of viruses into cells and interfere with various stages of viral replication processes or translation and polyprotein processing to prevent the release of the viruses to infect other cells (Lalani and Poh 2020). Natural bioactive flavonoids present in medicinal plants and herbs have been widely reported to have antiviral activity and are concentrated and modified for their better action (Villa et al. 2017).

Studies reported that flavonoids, such as genistein and ginkgetin, inhibited HIV and influenza A virus assembly and release, respectively. Ginkgetin inhibited the sialidase activity of the influenza A virus and thus inhibited virus assembly and release (Miki et al. 2007), while the genistein inhibited the Vpu protein involved in the formation of ion channels in infected cells and thus controlled the release of HIV (Sauter et al. 2014). Kaempferol was also reported to inhibit HIV replication in target cells (Behbahani et al. 2014) and block herpes simplex virus types 1 and 2 by attaching and entering the host cell (Zakaryan et al. 2017). Wu et al. (2015), who studied some flavonoids' antiviral effects found that quercetin, kaempferol, and epigallocatechin gallate were active against several influenza virus strains (Wu et al. 2016). The baicalin flavonoid was reported to inhibit the *dengue virus (DENV-2)* through a virucidal mechanism. It blocked the attachment of *DENV-2* to

the Vero cells (Moghaddam et al. 2015). Zandi et al. (2011) showed that quercetin also had direct antiviral activity against Dengue virus 2 (DENV-2) (Zandi et al. 2011). The baicalein, quercetin, and fisetin flavonoids were reported as active inhibitors of the chikungunya virus (Lani et al. 2016).

In the last two years, the ongoing COVID-19 pandemic, caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has forced the scientific community to seek alternatives that help fight the virus. In this sense, many studies have investigated the use of natural bioactive flavonoids present in medicinal plants, herbs and flavonoids extracted from agro-industrial by-products and have been widely reported to have antiviral activity against SARS-CoV-2 (Alzaabi et al. 2022; Santana et al. 2021). Several studies strongly highlighted that flavonoids, particularly quercetin, luteolin, myricetin derivatives, baicalein, baicalin, and epigallocatechin gallate, can display promising multi-target action against SARS-CoV-2, which stimulate their use in the current and expected future outbreaks (Alzaabi et al. 2022; Kaul et al. 2021; Liskova et al. 2021).

4.3.3 Health Benefits

The flavonoids' beneficial effects on cardiovascular diseases and diabetes have been reported as the most promising health benefits of these compounds. Furthermore, flavonoids have also been proved to protect against obesity, cancer, osteoporosis, neurodegenerative and hepatic diseases (Kaleem and Ahmad 2018; Mutha et al. 2021; Ballard and Maróstica 2019; Ekalu and Habila 2020).

4.3.3.1 Cardioprotective Effects

The positive effects of flavonoids ingestion on the cardiovascular system are well-known. Several works identified flavonoids as important cardioprotective agents with beneficial effects on cardiac functions, vasculature, and coagulation (Roohbakhsh et al. 2015; Nunes et al. 2016; Muñoz-Bernal et al. 2021). According to the literature, the cardioprotective properties of flavonoids are due to their action as antioxidant and anti-inflammatory agents, i.e. their ability to control oxidative stress and inhibit inflammation propagation, respectively (Ballard and Maróstica 2019; Romain et al. 2014). Furthermore, flavonoids have shown the capacity of improving coronary vasodilatation and inhibiting enzymes (e.g. NADPH oxidase, xanthine oxidase, myeloperoxidase [MPO], etc.) with a vital role in the development of atherosclerosis, hypertension, and heart failure (Ruiz-Cruz et al. 2017a; Ballard and Maróstica 2019). The main beneficial actions of flavonoids described in the literature and the primary mechanisms by which their protective effects on the human body occurred are summarised in Table 4.2.

All the classes of flavonoids summed up in Table 4.1 are shown to have the cardioprotective properties. From flavonols, quercetin is capable of inactivating

Table 4.2 Main beneficial effects of flavonoids on cardiovascular disease and diabetes (Ballard and Maróstica 2019; Ciumărnean et al. 2020; Muñoz-Bernal et al. 2021; Patel et al. 2018; Xiao 2022; Al-Ishaq et al. 2019)

Beneficial effects		Specific mechanisms
Cardiovascular disease	Antiplatelet	Decrease platelet adhesion Stopping excessive platelet activation
	Antiatherogenic	Reduce the oxidation of low-density lipoproteins (LDL) Lower plasma lipid levels
	Antihypertensive	Modulate the renin–angiotensin–aldosterone system Increase the concentration of endothelial NO
	Anti-ischaemic	Reduce cell suffering caused by myocardial or brain ischaemia Increase the concentration of endothelial NO
Diabetes	Antihyperglycaemic	Reduce serum glucose and fasting blood glucose levels by up- and down-regulation of different metabolic pathways Protect pancreatic β cells activating insulin signalling Improve glucose uptake and insulin resistance by regulation of various metabolic routes Inhibit glycogenolysis and gluconeogenesis by several metabolic pathways Reduce the absorption of glucose in the small intestine by inhibition of carbohydrate metabolising enzymes: α-glucosidase and α-amylase
	Hypolipidaemic	Reduce lipid peroxidation Reduce hyperlipidaemia Inhibit adipogenesis Reduce the level of TBARS (a by-product of lipid peroxidation) Normalise adiponectin level Increase the activity of lactate dehydrogenase (LDH)
Cardiovascular disease & diabetes	Antioxidant	Creation of stable flavonoids radicals Elimination of reactive oxygen species (ROS) Increasing the protection of antioxidant systems
	Anti-inflammatory	Inhibit prostaglandin synthesis Inhibit enzymes: nitric oxide (NO) synthase and phosphodiesterases Reduce inflammation by different inflammation metabolic pathways

reactive oxygen species (ROS) by neutralising the oxidised low-density lipoprotein (ox-LDL) and attenuating the TLR4/NF-κB signalling pathway in endothelial cells, thereby regulating the inflammatory process of atherosclerosis (Bhaskar et al. 2016). On the other hand, flavanones such as apigenin inhibited the endothelial receptor for ox-LDL, i.e., the lectin-like oxidised LDL receptor-1 (LOX-1), reducing atherosclerosis progression (Ballard and Maróstica 2019). Still, it was found that quercetin can regulate the expression of aortic nicotinamide-adenine dinucleotide phosphate (NADPH) oxidase, which is the main ROS source in vascular cells, preventing

atherosclerosis (Xiao et al. 2017). On the other hand, the flavanone luteolin exhibited in vitro xanthine oxidase inhibition activity, and the isoflavone daidzein may decrease MPO and catalase activity (Ballard and Maróstica 2019). Daidzein was reported to be able to interfere with the inducible nitric oxide synthase (iNOS) expression pathway resulting in the reduction of NO production and platelet aggregation in animal models (Alshehri et al. 2021). In other studies, quercetin exhibited a similar role as a blood pressure reducing agent, also regulating the bioavailability of the vasodilator (NO). However, it was shown to improve vascular function by directly acting on the vascular smooth muscle or/and inhibiting acetylcholinesterase (ACE) (Patel et al. 2018; Larson et al. 2012). The flavanone hesperidin also demonstrated vasculoprotective action, increasing the NO production in rats (Roohbakhsh et al. 2015) and stage 1 hypertension in persons (Valls et al. 2021). Another antihypertensive compound is the flavanol epigallocatechin gallate, which appeared to improve the endothelial function of hypertensive mice (Mohd Sabri et al. 2019).

Regarding flavonoids with action against myocardial infarction (MI) and minimising ischaemia-reperfusion injury (IRI), the flavonol fisetin and the isoflavone daidzein could be pointed out. Rodius et al. (2020) demonstrated that fisetin protects against cardiac cell death by reducing oxidative damage. On the other hand, daidzein can reduce the damage from MI by inhibiting the release of TNF-α and IL-6 cytokines (Ballard and Maróstica 2019). Moreover, in a recent study, daidzein positively affected cardiac fibrosis by reducing in vitro TGF-β1-induced cardiac fibroblast activation and ameliorating MI-induced cardiac fibrosis in vivo (Shu et al. 2022) (Table 4.2).

Grape and citrus by-products flavonoids have been highlighted for their cardioprotective effects (Roohbakhsh et al. 2015; Muñoz-Bernal et al. 2021; Ling et al. 2020). Studies using flavonoid extracts obtained from these food by-products as nutraceuticals or to fortify food products have been developed (Romain et al. 2014; Rizza et al. 2011). Ling et al. (2020) verified that a high purity flavonoids extract from the citrus peel (25–100 mg/kg) may be a potential activator of PPARα and PPARγ for lowering blood lipids and could improve liver function by antioxidant and anti-inflammatory effects on hamsters in high-fat diet (Ling et al. 2020). On the other hand, red wine supplemented with Vineatrol® (a vine-shoot extract rich in resveratrol and its oligomers) was tested on hyperlipidaemia hamsters showing the capacity to reverse the atherogenic process induced by a high-fat diet by improving metabolic, oxidative, and inflammatory markers (Romain et al. 2014).

Nowadays, flavonoids application in cardiovascular disease prevention and progression is common in the form of a micronised purified flavonoid fraction under the commercial name Daflon®. Daflon ® is a flavonoid vasoprotector venotonic agent that contains 90% diosmin and 10% flavonoids expressed as hesperidin, being among the venoactive drugs more widely prescribed in Europe (Gouda and Babiker 2020).

4.3.3.2 Antidiabetic Activity

Diabetes is one of the most widely prevalent metabolic disorders. Diabetes is characterised by hyperglycaemia, and frequently it is accompanied by other risk factors such as hypertension and obesity (Kaleem and Ahmad 2018; Mutha et al. 2021). The combination of diabetes, hypertension, and obesity was known by the medical term metabolic syndrome. Metabolic syndrome is a multifactorial disease with multiple risk factors for coronary heart disease and diabetes, fatty liver, and several cancers.

Nowadays, diabetes is treated by several antidiabetic agents (e.g. biguanides, sulfonylureas, glinides, and α-glucosidase inhibitors) in conjunction with insulin to control blood sugar levels (Kaleem and Ahmad 2018). Several studies have reported the antidiabetic activity of flavonoids. The main beneficial antidiabetic actions of flavonoids described in the literature are summarised in Table 4.1.

All the classes of flavonoids are shown to have antidiabetic activity, principally flavonoids aglycones and their O-glycosides/C-glycosides (Xiao 2022; Dinda et al. 2020). Among the flavonols, quercetin and its glycosides, rutin, kaempferol and its glycosides, myricetin, fisetin, and morin have been proved to have significant antidiabetic activities (Ballard and Maróstica 2019; Xiao 2022; Al-Ishaq et al. 2019). For example, Lee et al. (2021) investigated the antidiabetic effects of rutin and the rutin-rich Tartary buckwheat in a murine model of type 2 diabetes (T2D) glucagon-like peptide-1 (GLP-1) receptor-knockout mice and secretin tumour cell line (STC-1) cells. Blood glucose-lowering effects were observed after the consumption of 10% buckwheat or 0.1% rutin in rat diets. Moreover, an improvement in β-cell function and increased levels of GLP-1 closely associated with the blood-glucose-lowering effects were also detected (Lee et al. 2021). Anthocyanins are another flavonoid class with a positive antihyperglycaemic effect by stimulating GLP-1 secretion. Studies with cyanidin, delphinidin, and 3-rutinoside-rich blackcurrant extract increased GLP-1 levels in mice and promoted GLP-1 secretion from GLUTag cells. The modulation of the GLP-1 metabolism by anthocyanins supported their beneficial antidiabetic actions to improve glucose tolerance and homeostasis (Tani et al. 2017; Cremonini et al. 2021). Besides that, delphinidin and cyanidin showed to decrease inflammation and control redox signalling pathways by improving insulin resistance in high fat-fed mice (Daveri et al. 2018). Other anthocyanins with potential health benefits in the prevention of diabetes are cyanidin, peonidin, delphinidin, malvidin, pelargonidin, and petunidin (Dinda et al. 2020; Mechchate et al. 2021).

One antidiabetic effect that has been attracting significant attention is the prevention and treatment of diabetic cardiac complications. Recently, a new antidiabetic formulation using rutin and other plant flavonoids class compounds, namely catechins (catechin and epicatechin), was optimised to obtain a multi-targets antidiabetic formulation. The combinations were tested on alloxan-induced diabetic mice for 28 days. The binary combination between rutin and epicatechin (25% and 75%, respectively) showed to be the optimum combination that prevented hyperglycaemia

and hypoglycaemia (Mishra et al. 2013). Epicatechin also exhibited promotion activity of GLP-1 in vitro through their capacities to activate protein kinase A (PKA) (Mickymaray et al. 2020). Other plant-derived catechins with several antidiabetic effects are gallocatechin, epigallocatechin, catechin gallate, and epigallocatechin-3-gallate (EGCG) (Hernández-Hernández et al. 2019). Othman et al. (2017) treated streptozotocin-nicotinamide-induced diabetic rats with EGCG (2 mg/kg) to investigate its potential protective effect on diabetes-induced heart injury. Besides the antihyperglycaemic and antidyslipidaemic actions (reduction in glucose and lipid profile levels with an elevation in insulin levels), EGCG protects against diabetic cardiac injury through ameliorating the increase in the metabolic risk factors, oxidative stress, inflammation, and apoptosis (Othman et al. 2017).

On the other hand, the flavone luteolin was also a potential therapeutic agent for diabetic cardiomyopathy. According to Li et al. (2019), luteolin significantly reduced high glucose (HG)-induced inflammatory phenotype and oxidative stress in cultured H9C2 cardiomyocytes. In vivo model, luteolin inhibited cardiac fibrosis, hypertrophy, and dysfunction in mice with type 1 diabetes (Li et al. 2019). Other dietary flavones such as luteolin 7-O-glucoside, diosmin, apigenin, baicalein, wogonin, and tangeretin have significant antidiabetic effects (Dinda et al. 2020).

Regarding flavanones, naringin and its aglycone naringenin, hesperidin, and eriodictyol are well-known for their antidiabetic activities. These flavanones, commonly present in grape and citrus fruits, have been extracted and tested for several antidiabetic activities. More recently, naringenin and hesperetin showed to protect pancreatic β cells in vivo and in vitro independently of their antioxidant activity and are closely related to their inhibitory effect on histone acetylation. This protecting result of naringenin and hesperetin on pancreatic β cells was studied under a high glucose environment using in vivo (diabetic *db/db* mouse) and in vitro (rat insulinoma INS-1 cell line) models (Wang et al. 2021).

At last, the intake of isoflavones such as genistein, daidzein, prunetin, formononetin, and puerarin has been associated with a reduced risk of diabetes. Daidzein is one of the most important and highly studied isoflavones. Nevertheless, recently, its metabolites like desmethylangolensin (DMA), dihydrodaidzein (DHD), and cis-4-OH-equol, which are formed by intestinal bacteria, have attracted attention. In vitro, in vivo, and clinical studies have revealed that the antidiabetic action of daidzein and its metabolite equol occurs by regulation of glucose metabolism, lipid metabolic pathway during the diabetic condition, and suppression of inflammation pathogenesis of insulin resistance, diabetes, and cardiovascular disease (Das et al. 2018).

Fruits and their by-products showed to be good sources of flavonoids with antidiabetic properties (Tanveer et al. 2017; Gerardi et al. 2020; Rodríguez-González et al. 2017). The administration of proanthocyanidins obtained from persimmon peel to streptozotocin-induced diabetic rats showed a decrease in lipid peroxidation level, inhibited the reactive oxygen species generation, and reduced the serum glucose (Tanveer et al. 2017). Mango juice by-product powder significantly reduced serum glucose of streptozotocin-induced diabetic rats and insulin-mimetic effects in 3T3-L1 adipocyte cells. Despite the antidiabetic effects of mango by-product

powder being associated with its high soluble fibre content, it was also related to its richness in several polyphenolic compounds, including flavonoids such as quercetin, epicatechin gallate, and catechin gallate (Rodríguez-González et al. 2017). More recently, the supplementation of hypertensive and diabetic rats with wine pomace products rich in flavonols (mainly kaempferol-3-O-rutinoside) and anthocyanidins (mainly petunidin) exhibited a protective role against endothelial dysfunction and vascular remodelling. Wine pomace product inhibited the development of pathological vascular changes caused by hypertension and diabetes, decreased ROS in aortic tissue, and increased NO synthase activation (Gerardi et al. 2020).

The interaction of flavonoids and gut microbiota can reduce the occurrence of diabetes and ameliorate their related complications. On the other hand, gut microbiota dysbiosis has been associated with the occurrence and development of diabetes. In this sense, flavonoids and their metabolites could be used to regulate the gut microbiota composition to prevent the progression of diabetes or ameliorate its complications. However, more research is required on this thematic (Han et al. 2022).

4.3.4 Flavonoids as Colourant

Consumers are increasingly aware of the importance of food choices to their health and well-being. Besides, consumers looking for functional food with bioactive properties such as antioxidant, antiviral, cardioprotective, and antidiabetic activities earlier in this chapter also wanted more natural food formulations. In this sense, the food industry has been studying flavonoids to adapt organoleptic, sensorial, and conservation properties of food products (Carpena et al. 2021). Consumers consider colour the most significant attribute of food choice in purchasing among organoleptic food properties. They relate colour with the freshness and quality of the product.

The food colourant market is expanding with a growing annual rate of 4.6%, principally the natural colourants. Synthetic colourants have been associated with long-term adverse health impacts. Therefore, natural colourants have gained the attention of researchers and industrial food technologists (Prajapati and Jadeja 2022).

Flavonoids are between the natural compounds used as colouring agents, mainly anthocyanins and also flavonols. These compounds allowed to obtain a range of colours between cream, yellow, pink, red, blue, and black (Carpena et al. 2021). A natural flavonoid-based colouring agent is already used in the food sector, namely anthocyanins (E163). The regulatory authorities approved this flavonoid-based red pigment as a food additive, and it is extracted from grape skins and blackcurrant pomace. Until now, natural food colouring studies have strongly focused on anthocyanins. However, these compounds are extremely unstable and sensitive to various factors such as pH, temperature, oxygen, and light (Bernardes et al. 2019). For example, anthocyanins are red in acidic pH, while in basic conditions, they look blue and are purple in neutral pH (Carpena et al. 2021). In this sense, strategies for stabilising these pigments have been developed. Bernardes et al. (2019) developed

inulin-coated microcapsules to protect anthocyanins-rich extract obtained from jussara (Euterpe edulis Martius) fruit. These microcapsules were incorporated into a gelatine model system presenting better gelatine colour parameters throughout its storage (Bernardes et al. 2019). Besides microencapsulation, the addition of other antioxidants and co-pigmentation were other strategies that have been used to stabilise anthocyanins (Silva et al. 2017; Pan et al. 2014). The addition of antioxidants more susceptible to oxidation than anthocyanins will be oxidised before them, protecting the anthocyanins' red colour. In co-pigmentation, the colourants and other colourless organic compounds (self-association, inter- and intramolecular association), or metallic ions (ion complexation), form associations, leading to an increased colour intensity (Pan et al. 2014). Intermolecular co-pigmentation using other flavonoids classes has been suggested as suitable colour enhancers and stabilisers of anthocyanins (Pan et al. 2014; Erşan et al. 2022). Pan et al. (2014) applied a co-pigmentation strategy to blueberry juice using vitexin, orientin, and flavonoid C-glycosides that allowed to obtain a more saturated and stable colour on blueberry juice and stabilise anthocyanins through shelf-life (Pan et al. 2014). Another study applied rooibos extracts to anthocyanin-rich strawberry model solutions, demonstrating that flavonoid-rich fractions (luteolin, apigenin, and quercetin glycosides) significantly enhanced strawberry products' colour (Erşan et al. 2022). Furthermore, intramolecular co-pigmentation, in which the central anthocyanin chromophore and aromatic acyl residues interact covalently with added compounds, has been employed in red wines and model wine solutions. Compounds such as catechins and procyanidins have been added to wines to obtain polymeric pigments responsible for most aged wine colours (Escott et al. 2018; Liu et al. 2019).

4.3.5 Flavonoids on Cosmetic

Flavonoids have been gaining popularity as cosmetic ingredients due to their several activities: antioxidant, anti-inflammatory, antimicrobial, UV-protecting, and even wound healing (Ruiz-Cruz et al. 2017a; Orhan et al. 2010). Mostly, antioxidant and UV-protecting activities of flavonoids called the attention of the cosmetic industry. Flavonoids can absorb UVA and UVB rays and alleviate the effects of oxidative stress, improving skin appearance. In a recent study, anthocyanin-rich extracts from blackberry and raspberry showed promising natural cosmetic ingredients as sunscreen agents and prevent skin ageing. When incorporated into cosmetic formulations, these extracts showed in vitro solar protection factor and antioxidant activity when stored in opaque packages (Cefali et al. 2019).

On the other hand, quercetin was shown to inhibit skin damage from UV irradiation that induced inflammatory cytokine production in primary human keratinocytes cells (Vicentini et al. 2011). The anti-inflammatory activity of flavonoids also has a health promising effect on the skin with great interest to cosmetics, reducing inflammatory symptoms induced by external agents and as a possible therapeutic effect on psoriasis and atopic dermatitis (Wadhwa et al. 2022; Wang

et al. 2022; Chen et al. 2017). According to Chen et al. (2017), quercetin showed anti-psoriasis effects in the psoriasis-induced mice model intimately associated with its antioxidant and anti-inflammatory activity (Chen et al. 2017). On the other hand, baicalin ameliorated induced atopic dermatitis-like skin lesions in mice and has other positive effects on the skin regarding restoring the epidermal barrier function and modulation of the gut microbiota (Wang et al. 2022).

The healing activity of flavonoids is another property of appeal to cosmetics. Fruit peel and pulp extracts rich in flavonoids have been shown to aid the healing of the skin after cutaneous infections such as acne, contributing to skin recovery. In the case of the cashew pulp extract, no in vitro cytotoxic effect was detected on keratinocytes cells; however, it did not have antibacterial activity against *Propionibacterium acnes* (Cefali et al. 2021).

Some flavonoids have already been employed in commercial cosmetic products, as is the case of quercetin and baicalein. Quercetin and baicalin were used on Sesderma C-Vit ® and Vichy - Slow Âge ® to neutralise cellular damage produced by ROS and correct the signs of ageing (Adamska-Szewczyk and Zgórka 2019).

4.4 Conclusion

Flavonoids have been raising a growing interest in the industry due to their several biological activities in human health and due to their organoleptic properties in food products. Nowadays, flavonoids are commonly used as functional ingredients or colourants in food products, tablets, supplements, and capsules in the pharmaceutical and nutraceutical industries as well as bioactive ingredients in cosmetic formulations. The search for new flavonoid-rich sources has been increasing to answer growing market demand, highlighting the flavonoids richness of the food by-products. In the last years, several works focused on evaluating the flavonoids content of food by-products. In many cases, by-products contain a higher flavonoid content than the raw source material. However, it is still required to develop and improve the extractive methodologies to maximise extraction yields and simultaneously minimise the extraction of undesirable compounds using sustainable processes. Moreover, more studies are required to achieve functional ingredients, nutraceuticals, and drug flavonoids based on food by-products to stop the wastage of rich sources of bioactive compounds.

The benefits of flavonoids against cardiovascular diseases and diabetes described above are well-demonstrated by in vitro and in vivo studies. However, long-term clinical studies are insufficient and even more regarding the extract of flavonoids from food by-products. Some limitations still exist regarding the bioavailability and dose standardisation of flavonoids to confirm their effectiveness in disease prevention and treatment. Besides that, additional studies to improve flavonoids stability and bioavailability are also needed to guarantee an effective and efficient flavonoids application in food, cosmetic, and pharmaceutical formulations.

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Chapter 5
Terpenes

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Abstract Terpenes are the largest and most diverse group of naturally occurring compounds found in plants. They can be classified according to the number of isoprene units, the most common being monoterpenes (C_{10}), sesquiterpenes (C_{15}), diterpenes (C_{20}), and triterpenes (C_{30}). Besides being the principal constituents of essential oils and playing fundamental roles in plants, many terpenes are extensively used in pharmaceutical and industrial applications ranging from flavours to fragrances and medicines. Several studies have already demonstrated the diversity of terpenes' biological properties, including cancer chemopreventive effects, antimicrobial, antiviral, analgesic, anti-inflammatory, antifungal, antiparasitic, and other activities. This chapter compiles the various terpenes isolated from plants, their sources, biological activities and beneficial health effects, mechanism of action, extraction and applications, and the future perspective for using the terpenes as lead molecules in several areas of the industry.

5.1 Introduction

From a structural and chemical perspective, terpenes are the largest and most diverse class of natural compounds, including more than 50,000 known molecules (Cox-Georgian et al. 2019; Pasquini et al. 2021; Torres-Fajardo and Higuera-Piedrahita 2021). They originate from mevalonic acid and are composed almost

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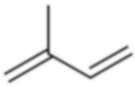

Fig. 5.1 The structural form of an isoprene unit

entirely of carbon, oxygen, and hydrogen. Their presence in different parts of the plant (leaves, flowers, stems, buds, fruits, pods, seeds, roots, and barks) contributes to its aroma, colour, and flavour (Cox-Georgian et al. 2019; Uwineza and Waśkiewicz 2020). Structural diversity in terpenoids from natural products arises from the coupling chemistry employed to link C_5 isoprenoid precursors. Moreover, different terpenes or derived compounds have been described to perform distinct functions ranging from primarily structural (such as cholesterol in maintaining the membrane structure) to more functional ones such as carotenoid pigments or gibberellin a plant-derived terpene responsible for the regulation of cell growth and defence (Mostofian et al. 2020; Wang et al. 2019a). In addition, due to their allelopathic effects, terpenes have a natural role in attracting pollinators, healing plants' injured tissues from herbivore attacks and naturally repelling insects and parasites (War et al. 2012; Maffei 2010; Nejia et al. 2013). Despite terpenes being defined as hydrocarbons having the five-carbon isoprene unit as their building block (Fig. 5.1), many authors use the term 'terpenes' more broadly to include also the terpenoids. These terpenes have their carbon skeleton modified by oxidation and rearrangement since methyl groups are generally moved or removed when oxygen atoms are added to the hydrocarbon molecules. Therefore, terpenes are simple hydrocarbons while terpenoids contain oxygen in distinct functional groups, such as alcohols, aldehydes, ketones, acids, esters, and ethers, increasing terpene's chemical and functional diversity (Pasquini et al. 2021; Mewalal et al. 2017; Mosquera et al. 2021).

Most of the natural terpenes have the general formula $(C_5H_8)n$, and their thermal decomposition gives isoprene as one of the products (Fig. 5.1) (Mewalal et al. 2017; Pichersky and Raguso 2018; Caputi and Aprea 2011; Hanuš and Hod 2020). They can be classified based on the value of n (number of isoprene units) or the number of carbon atoms present in the structure. Mono- and sesquiterpenes (C_{10} and C_{15}, respectively) and their derivatives are highly volatile compounds and frequently the main constituents of essential oils (EOs). At the same time, di- and triterpenes (C_{20} and $C_{30,}$ respectively) and their derivatives are more complex compounds being less volatile or even assumed as non-volatile. They are found in EOs at low concentrations, mainly from plant gums and resins (Bicas et al. 2009; Haberstroh et al. 2018; Yáñez-Serrano et al. 2018). Terpenes can also be classified as acyclic with an open structure, cyclic with one ring structure, and bi-, tri-, and tetra-cyclic with two, three, and four rings.

Plants synthesise diverse compounds unique to this class of chemicals and contribute to the vast number of existing terpenes and terpenoids. Some plants store or emit these compounds and deposit them in specific organs, such as resin ducts, performing a defensive function or emitting immediately after their biosynthesis. Abiotic and biotic stress factors such as drought, extreme temperature,

pollution, or pathogen attack can interfere and rearrange the biosynthesis and emission of terpenes. However, the response may depend on the stressor type and intensity (Pasquini et al. 2021; Haberstroh et al. 2018; Blanch et al. 2007).

Besides their fundamental role in plants, many terpenes are widely used in pharmaceutical and industrial applications ranging from flavours to fragrances (Cox-Georgian et al. 2019). Extensive application of chemical techniques and biological tests has led to the identification, characterisation, and extraction of significant components of broad interest, especially concerning the recovery of terpenes with particular interest for cosmetic and other industries (Salha et al. 2021). Several studies have already demonstrated the diversity of their biological properties, including cancer chemopreventive effects, antimicrobial, antiviral, analgesic, anti-inflammatory, antifungal, antiparasitic, and other activities (Yang et al. 2020). Recently, there has been an increased interest in these compounds obtained from industries' crops since some specific terpenes have been identified as specialties biofuels (Mewalal et al. 2017; Greay and Hammer 2011; Guimarães et al. 2014; Tetali 2019).

This chapter compiles some of the major roles of terpenes and terpenoids isolated from plants. For different compounds, their biological activities and beneficial health effects are emphasised. Moreover, general aspects regarding biosynthesis and extraction are also included.

5.2 Chemistry and Biosynthesis of Terpenes

5.2.1 Classification

These compounds are part of the secondary metabolism of plant species and consist of isoprene units: 2-methylbuta-1,3-diene, (C_5H_8) (Fig. 5.1) linked to each other in numerous ways, most commonly by head to the tail arrangement but other arrays can be found (Bicas et al. 2009; Rubulotta and Quadrelli 2019).

According to the number of isoprene units, terpenes can be classified as hemiterpenes when having a single isoprene unit (C_5), monoterpenes (C_{10}) when two isoprene units are linked, sesquiterpenes (C_{15}), diterpenes (C_{20}), sesterterpenes (C_{25}), triterpenes (C_{30}), and tetraterpenes (C_{40}) (Table 5.1 and Fig. 5.1). When more than eight isoprene units are connected, they are named polyterpenes (Torres-Fajardo and Higuera-Piedrahita 2021; Guimarães et al. 2014; Tetali 2019; Rubulotta and Quadrelli 2019; Harman-Ware 2020a). The isopropyl part of 2-methylbutane is defined as the *head* and the ethyl residue as the *tail*. In mono-, sesqui-, di-, and sesterterpenes, the isoprene units are linked from *head-to-tail*; tri- and tetraterpenes contain one *tail-to-tail* connection the centre (Pichersky and Raguso 2018). Examples of these compounds are shown in Fig. 5.2.

Table 5.1 Classification of terpenes based on the isoprene units

Classification	Carbon atoms	N° isoprene units	Examples
Hemiterpene	C_5	1	Isoprene
Monoterpene	C_{10}	2	α-pinene, limonene, sabinene, myrcene, carene, cymene, linalool, nerol, geraniol, menthol
Sesquiterpene	C_{15}	3	β-caryophyllene, β-farnesene, longifolene, δ-cadinene, humulene, germacrene D, artemisin
Diterpene	C_{20}	4	Camphorene, cafestol, kahweol, cambrene, taxanes
Sesterterpene	C_{25}	5	Merochlorin A, ophiobolins, manoalide
Triterpene	C_{30}	6	Squalene
Tetraterpene	C_{40}	8	Carotenoids
Polyterpene	C_n	n	cis-1,4-polyisoprene (rubber)

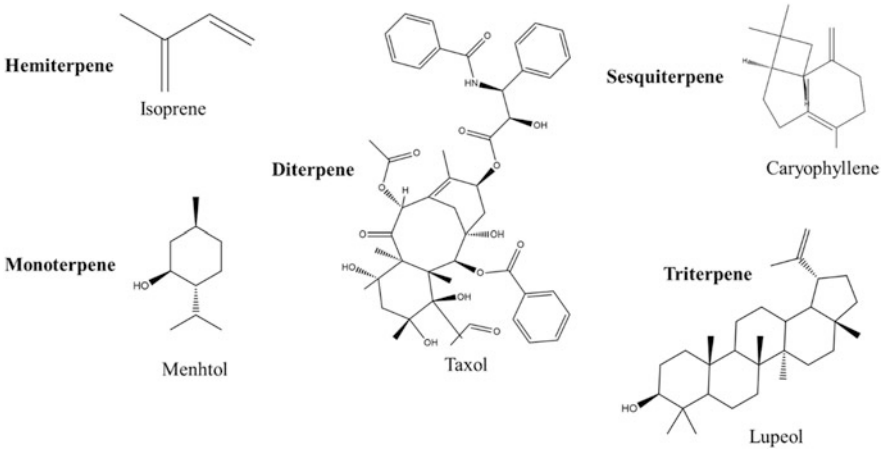

Fig. 5.2 Structural forms of volatile, semi, and non-volatile terpenes

5.2.2 Biosynthesis

Overall, all terpenes originate, in part, from the C_5 substrate dimethylallyl pyrophosphate (DMAPP) and isopentenyl diphosphate (IPP), typically by initially condensing DMAPP with one or more IPP molecules. The type of compound formed is associated with the synthesis pathway (Petrović et al. 2019). Tri- and sesqui-terpenes are most likely synthesised from the precursors produced in the mevalonate pathway (MVA) in the cytoplasm, while hemi-, mono-, di-, and tetra-terpenes are generally synthesised from the precursors produced in the methylerythritol 4-phosphate (MEP) pathway in the chloroplast (Fig. 5.3) (Mewalal et al. 2017; Tetali 2019). Both pathways use IPP and its respective isomer DMAPP to form the isoprene unit (C_5). The main difference is that, while in the MVA pathway, the activated building

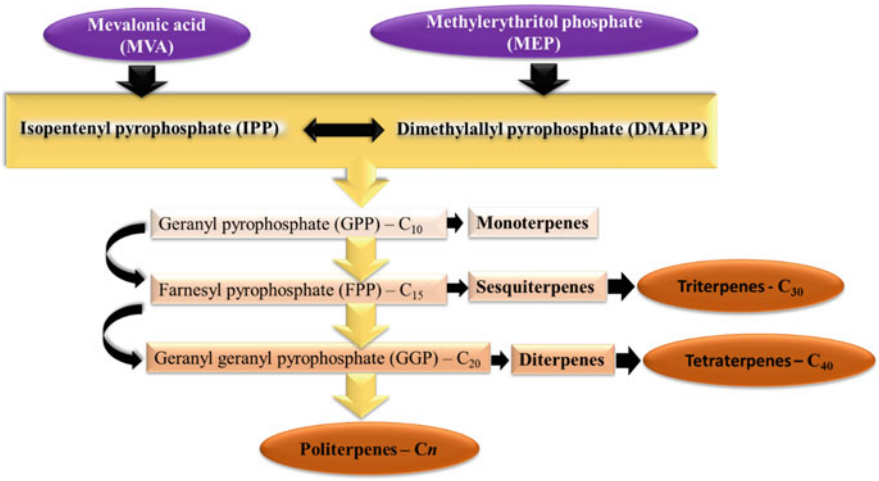

Fig. 5.3 Principal pathways of the biosynthesis of terpenes

blocks are synthesised from acetyl-CoA, in the MEP pathway, they are obtained from pyruvate with glyceraldehyde 3-phosphate. Molecules of IPP and DMAPP condense to form geranyl pyrophosphate (GPP) with 10 carbons, and this reaction is catalysed by GPP synthase. Then, a molecule of IPP will react with the GPP to form a molecule of farnesyl pyrophosphate (FPP), presenting 15 carbons. The FPP can further react with a molecule of IPP and form a geranylgeranyl pyrophosphate (GGPP) with 20 carbons. Each combination will cause the release of pyrophosphate. Overall, these compounds are considered linear precursors of all terpenes (Mewalal et al. 2017; Rubulotta and Quadrelli 2019; Harman-Ware 2020a; Petrović et al. 2019).

5.3 Extraction Technologies

In general, extraction methods can be classified as conventional and non-conventional. The first group encompasses maceration, Soxhlet, steam distillation, and hydrodistillation; they require long extraction times, high amounts of solvents, and high energy costs. The second group encompasses supercritical fluid extraction, ultrasound-assisted extraction, and microwave-assisted extraction. Non-conventional methods are considered eco-friendly as they are associated with lower energy costs and the avoidance of toxic solvents and therefore have a lower environmental impact.

5.3.1 Conventional Extraction Methods for Low Molecular Weight (LMW) Terpenes

LMW terpenes (mono- and sesquiterpenes) are the main constituents of essential oil (Tongnuanchan and Benjakul 2014). The techniques most frequently used for their extraction are steam distillation and hydrodistillation, as both are considered the main methods to obtain essential oil from different anatomical parts of plants (Salha et al. 2021).

5.3.1.1 Steam Distillation

Steam distillation is one of the ancient and officially approved methods for isolating essential oils from plant materials (Li et al. 2014) being used in commercial-scale production due to the low cost associated with the process (Giacometti et al. 2018). In this conventional type of extraction, the plant material is exposed to a distillation process with boiling water without the water entering in contact with the plant material. Heat plays a fundamental role in this method since the steam passes through the sample, destroying its morphology and releasing the volatile aromatic compounds, which are then carried by vapour that is then condensed, resulting in a separation of phases of distilled water-essential oil (Tongnuanchan and Benjakul 2014; Pateiro et al. 2018). Steam distillation can also be carried out under pressure, depending on the characteristics of the sample (Li et al. 2014). This method is used to create fine perfumes, obtain distillates that refine the flavours and aroma of food and drinks, and produce medicines from plants (Salha et al. 2021). Different studies reveal that steam distillation can extract significant quantities of terpenes, leading to good responses on bioactive assays (Salha et al. 2021; El Kharraf et al. 2021; Ragab et al. 2019).

5.3.1.2 Hydrodistillation

Hydrodistillation is a variant of steam distillation. Instead of using steam that passes through the sample, it relies on a solid–liquid maceration of plant material in the water that is heated to ebullition. The heat allows the release of molecules of interest/volatile compounds present in the plant cells, which are carried by water vapour in an azeotropic mixture and collected at the end of the process (Tongnuanchan and Benjakul 2014). This process obtains two phases, namely water and an oil-rich phase. Hydrodistillation with Clevenger® apparatus is described in the European Pharmacopoeia as a method adequate for volatile oil extraction from different herbal products such as medicinal plants and dried spices (Li et al. 2014; Giacometti et al. 2018; Tavakolpour et al. 2017). This method is one of the most widely used at laboratory scale, being described in several studies to extract essential oil from

different plant matrices (Fagbemi et al. 2021; Jafari-Sales and Pashazadeh 2020; Baker et al. 2021).

5.3.1.3 Enfleurage method

Enfleurage is the oldest perfumery process commonly employed in the south of France. It is a traditional method of extraction of flowers' essential oil by their contact with odourless cold fat or vegetable oil. In this way, the essential oil and aromas are absorbed by the fat/oil (Ali et al. 2015), preserving the essential oil from hydrolysis during the extraction (Oktavianawati et al. 2019). Enfleurage extraction is generally a lengthy process since it requires three days to be complete. Moreover, the fat may be liquefied several times to enhance its organoleptic characteristics. The subsequent addition of alcohol to the fat/oil allows the absorbed essential oil to recover (Ali et al. 2015). Enfleurage has a greater yield of extraction when compared to other methods (Paibon et al. 2011). Formerly it was used for extracting essential oil of all flowers, but modern methods have shown that better results can be obtained more economically (Picot-Allain et al. 2021). However, some exceptions still remain such as the cases of jasmine (*Jasminum* L.) and tuberose (*Agave amica* L.), which do not tolerate other extraction methods (Barrales-Cureño et al. 2021; Roopashree and Naik 2019; Poucher 1993).

5.3.2 Conventional Extraction Methods for High Molecular Weight (HMW) Terpenes

Diterpenes, triterpenes, esters, and waxes are examples of high molecular weight molecules present in plant material. Di- and triterpenes are considered semi- or non-volatile terpenes compared with mono- and sesquiterpenes (LMW). Therefore, non-polar organic solvents are commonly used to extract HMW terpenes (Wang et al. 2014). However, for pharmaceutical, cosmetic, and food industries, the utilisation of organic solvents is limited and maximum residues are strictly defined for the allowed solvents (Choi and Verpoorte 2019). The maceration and Soxhlet extraction methods are used traditionally to obtain gums and resins containing terpenes of high molecular weight (Bensebia et al. 2016). However, cold press extraction is also described in the literature as an industrial method for extracting HMW terpenes (Putnik et al. 2017; Chemat et al. 2012).

5.3.2.1 Cold Press Extraction

Considered an old but evergreen extraction method, the cold press is commonly used in the industrial field to extract oil from different matrices (Chemat et al. 2012). The

cold pressing method consists of the physical process of crushing citrus peels, in which the essential oil is present. With the mechanical action of crushing, the oil is released and transferred to a natural sponge, from which it is finally removed. For essential oil extraction, the citrus genus is the most suitable for this type of operation. The oil obtained by this method adds better fruit odour characteristics than other oil extraction methods (Ali et al. 2015).

5.3.2.2 Maceration, Heat-Assisted Extraction, and Soxhlet Extraction

Maceration is a common solid–liquid extraction used in the nutraceutical field (Chemat et al. 2012). In this extraction method, the sample is soaked and agitated to increase cell permeability, releasing the compounds of interest, such as terpenes (Chia et al. 2020). This process is performed at ambient temperature or in the cold, and compounds are extracted based on diffusion and mass-transfer phenomena. However, to increase the extraction efficiency, the temperature can be applied. In that case, the process is called heat-assisted extraction (HAE) (Rocchetti et al. 2019). The constant agitation in dynamic maceration or heat-assisted extraction increases the solubilisation of the compounds of interest, generating higher extraction yields. Using different solvents or their mixtures can facilitate the mass transfer of the compounds of interest (Garcia-Vaquero et al. 2020). Soxhlet extraction occurs by adding the sample into a cellulose container inserted into the extraction chamber, situated on top of a collecting flask and under a reflux condenser, making this approach a hybrid continuous-discontinuous technique (Fernandez-Pastor et al. 2017; López-Bascón and Luque de Castro 2020).

The use of volatile non-polar solvents and high extraction times are generally perceived as disadvantages of these techniques (de Melo et al. 2020). In addition, these extraction methods have demonstrated poor selectivity. Another aspect being considered is that HAE and Soxhlet frequently use high temperatures, which can degrade thermo-labile bioactive compounds (Palmieri et al. 2020). However, there is the possibility of coupling the Soxhlet apparatus to other equipment to improve the extraction efficiencies such as ultrasound extraction and (Subramanian et al. 2016) microwave-assisted Soxhlet (Prados-Rosales et al. 2002) though the most used is still extraction by convection heat.

Because of the mentioned drawbacks of these methodologies, the search for more environmentally friendly extractions has driven the development of new extraction technologies aiming to reduce the use of toxic solvents and energy costs while improving the extracts' quality and yield.

5.3.3 Novel Non-Conventional Extraction Technologies

In order to reduce energy costs, waste generation, and the use of environmentally friendly solvents, new extraction technologies are being considered viable

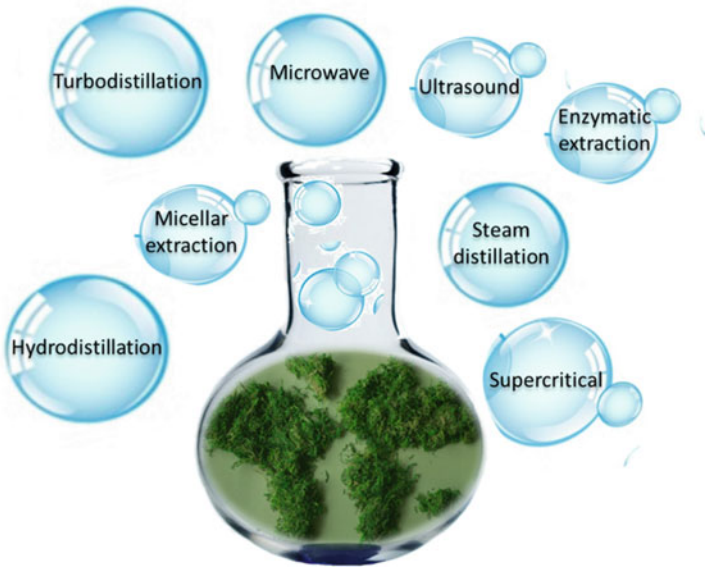

Fig. 5.4 Extraction methods considered as being eco-friendly

alternatives for the future (Fig. 5.4), being less aggressive than conventional technologies and many times more profitable in terms of extraction yield. Green extraction methods such as supercritical fluid extraction, ultrasound-assisted extraction (UAE), and microwave-assisted extraction (MAE) have increased the efficiency of the extraction processes of terpenes in general (Bensebia et al. 2016).

5.3.3.1 Supercritical Fluid Extraction (SFE)

Supercritical Fluid Extraction (SFE) is a suitable and efficient technology considered an environmentally friendly process. SFE allows the processing of plant material at low temperatures, limiting thermal degradation and avoiding toxic solvents (Tongnuanchan and Benjakul 2014). Supercritical fluids such as CO_2, propane, butane, or ethylene are used as solvents for various applications. CO_2 is the supercritical solvent of choice in the extraction of flavour and fragrance compounds since it is colourless, odourless, safe, pure, cost-effective, non-flammable, and recyclable at relatively low pressures near room temperature. Higher CO_2 density (above 500 kg/m^3) favours the yield of extraction of diterpenes, triterpenes, esters, and waxes (Jasna et al. 2009; Reverchon et al. 1995). In short, the solvent is forced to pass through a metallic filter submitted to cooling through heat exchangers. The cooled solvent is then pressurised using a high-pressure pump. In this stage, a co-solvent like ethanol may be mixed to enhance the solubilisation of compounds with greater polarity (Capuzzo et al. 2013). The solvent flows through the feedstock

to extract its soluble compounds in supercritical conditions. Subsequently, the extract is precipitated in the separation vessel due to depressurisation (Priyanka 2018).

5.3.3.2 Microwave-Assisted Extraction (MAE)

Microwave is a non-contact heat source that can achieve more effective and selective heating, resulting in a distillation process that is completed in minutes instead of hours as in conventional extractions (Li et al. 2014). Microwaves are electromagnetic spectrum radiation ranging in frequency from 300 MHz (radio radiation) to 300 GHz. The principal mechanism of microwave equipment is based on two principles. The first is related to the interaction of electric and magnetic fields, resulting in dielectric and magnetic losses leading to heating. The second principle refers to the dipole rotation that occurs when dipole molecules try to align themselves with the alternating electric field in a microwave-produced medium, with heat being produced due to this rotation (Vinatoru et al. 2017).

Microwave extraction is a technique that aims to achieve higher efficiency, which is given by its selective heating. The microwave extraction technique may or may not be accompanied by solvent addition to the extraction. In addition to the frequency used in the extraction and the type of solvent (or the absence of solvent), parameters such as temperature, time, and sample mass are essential in determining the extraction yield. Moreover, besides being used in microwave-assisted hydrodistillation for the extraction of essential oils, it also allows the combination with conventional organic solvents for the extraction of less volatile terpenes (Liu et al. 2018; Sarfarazi et al. 2020). The short extraction time and the possibility of low MAE temperatures make this an excellent technique for extracting terpenes from plants (Isidore et al. 2021).

5.3.3.3 Ultrasound-Assisted Extraction (UAE)

Ultrasound-assisted extraction is a solid–liquid extraction that uses waves with ultrasonic frequencies between 20 and 100 kHz, which enhances the extraction of bioactive compounds from plants (Wen et al. 2018). Waves at these frequencies generate cavitation and vibration, which are responsible for creating cycles of expansion and compression, causing the formation of bubbles that promote and facilitate cellulose destruction and the release of compounds of interest into the extraction solvent (Isidore et al. 2021; Villalva et al. 2021). In addition to frequency, the propagation of ultrasonic waves is related to power (W). Despite UAE not requiring the use of high temperatures commonly associated with other extraction methodologies, temperature and extraction time are also considered relevant parameters in this method (Garcia-Perez et al. 2021; Turrini et al. 2021; Dzah et al. 2020). To date, UAE has proven to be a valuable extraction technique for obtaining terpenes

present in the essential oils of botanical species (Chemat et al. 2012; Santos et al. 2019; Munekata et al. 2020).

5.3.3.4 Enzymatic-Assisted Extraction

Enzyme-assisted extraction has been intensively studied in the last decade. Since plants have a cell wall composed of resistant polysaccharides that can interfere in extracting bioactive compounds and essential oils (Tirgarian et al. 2019). Enzymes like cellulase, xylanase, and pectinase can degrade cell wall components, enabling more efficient extraction of bioactive compounds and improving the bioactive content of essential oils and extracts. Influential factors that determine the extraction efficiency of enzymatic-assisted extraction include solvent pH, reaction temperature, enzyme concentration, and enzyme type (Miljanović et al. 2020). Some authors have considered that enzymatic treatment also promotes changes in the molecular structure of compounds by converting phytochemicals into final products with higher bioavailability and bioactivities (Kitrytė et al. 2018; Martins et al. 2016a).

5.3.3.5 Micelle-Mediated Extraction

Also known as cloud-point extraction, it is a surfactant-based extraction technique. The micelle-mediated extraction procedure involves forming hydrophobic species by complexation with an organic ligand in a surfactant medium (Paleologos et al. 2005). The solution becomes cloudy and separates in two phases, i.e. aqueous phase and surfactant rich coacervate phase (Chatterjee et al. 2017). The distribution of solute is more in the surfactant rich phase due to solubilisation in the micelles, thereby leading to its extraction. Therefore, several studies have focused on the choice of the surfactant and chelating agent. Nonionic surfactants are the most common surfactants in cloud-point extraction (Hinze and Pramauro 1993).

5.3.4 Separation of Terpenes

Essential oils are complex mixtures as they are frequently composed of several terpenes and terpenoids. Because some terpenes are prone to oxidation phenomena, thus contributing to off-flavours and limiting essential oil's application in food, cosmetic, and pharmaceutical industries, they are frequently submitted to deterpenation (i.e. separation of terpenes from terpenoids) to maintain the quality of the final product. The separation of terpenes significantly improves the oil's stability, solubility, and storage requirements, thus allowing for their industrial applications (Ozturk et al. 2018).

5.3.5 *Identification and Quantification Techniques*

Various methods to identify and quantify terpenes have been widely studied. Most often, those require the use of techniques such as gas chromatography (GC), high-performance liquid chromatography (HPLC), capillary electrophoresis, thin-layer chromatography, and SFE chromatography to separate the compounds of interest (Kumar et al. 2016; Xu et al. 2018a). Jiang et al. (2016) proposed different protocols for the extraction/isolation and analysis of terpenoids according to their polarity and size. The analysis was mainly based on chromatographic techniques, such as HPLC and GC. Among those, GC coupled to mass spectrometry detection (GC-MS) is considered the gold-standard technique being the most frequently used to identify volatile terpenes (Louw 2021). On the other hand, for less volatile compounds, such as triterpenes, LC-MS-MS is preferred as an alternative to GC-MS due to its remarkable capacity in the identification of compounds, with the advantages of being also faster and requiring minimum sample preparation (Garg et al. 2020).

5.4 Pharmacological Activities

The presence of diverse functional groups such as phenols, esters, aldehydes, ketones, and alcohols in secondary metabolites produced plants are related to their variety of biological effects due to different action sites available (Masyita et al. 2022; Boukhatem 2020; Singh and Sharma 2015). Many of these bioactive compounds are volatiles obtained in the form of essential oils. Individually or as a mixture of compounds, they have been used in folk medicine for their disinfectant and preservative effects since ancient times. Nowadays, different terpenes and terpenoids are also used as active ingredients in modern pharmaceuticals (Pasquini et al. 2021; Yang et al. 2020; Petrović et al. 2019; Paduch et al. 2007). Many of these novel drugs are based on the knowledge of folk medicine. Two of the most successful terpenes in the market today are used against malaria (artemisinin and its derivatives) (Krishna et al. 2008) and different cancers (taxol and its derivatives) (Cox-Georgian et al. 2019; Yared and Tkaczuk 2012). Several studies have been demonstrating the bioactivity of different terpenes, including those related to antitumour, anti-inflammatory, antiviral, antimicrobial, antiparasitic, antioxidant, antidepressant, antidiabetic, and against cardiovascular diseases, among other pharmacological activities (Cox-Georgian et al. 2019; Yang et al. 2020; Paduch et al. 2007). Additionally, they can be employed as a natural alternative to ward off insects for their anti-insect properties (Salha et al. 2021; Petrović et al. 2019; Singh and Sharma 2015). It has been estimated that the market of terpene-based pharmaceuticals grows every year, with sales of several million dollars (Wang et al. 2005; Kim et al. 2019). Accordingly, the research on the biological potential of terpene-core compounds is of increasing interest to develop new drugs in contemporary medicine. Several terpenes and/or their derivatives presenting biological activity, namely

mono-, sesquiterpenes, and their derivatives, are frequently found as complex mixtures in different essential oils. Therefore, many studies have focused on separating and isolating these compounds towards a deep knowledge of their specific activities. These studies also showed that the combination of specific compounds could lead to improved results concerning biological potential, probably due to a synergic effect (Boukhatem 2020; Singh and Sharma 2015). Moreover, it is essential to highlight that those different enantiomers will have different biological potentials, and the configuration should be considered (Zhu et al. 2017). Some of the most common terpenes and their described biological potential are compiled in Tables 5.1, 5.2, 5.3, and 5.4.

5.4.1 Monoterpenes with Relevant Pharmacological Activities

Figure 5.5 shows the chemical structures of different monoterpenes that have been associated with relevant biological activities. Several studies so far have evidenced a correlation between the antitumour activity of some essential oils and their monoterpene composition, showing that these compounds can play an important role in cancer prevention and treatment. The antitumoural activity can occur through multiple mechanisms in different steps, such as preventing the interaction of carcinogens in the initiation stage, inhibiting the development and migration of cancer cells, and inducing cancer cell apoptosis and, therefore, tumour regression. According to Paduch et al. (2007), the post-translational isoprenylation of proteins that regulate the growth of cells is the most important mechanism influenced by monoterpenes. Up until now, various works have reported that different monoterpenes exhibit antitumour activity. Limonene and perillyl alcohol, as well as their derivatives and stereoisomers, have been described to have a potent antitumour activity (da Silva et al. 2021) that allows the apoptosis of different tumours, including prostate, pancreas, breast, colon, and liver, both in vitro (Rabi and Bishayee 2009; Shi and Gould 2002; Bardon et al. 2002; Crowell 1999) and in vivo tests, specifically in nude mice (Lu et al. 2004; Gould et al. 1994) and human patients with early and advanced cancer (Boukhatem 2020; Vigushin et al. 1998; Miller et al. 2013; Zielińska-Błajet and Feder-Kubis 2020; Chebet et al. 2021). Also, by inducing apoptosis and cell cycle arrest, thymoquinone, naturally found in *Nigella sativa* (black cumin), exhibits activity against breast, skin, brain, bile duct, and non-small cell lung cancers both in studies in vitro (cancer cell lines) and in vivo (rats) (Majdalawieh et al. 2017; Khader and Eckl 2014). The main compound from *Cannabis sativa* essential oil, β-myrcene, presents a significant antiproliferative action against some cancer cell lines, such as breast and human lung carcinoma and leukaemia (Bai and Tang 2020; Tomko et al. 2020; Surendran et al. 2021). In a study with camphene isolated from *Piper cernuum* (Piperaceae), the growth of a subcutaneous tumour was inhibited in both human tumour cell lines and B16F10-Nex2 murine melanoma, suggesting its promising role in tumour therapy (Girola et al. 2015). Another study demonstrated that carvone could reduce pulmonary

Table 5.2 Relevant monoterpenes and their bioactivities

Terpene/terpenoids	Examples of plant sources	Families	Described activity	References
Perillyl alcohol	*Lavandula* spp., *Cymbopogon* spp., *Salvia* spp., *Mentha* spp.	Lamiaceae, Poaceae	Antitumour	Yang et al. (2020), Petrović et al. (2019), Singh and Sharma (2015)
Geraniol	*Cymbopogon* spp., *Rosa* spp.	Poaceae, Rosaceae	Antitumour Antimicrobial Anti-inflammatory Antioxidant Anti-insect	Salha et al. (2021), Yang et al. (2020), Petrović et al. (2019), Paduch et al. (2007)
Geraniol derivative			Antimicrobial	Salha et al. (2021), Yang et al. (2020)
D-limonene	*Citrus* spp.	Pinaceae, Rutaceae	Antitumour Antimicrobial Anti-inflammatory Antiparasitic Antiviral Anti-insect Cardiovascular activity Antidiabetic	Cox-Georgian et al. (2019), Pasquini et al. (2021), Yang et al. (2020), Petrović et al. (2019), Singh and Sharma (2015), Paduch et al. (2007), Ivanova-Petropulos et al. (2015)
Thymoquinone	*Nigella* spp., *Monarda* spp.	Lamiaceae Ranunculaceae	Antitumour	Cox-Georgian et al. (2019)
Camphor	*Cinnamomum* spp., *Dryobalanops* spp., *Ocotea* spp., *Rosmarinus* spp., *Heterotheca* spp.	Lauraceae, Dipterocarpaceae, Lamiaceae, Asteraceae	Antitumour Antiviral	Cox-Georgian et al. (2019)
β-myrcene	*Adenandra* spp., *Laurus* spp., *Humulus* spp., *Myrcia* spp. *Rosmarinus* spp., *Cannabis* spp.,*Cananga* spp., *Thymus*	Rutaceae, Lauraceae, Cannabaceae, Zingiberaceae, Myrtaceae, Annonaceae	Antitumour Antimicrobial Anti-inflammatory	Cox-Georgian et al. (2019), Pasquini et al. (2021), Isidore et al. (2021)

	spp., *Petroselinum* spp., *Amomum* spp., *Humulus* spp.		Antiparasitic Antioxidant Anti-insect	
Thujaplicin	*Chamaecyparis* spp., *Thuja* spp., *Thujopsis* spp., *Juniperus* spp., *Cedrus* spp., *Cupressus* spp.,*Calocedrus* spp., *Platycladus* spp., *Tetraclinis* spp.	Cupressaceae	Antitumour	Cox-Georgian et al. (2019)
α-terpinene	*Cuminum* spp., *Melaleuca* spp., *Origanum* spp., *Cannabis* spp.	Apiaceae, Myrtaceae, Lamiaceae, Cannabaceae	Antitumour Antiviral Antimicrobial	Cox-Georgian et al. (2019), Yang et al. (2020), Paduch et al. (2007)
Thymohydroquinone	*Nigella* spp., *Thymus* spp.	Lamiaceae, Ranunculaceae	Antitumour	Cox-Georgian et al. (2019)
Carvone	*Carum* spp., *Mentha* spp., *Anethum* spp.	Apiaceae, Lamiaceae	Antitumour Antimicrobial Antiviral	Cox-Georgian et al. (2019), Yang et al. (2020), Paduch et al. (2007)
Camphene	*Piper* spp.	Piperaceae	Antitumour	Cox-Georgian et al. (2019), Salha et al. (2021), Ivanova-Petropulos et al. (2015)
p-cymene	*Artemisia* spp., *Protium* spp., *Origanum* spp., *Thymus* spp.	Asteraceae, Burseraceae, Lamiaceae	Antitumour Antimicrobial Anti-inflammatory Antiparasitic	Cox-Georgian et al. (2019), Pasquini et al. (2021), Ivanova-Petropulos et al. (2015)
Carveol	*Mentha* spp.	Lamiaceae	Antitumour Antiviral	Cox-Georgian et al. (2019)
α-pinene	*Rosmarinus* spp., *Pinus* spp., *Salvia* spp., *Sideritis* spp.	Lamiaceae, Pinaceae, Asteraceae	Antitumour Antiparasitic Antimicrobial Anti-inflammatory Antiviral	Cox-Georgian et al. (2019), Pasquini et al. (2021), Paduch et al. (2007), Ivanova-Petropulos et al. (2015)

(continued)

Table 5.2 (continued)

Terpene/terpenoids	Examples of plant sources	Families	Described activity	References
β-pinene	*Rosmarinus* spp.	Lamiaceae	Antitumour Antimicrobial Anti-inflammatory Antiparasitic Antiviral Antidepressant	Cox-Georgian et al. (2019), Pasquini et al. (2021), Paduch et al. (2007), Ivanova-Petropulos et al. (2015)
1,8-cineole	*Rosmarinus* spp., *Hyssopus* spp., *Laurus* spp. *Ocimum* spp. , *Salvia* spp., *Thymus* spp., *Verbena* spp.	Lamiaceae, Verbenaceae	Antitumour Antimicrobial Anti-inflammatory Antiparasitic Antioxidant Antiviral	Cox-Georgian et al. (2019), Pasquini et al. (2021), Yang et al. (2020), Boukhatem (2020), Singh and Sharma (2015), Paduch et al. (2007), Ivanova-Petropulos et al. (2015)
Citral	*Backhousia* spp., *Litsea* spp., *Cymbopogon* spp., *Leptospermum* spp., *Ocimum* spp., *Citrus* spp., *Lindera* spp.	Myrtaceae, Lauraceae, Poaceae, Lamiaceae	Antitumour Antimicrobial Anti-inflammatory	Petrović et al. (2019), Boukhatem (2020)
Sabinene	*Salvia* spp.	Lamiaceae	Antimicrobial Antiviral	Cox-Georgian et al. (2019), Yang et al. (2020)
Menthol	*Mentha* spp.	Saccharomycetaceae, Lamiaceae	Antimicrobial Antiparasitic Antioxidant	Yang et al. (2020), Petrović et al. (2019), Paduch et al. (2007)
Citronellal	*Melissa* spp.	Lamiaceae	Antimicrobial	Salha et al. (2021), Boukhatem (2020)
Citronellol			Antimicrobial Cardiovascular activity	Salha et al. (2021), Boukhatem (2020), Paduch et al. (2007), Ivanova-Petropulos et al. (2015)

Neral	*Melissa* spp.	Lamiaceae	Antimicrobial	Salha et al. (2021), Boukhatem (2020), Paduch et al. (2007)
Thymol	*Origanum* spp., *Thymus* spp.	Lamiaceae	Antimicrobial Anti-inflammatory Antiparasitic Antioxidant Antiviral	Pasquini et al. (2021), Salha et al. (2021), Yang et al. (2020), Petrović et al. (2019), Boukhatem (2020), Paduch et al. (2007), Ivanova-Petropulos et al. (2015)
Eugenol	*Myristica* spp. *Syzygium* spp.	Myrtaceae Myristicaceae	Antimicrobial Antiviral Anti-insect	Pasquini et al. (2021), Salha et al. (2021), Boukhatem (2020), Al-Salihi and Alberti (2021)
Linalool	*Citrus* spp., *Hyssopus* spp., *Lavandula* spp., *Mentha* spp., *Ocimum* spp., *Origanum* spp., *Quercus* spp., *Salvia* spp., *Thymus* spp., *Verbena* spp.	Lauraceae Rutaceae Lamiaceae Fagaceae Verbenaceae	Antimicrobial Anti-inflammatory Antiparasitic Antioxidant Cardiovascular activity Antidepressant	Pasquini et al. (2021), Ivanova-Petropulos et al. (2015)
α-terpineol	*Pinus* spp.	Pinaceae	Antimicrobial Antiviral	Yang et al. (2020), Al-Salihi and Alberti (2021)
Terpinen-4-ol	*Melaleuca* spp.	Myrtaceae	Antimicrobial Anti-inflammatory Antiviral	Cox-Georgian et al. (2019), Salha et al. (2021), Al-Salihi and Alberti (2021)
γ-terpinene			Antimicrobial	Ivanova-Petropulos et al. (2015)
Carvacrol	*Origanum* spp., *Thymus* spp., *Lepidium* spp., *Citrus* spp.	Lamiaceae, Paeoniaceae, Brassicaceae, Rutaceae	Antimicrobial Antioxidant Cardiovascular activity	Petrović et al. (2019), Ivanova-Petropulos et al. (2015)

(continued)

Table 5.2 (continued)

Terpene/terpenoids	Examples of plant sources	Families	Described activity	References
β-phellandrene			Anti-inflammatory	Siqueira et al. (2016)
β-ocimene	*Ocimum* spp., *Artemisia* spp.	Lamiaceae, Asteraceae	Anti-inflammatory	Petrović et al. (2019)
γ-terpinene	*Foeniculum* spp., *Lavandula* spp., *Mentha* spp., *Origanum* spp., *Petroselinum* spp., *Salvia* spp., *Thymus* spp.	Apiaceae, Lamiaceae	Antiparasitic Antiviral	Pasquini et al. (2021), Yang et al. (2020)
β-terpinolene	*Melaleuca* spp.	Myrtaceae	Antioxidant	Guo et al. (2021)
Borneol			Antiviral	Yang et al. (2020), Paduch et al. (2007)
Myrtenol			Cardiovascular activity	Petrović et al. (2019)

Table 5.3 Relevant sesquiterpenes and their bioactivities

Terpene/ terpenoids	Examples of plant sources	Families	Activity	References
Costunolide	*Saussurea* spp.	Costaceae	Antitumour Anti-inflammatory	Yang et al. (2020), Paduch et al. (2007)
Artemisinin	*Artemisia* spp.	Asteraceae	Antitumour Antimicrobial Antiparasitic	Yang et al. (2020), Petrović et al. (2019), Paduch et al. (2007)
Farnesol	*Cymbopogon* spp., *Matricaria* spp.	Asteraceae, Poaceae	Antitumour Antimicrobial	Boukhatem (2020)
Caryophyllene	*Cannabis* spp.	Cannabaceae	Antitumour Anti-inflammatory Antiparasitic Antiviral Antidepressant	Cox-Georgian et al. (2019), Pasquini et al. (2021), Ivanova-Petropulos et al. (2015)
Caryophyllene oxide	*Cannabis* spp.	Cannabaceae	Antitumour Anti-inflammatory	Isidore et al. (2021)
helenalin	*Arnica* spp., *Inula* spp.	Asteraceae	Antitumour	Petrović et al. (2019)
eupalinin	*Eupatorium* spp.	Asteraceae	Antitumour	Petrović et al. (2019)
angeloylenolin	*Centipeda* spp.	Asteraceae	Antitumour	Petrović et al. (2019)
coronopilin	*Ambrosia* spp.	Asteraceae	Antitumour	Petrović et al. (2019)
eudesmanolide	*Inula* spp.	Asteraceae	Antitumour	Petrović et al. (2019)
cnicin	*Centaurea* spp.	Asteraceae	Antitumour Antimicrobial	Petrović et al. (2019)
artemisin	*Artemisia* spp.	Asteraceae	Antitumour	Petrović et al. (2019)
Patchouli alcohol	*Pogostemon* spp.	Lamiaceae	Antimicrobial	Yang et al. (2020)
Nerolidol			Antimicrobial Antioxidant	Ivanova-Petropulos et al. (2015)
Cadinene			Antimicrobial	Ivanova-Petropulos et al. (2015)
Spathulenol			Antimicrobial	Ivanova-Petropulos et al. (2015)
tanachin and tavulin	*Tanacetopsis* spp.	Asteraceae	Antimicrobial	Petrović et al. (2019)
centaurepensin A and derivatives	*Centaurea* spp.	Asteraceae	Antimicrobial Antiviral	Petrović et al. (2019)

(continued)

Table 5.3 (continued)

Terpene/ terpenoids	Examples of plant sources	Families	Activity	References
paeoniflorin and derivatives	*Paeonia* spp.	Paeoniaceae	Anti-inflammatory	Yang et al. (2020)
arglabin Artemisin	*Artemisia* spp.	Asteraceae	Antiparasitic	Petrović et al. (2019)
Germacrene D			Antioxidant Antiviral	Ivanova-Petropulos et al. (2015)
Artesunate			Antiviral	Yang et al. (2020)
Germacrone	*Eryngium* spp.	Apiaceae	Antiviral	Al-Salihi and Alberti (2021)
Patchoulol	*Pogostemon* spp.	Lamiaceae	Antiviral	Cox-Georgian et al. (2019), Al-Salihi and Alberti (2021)
β-santalol	*Santalum* spp.	Santalaceae	Antiviral	Al-Salihi and Alberti (2021)
13-acetyl solstitialin A	*Centaurea* spp.	Asteraceae	Antiviral	Petrović et al. (2019)
Maaliol	*Valeriana* spp.	Caprifoliaceae	Antidepressant	Cox-Georgian et al. (2019)

Table 5.4 Relevant diterpenes and their bioactivities

Terpene/ terpenoids	Examples of plant sources	Families	Activity	References
Paclitaxel/taxol	*Taxus* spp.	Taxaceae	Antitumour	Yang et al. (2020), Petrović et al. (2019)
Trichotomone	*Clerodendrum* spp.	Lamiaceae	Antitumour	Petrović et al. (2019)
Andrographolide	*Andrographis* spp.	Acanthaceae	Antimicrobial Antidiabetic	Yang et al. (2020), Cox-Georgian et al. (2019)
Phytol	*Cannabis* spp., *Jasminum* spp., *Olea* spp.	Cannabaceae, Oleaceae	Antimicrobial	Petrović et al. (2019)
Cassipourol β-sitosterol α-Amyrin	*Platostoma* spp.	Lamiaceae	Antimicrobial	Petrović et al. (2019)
Triptolidenol	*Tripterygium* spp.	Celastraceae	Anti-inflammatory	Yang et al. (2020)
Triptolide			Anti-inflammatory	Yang et al. (2020)
Putranjivain A	*Euphorbia* spp.	Euphorbiaceae	Antiviral	Yang et al. (2020), Paduch et al. (2007)
Stevioside	*Stevia* spp.	Asteraceae	Antidiabetic	Yang et al. (2020), Paduch et al. (2007)

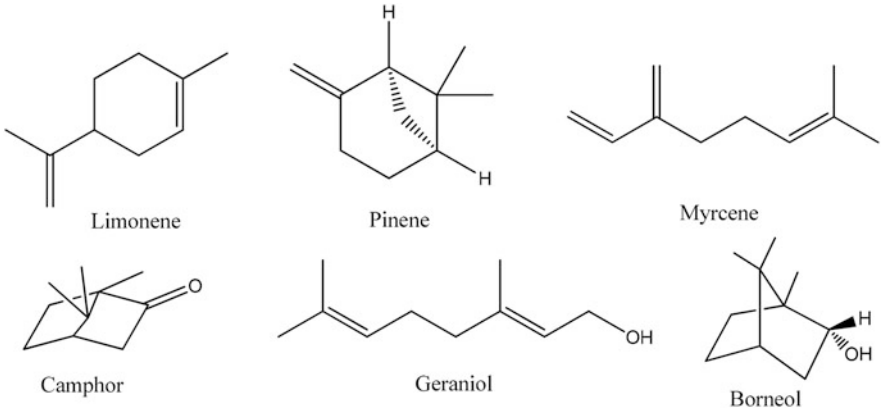

Fig. 5.5 Example of monoterpenes with relevant biological activity described in literature

adenoma and fore-stomach cancer formation against different cell lines (Patel and Thakkar 2014; Pina et al. 2022). Geraniol has been demonstrated to have both preventive and therapeutic potential against different types of tumours (lung, colon, prostate, pancreatic, and liver) when tested in different cancer cell lines but also in vivo using rats (Cho et al. 2016; Galle et al. 2014; Carnesecchi et al. 2001; Kim et al. 2011; Burke et al. 1997). As demonstrated by different studies, this can be due to the inhibition of the expression of the HMG-CoA (3-hydroxy-3-methylglutaryl coenzyme A) reductase gene in tumour cells. Therefore, geraniol has been suggested as a potential multitarget drug in chemotherapy since it can intervene in different processes such as cell proliferation, apoptosis, autophagy, and metabolism (Yang et al. 2020). By inhibiting the NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) activity, terpineol and its isomers have shown antitumoural activity when tested in cell lines (breast, lung, prostate, ovarian, and leukaemia) (Khaleel et al. 2018; Hassan et al. 2010). Similarly, the isomers thymol and carvacrol have also evidenced interesting antitumour properties. Several studies in vitro and in vivo have shown that thymol and carvacrol induced apoptosis, cytotoxicity, cell cycle arrest, and antimetastatic activity, and also displayed different antiproliferative effects and inhibition of signalling pathways (Sampaio et al. 2021; Islam et al. 2019; Fan et al. 2015). Linalool, a monoterpene found in many aromatic plants, has also demonstrated a high potential as an antitumoural compound since it promotes autophagy and apoptosis of tumour cells (prostate, breast, colorectal, and liver) either in mice or in vitro using different cell lines (Yang et al. 2020; Iwasaki et al. 2016; Pan and Zhang 2019; Chang and Shen 2014; Cerchiara et al. 2015; Xiu-Bin et al. 2015). In addition to its antitumoural effects, linalool and its derivatives also have anti-inflammatory activity since they can relieve the symptoms of inflammation by affecting the nuclear translocation of NF-κB and related pathways (Petrović et al. 2019; Ma et al. 2015). According to Ma et al. (2015), linalool inhibits acute lung inflammation in mice by producing interleukin-6 (IL-6), IL-1β, IL-8, tumour necrosis factor-alpha (TNF-α), and monocyte chemoattractant

protein-1 (MCP-1), which are involved in neuroinflammatory processes and several diseases. Other monoterpenes such as 1,8-Cineole, the main compound of *Eucalyptus* sp., and terpinen-4-ol, the main compound of *Melaleuca alternifolia*, have also demonstrated anti-inflammatory activity as they can suppress the production of several pro-inflammatory substances such as prostaglandins, tumour necrosis factor-alpha (TNF-α), and interleukin 1 beta (IL-1β) (Salha et al. 2021; Paduch et al. 2007). Paeoniflorin and its derivatives can inhibit the production of inflammatory factor nitric oxide (NO), interleukin 6 (IL-6), and TNF-α induced by lipopolysaccharides (Yang et al. 2020; Singh and Sharma 2015). The reduction of pro-inflammatory interleukins production is also the mechanism of action of α-pinene, explaining the anti-inflammatory activity of plants whose essential oil is rich in this compound, such as pine trees (Kim et al. 2015a). Besides the mentioned monoterpenes, borneol, β-myrcene, citronellol are also known for their anti-inflammatory activities (Salha et al. 2021; Paduch et al. 2007).

Terpenes have shown several other interesting biological properties, such as neuroprotection, and some are being studied to treat depression. According to Cox-Georgian et al. (2019), antidepressant properties are evidenced for linalool and pinene (active principles of *Litsea glaucescens*, *Tagetes lucida* and flowers of lavender) by interacting with the serotonin-1A receptors ($5HT_{1A}$) of the serotonergic pathway (Bonilla-Jaime et al. 2015). When inhaled, the monoterpenes car-3-ene, borneol, verbenol, and pinocarveol have also evidenced antidepressant properties (Pasquini et al. 2021). Some studies showed that myrcene, linalool, citronellol, 1,8-cineole, α- and γ-terpinene have neuroprotective functions owing to their antioxidant effect (Salha et al. 2021; Petrović et al. 2019; Singh and Sharma 2015). Furthermore, borneol can act as an essential neuroprotective agent against Alzheimer's disease due to its free radical scavenging activity (Yang et al. 2020). Several studies indicate that β-myrcene, menthol, camphor, citronellol, terpineol and its isomers, linalool and linalyl acetate, have analgesic or sedative properties. Therefore, menthol is frequently used topically as an analgesic agent, with the advantage of presenting antipruritic, counterirritant, and antiseptic activity. Similarly, camphor is traditionally used in different preparations, including liniments and cream formulations (Cox-Georgian et al. 2019; Salha et al. 2021; Singh and Sharma 2015; Paduch et al. 2007). Several monoterpenes, including terpineol and its isomers, 1,8-cineole, d-limonene, pinene, nerol, and geranial, as well as the essential oils containing them, have shown antimicrobial activity, which is most probably related to the lipophilic structure of these compounds (Pasquini et al. 2021). These compounds can easily pass through lipid bilayers, causing morphological changes in the cell membrane and cytoplasm, resulting in the leakage of intracellular material (Carson and Riley 1995). Especially terpinen-4-ol, 1,8-cineole and linalool possess antibacterial activity against bacteria isolated from the oral cavity, skin, and respiratory tract (Pasquini et al. 2021; Zengin and Baysal 2014; Wang et al. 2019b). The antimicrobial potential of menthol, particularly against *Escherichia coli* and *Candida albicans* has also been demonstrated (Gupta et al. 2021). Carvone and perillaldehyde were also able to inhibit the growth of *C. albicans,* with the former

being effective against *Listeria monocytogenes*, *Enterococcus faecium*, and *Escherichia coli* (Yang et al. 2020; Singh and Sharma 2015).

Antifungal activity was also evidenced by α-terpinene, which exhibited activity similar to commercial drugs, and by some terpenoids such as citronellal, citronellol, nerol, geraniol, borneol, and geranial (Salha et al. 2021; Paduch et al. 2007; Abbaszadeh et al. 2014). Moreover, monoterpene mixtures, such as the one including terpinen-4-ol, α-pinene, β-pinene, 1,8-cineole linalool, and α-terpineol, have also the capacity of limiting fungi development (Marei et al. 2012; Wang et al. 2019c).

In addition, paeoniflorin, a monoterpene glycoside isolated from the root bark of *Paeonia suffruticosa,* showed in vitro antifungal activity against *Candida albicans* and was effective against carbapenem-resistant *Klebsiella pneumoniae*, which are emerging and highly resistant pathogens that can have a significant impact in clinical practice but also in food production (Qian et al. 2020). Besides their interest as antibacterial and antifungal compounds, some volatile terpenes are known for their antiviral potential. Among them are 1,8 cineole, thymol, α-terpineol, α-and γ-terpinene, carvone, carveol, α- and β-pinene, camphor, β-ocimene, *p*-cymene, citral, and isoborneol. This last compound has great antiviral activity against Herpes simplex Virus-1 (HSV-1) by inhibiting the virus replication and the glycosylation of viral proteins (Cox-Georgian et al. 2019; Pasquini et al. 2021). Another relevant activity associated with monoterpenes is their antiparasitic activity. For example, d-limonene, α-pinene, and β-pinene have antiplasmodial properties, especially against *Plasmodium falciparum* (Petrović et al. 2019*;* Boukhatem 2020), while thymol and menthol have anti-leishmanial and trypanocidal activity (Luna et al. 2019). The antiparasitic functions of terpenes are related to their interaction with Fe (II) groups of the infected erythrocytes, releasing free radicals that can kill parasites (Rodrigues Goulart et al. 2004). Monoterpenes are also considered very interesting as natural anti-insect compounds. Besides its anti-inflammatory, antioxidant, and analgesic activities, citronellol, neral, and geranial, as well as thymol and menthol and their derivatives, have anti-insect properties and thus can be potential alternatives to synthetic insecticides (Reis et al. 2016; Al Dawsari and Alam 2022).

5.4.2 *Sesquiterpenes with Relevant Pharmacological Activities*

The chemical structures of some relevant sesquiterpenes are shown in Fig. 5.6. Several sesquiterpenes, such as caryophyllene, caryophyllene oxide, germacrene, and patchoulene, have been attracting attention due to their interesting biological activity, including anti-inflammatory, analgesic, antiparasitic, and antitumour properties. A pharmacological study revealed that β-caryophyllene isolated from the leaves of *M. koenigii* exhibited promising antimalarial activity against the chloroquine-sensitive strain of *Plasmodium* sp. (Kamaraj et al. 2017). Additionally, several studies highlighted its anti-inflammatory and tissue-protective properties by

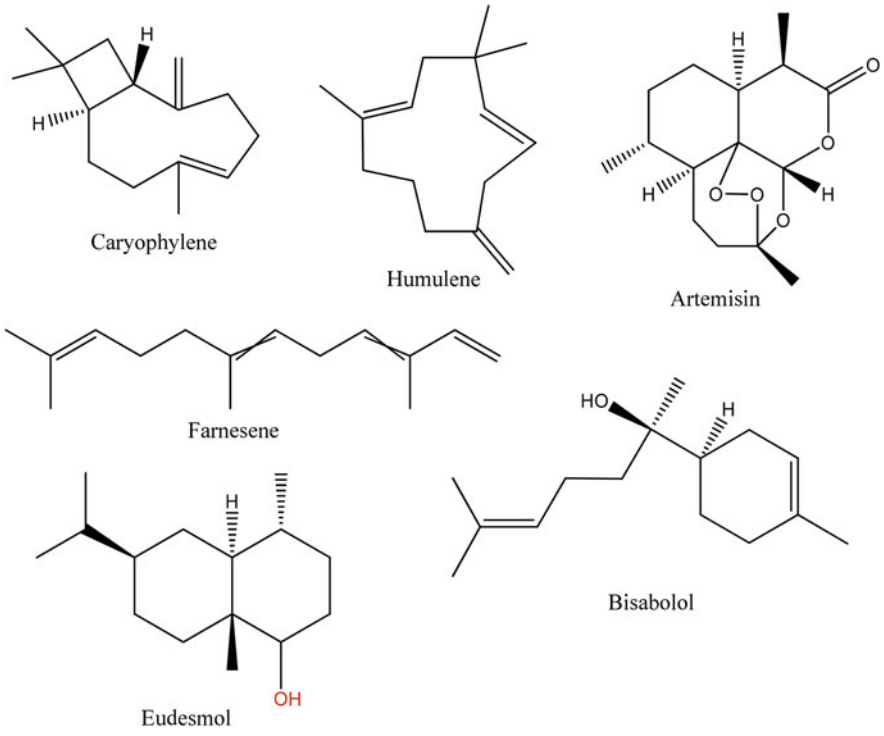

Fig. 5.6 Example of sesquiterpenes with relevant biological activity described in literature

modulating numerous signalling pathways and inhibiting inflammatory mediators, including cytokines (Jha et al. 2021). Its isomer humulene, or α-caryophyllene, given orally or by aerosol, exhibited anti-inflammatory properties in a murine model by reducing the inflammatory mediators (Rogerio et al. 2009). Caryophyllene oxide is also known for its similar properties since it evidenced anti-inflammatory activity in vivo and central and peripheral analgesia (Chavan et al. 2010). β-patchoulene also showed relevant anti-inflammatory activity in mice models with acute inflammation (Zhang et al. 2016a) being able to mediate a gastroprotective effect against the oxidative stress associated with a gastric ulcer, both in vitro and in vivo (Cox-Georgian et al. 2019). Other terpenoids with anti-inflammatory therapeutic potential are parthenolides (from *Tanacetum parthenium* L.), which suppress inflammation by inhibiting the activity of NF-κB (Mathema et al. 2012).

Besides having anti-inflammatory activity, β-caryophyllene can reduce alcohol-induced liver injury and has demonstrated neuroprotective properties against Parkinson's disease by acting on specific G-protein-coupled receptors, namely the cannabinoid CB2R, which is a receptor that reduces neuropathic pain and glial upregulation (Viveros-Paredes et al. 2017; Sharma et al. 2015; Navarro et al. 2016). Farnesene has two significant isomers, α and β, the former having four

stereoisomers and the last two (Tang et al. 2021). (E)-β-farnesene is a typical volatile component of many higher plants, being also an alarm pheromone in several insect species with the capacity of inducing a flee response in aphids (Bhatia et al. 2015; Zhang et al. 2017). Besides that, neuroprotective effects against H_2O_2-induced cell death in primary cultured cortical neurons have been demonstrated in previous studies (Turkez et al. 2014). Farnesol, an intermediate in the biosynthesis of farnesene, has been shown to inhibit tumorigenesis in animal models suggesting that it functions as a chemopreventative and antitumour agent in vivo (Joo and Jetten 2010). Similarly, β-eudesmol, a hydroxylated sesquiterpene, presented antitumour activity using in vitro and in vivo experimental models (Ma et al. 2008; Srijiwangsa et al. 2018). Moreover, this compound can inhibit angiogenesis, as demonstrated in mice implanted with subcutaneous Matrigel plugs and in mice adjuvant-induced granuloma (Small 1992). Other sesquiterpenes such as helenalin (*Arnica* sp.), eupalinin (*Eupatorium chinense* L.), inuviscolide (*Inula viscosa* L.), angeloylenolin (*Centipeda minima* L.) were also reported as antitumour agents by promoting autophagy in cancer cells, inhibiting the proliferation by cell cycle arrest and apoptosis (Petrović et al. 2019; Paduch et al. 2007). A similar mechanism of action mediated via G_2/M cell cycle arrest and promotion of apoptosis was reported for germacrone, which showed an antitumour effect on a human hepatoma cell line (He et al. 2019). Bisabolol, a compound widely used in pharmaceutical and cosmetic industries, and its isomers α-bisabolol and epi-α-bisabolol have evidenced antitumour effects against pancreatic cancer by inducing apoptosis and suppressing Akt (Protein kinase B) activation (Seki et al. 2011; Maurya et al. 2014). Moreover, Raut, Karuppayil (Raut and Karuppayil 2014) suggested that α-(-)-bisabolol may be a useful therapeutic candidate for treating skin inflammation. Another study suggests the use of α-bisabolol and nerolidol as potential antifungal agents against *Trichophyton* spp. (de Medeiros et al. 2021). This compound also shows potential to be incorporated in oral healthcare products since it evidenced antimicrobial activity against *Solobacterium moorei*, a Gram-positive bacteria associated with halitosis (Forrer et al. 2013).

Besides its antitumoural activity, germacrone also possesses antiviral activity as it was shown to inhibit influenza virus, porcine parvovirus, feline calicivirus, and porcine reproductive and respiratory syndrome virus replication. Germacrone has inhibitory effects on influenza A virus subtype H1N1 (H1N1), Influenza A virus subtype H3N2 (H3N2), and influenza B viruses in the early stages of the viral cycle and can protect mice from a fatal infection (He et al. 2019).

The artemisinins are a class of sesquiterpene lactones extracted from the sweet wormwood plant (*Artemisia annua*), being a successful example of drugs available in the market (Eckstein-Ludwig et al. 2003). They are potent antimalarial drugs since they can kill all stages of the malaria parasite (*Plasmodium falciparum*) and are thus widely used worldwide. The action mechanism of the artemisinin is thought to occur through haem-dependent activation of an endoperoxide bridge that occurs within the parasite's food vacuole (Ismail et al. 2016). Recently, artemisinin and its derivatives have attracted attention. Several in vivo studies confirmed that they have significant therapeutic effects on metabolic diseases, especially diabetes, by promoting insulin secretion and protecting pancreatic ß cells (Jiang et al. 2020).

5.4.3 *Diterpenes with Relevant Pharmacological Activities*

The chemical structures of some relevant dizer-enes are shown in Fig. 5.7. This class of compounds shows significant biological activities, including anti-inflammatory, anticancer, analgesic, antimicrobial, and antifungal activities. Some diterpenes also have cardiovascular activity, such as forskolin (Salehi et al. 2019), marrubenol (El Bardai et al. 2004), and kaurene (Ambrosio et al. 2006). They cause relaxation of smooth muscles in the walls of blood vessels by inhibiting Ca^{2+} channels (Salehi et al. 2019). Likewise, tanshinone IIA, an active ingredient isolated from the rhizome of *Salvia miltiorrhiza* Bunge, can act on Ca^{2+} influx and increase endothelial nitric oxide synthase protein expression and phosphorylation, leading to vasodilatation and blood pressure reduction (Shang et al. 2012). Recently, it has also been suggested that tanshinone IIA can prevent the formation of atherosclerosis and the damage and hypertrophy of the heart (Wang et al. 2017; Chen and Xu 2014). Due to the low intestinal absorption of this lipophilic compound, sodium tanshinone IIA

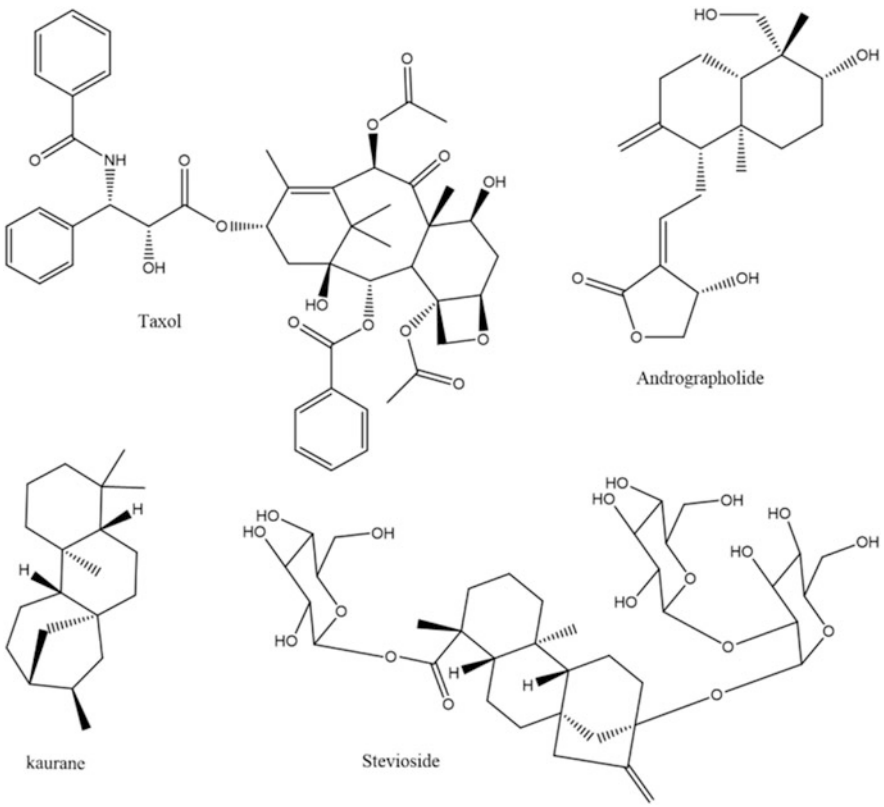

Fig. 5.7 Example of diterpenes with relevant biological activity described in the literature

sulfonate has been developed, aiming to increase its bioavailability (Shang et al. 2012).

Kaurenoic and pimaradienoic acids, isolated from the roots of *Viguiera* sp., have been evaluated on vascular smooth muscle contractility of rat aorta and showed pronounced antispasmodic and relaxant activity (Tirapelli et al. 2005). Resiniferatoxin, a vanilloid isolated from the *Euphorbia resinifera* latex, is used in clinical trials for bladder hyperreflexia and diabetic neuropathy (Appendino and Szallasi 1997).

Andrographolide, a diterpene-lactone isolated from herbaceous plant *Andrographis paniculata*, has promissory effects for treating diabetes (Nugroho et al. 2013). It also has potent antibacterial activity against most Gram-positive bacteria, showing interesting inhibitory properties on *P. aeruginosa* and *S. aureus* biofilms (Zhang et al. 2020).

The diterpenoid stevioside is an active ingredient isolated from *Stevia rebaudiana* (Bertoni) Hemsl. It possesses insulinotropic, glucagonostatic, and antihyperglycaemic effects, therefore acting as an antihyperglycaemic agent and inducing blood pressure reduction (Gregersen et al. 2004; Jeppesen et al. 2000). It has been shown that stevioside and the aglucon steviol potentiate insulin secretion in a glucose-dependent way in vitro (Gu et al. 2019). A clinical trial using stevia supplementation on glycaemic control improved cardiometabolic risk in diabetic patients (Rashad et al. 2019).

Paclitaxel is a tetracyclic diterpenoid isolated from *Taxus* sp, a known anticancer drug currently used in medicine to treat several kinds of tumours. Paclitaxel and its analogue docetaxel act as an antimitotic agent by binding to the microtubules (Yared and Tkaczuk 2012; Galletti et al. 2007).

The abietane-type diterpenoid ferruginol also displays antitumour properties in human ovarian cancer and malignant melanoma cell models by inhibiting cell migration and inducing apoptosis. Nevertheless, up until now, there is only one commercial drug composed of abietane-derived diterpenoids, ecabet sodium, which is used to treat reflux oesophagitis and peptic ulcer disease (González-Cardenete et al. 2021). In addition, abietane analogues such as ferruginol and dehydroabietinol exhibit inhibitory activity in vitro towards some *Mycobacterium* species and have been shown to have antiparasitic activity in the antipromastigote assay (González-Cardenete et al. 2021). Triptolidenol, triptolide, tripterine, and triptonide, which are isolated from *Tripterygium wilfordii*, are effective in inflammation and auto-immune disorders. Its primary mechanism consists in inhibiting the production of inflammatory cytokines (Jin et al. 2021).

The diterpene pepluanone, isolated from *Euphorbia peplus*, reduced inflammatory processes by inhibiting cell signalling (Barile et al. 2007), while putranjivain A, isolated from *Euphorbia jolkini*, demonstrated an antiviral effect against Herpes simplex virus-2 (HSV-2) in Vero cells by inhibiting cell penetration (Cheng et al. 2004). Andrographolide also showed promising antiviral activity due to inhibiting Chikungunya virus infection and reduced virus production (Wintachai et al. 2015). Other diterpenes with very interesting biological activity are the ginkgolides which show antagonistic activity towards platelet-activating factors, resulting in

antithrombotic effects (Cui et al. 2019). A clinical study showed that ginkgolide effectively improved neurological deficit after recombinant tissue plasminogen activator therapy in acute ischaemic stroke patients (Zhang et al. 2021).

5.4.4 *Triterpenes with Relevant Pharmacological Activities*

The chemical structures of some relevant triterpenes are shown in Fig. 5.8. The majority of triterpenes found in nature are cyclic triterpenes that can be further converted to various metabolites, including saponins. Saponins are not as abundant as most low molecular weight terpenes. However, this compound class presents a large diversity since they present different active sites allowing for the glycosylation of saponin aglycones (Dinda et al. 2010). Among saponins for which broad-spectrum pharmacological bioactivities have been reported, cucurbitacins are the most widely known (Alghasham 2013). Different studies have shown that these compounds, present in plants from the Cucurbitaceae family and used in traditional Chinese medicine, have different biological properties with particular relevance for their anticancer, anti-inflammatory, and hepatoprotective activity (Jing et al. 2020; Liu et al. 2021). Cucurbitacins exhibit anticancer activity through a variety of mechanisms, including the promotion of apoptosis through the Janus kinase/signal

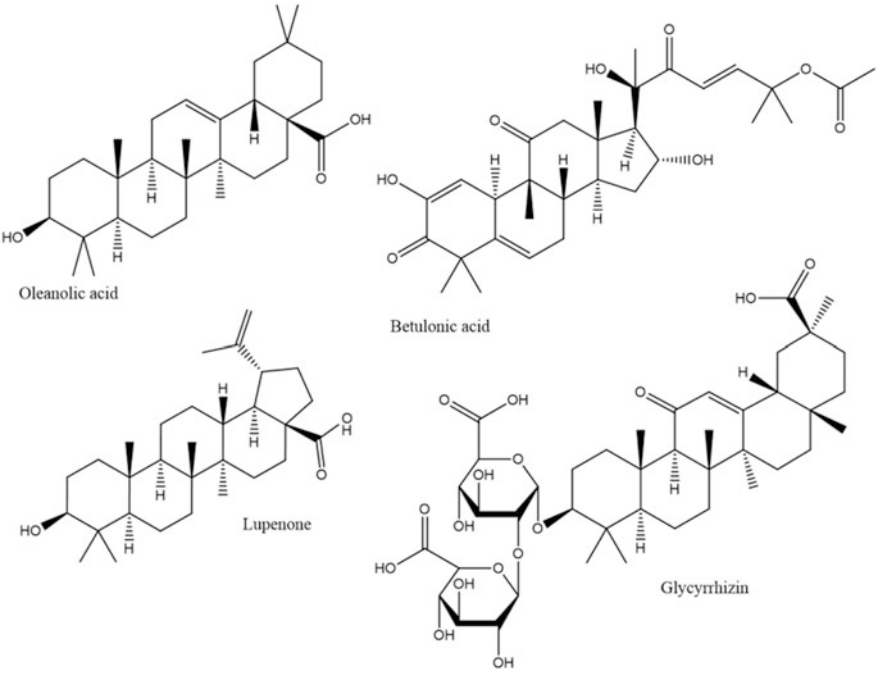

Fig. 5.8 Example of triterpenes with relevant biological activity described in the literature

transducer and activator of transcription (JAK/STAT3) and Mitogen-Activated Protein Kinase (MAPK) pathways, the promotion of cell cycle arrest by inhibiting cyclins and by inhibiting the invasion and migration of cancer cells (Jing et al. 2020). These highly oxidised tetracyclic triterpenes have shown synergistic effects when combined with some drugs and also have been proposed as potential chemotherapy before the onset of lung cancer (Jing et al. 2020; Liu et al. 2021).

Lupeol, a compound found in a wide range of fruits, vegetables, and medicinal herbs, manifests chemopreventive effects and, at the same time, shows direct antioxidant activity in vitro and on animal models due to decreased lipid peroxidation and increased enzymatic and non-enzymatic antioxidants (Chaturvedi et al. 2008; Palanimuthu et al. 2012).

Ursolic acid can induce tumour cell apoptosis and has apparent antitumour effects on the NCI-H292 human lung cancer cells in vitro (Chen et al. 2019). In another in vitro study with human osteosarcoma (143B cells), apoptosis was induced by inactivating Wnt/β-catenin signalling through upregulating p53 (Zhang et al. 2016b).

Another natural triterpenoid, pomolic acid (isolated from *Chrysobalanus icaco* and *Licania tomentosa*), is cytotoxic against K562 cell line (human erythroleukaemia) and possesses anti-multidrug resistance properties (Fernandes et al. 2003).

Jenisseensosides (glucosides of quillaic acid) isolated from the plant *Silene jenisseensis* and *Silene fortunei*, can stimulate the proliferation of the Jurkat cell line when in low concentrations, while in higher doses, they regress to apoptosis (Lacaille-Dubois et al. 1997; Gaidi et al. 2002). Avicins, saponins isolated from *Acacia victoriae,* have preventive effects against H-*ras* gene mutation and changes in carcinogenesis (Hanausek et al. 2001).

Oleanolic acid and its isomer, ursolic acid, are pentacyclic triterpenoid compounds isolated from plants that frequently occur simultaneously in nature, either in free acid form or as an aglycone precursor of triterpenoid saponins. Because they share similar structural features, they show similar pharmacological activities (Jesus et al. 2015). Oleanolic and ursolic acid have shown immunomodulatory activity induced by activating macrophages infected in vitro with *Mycobacterium tuberculosis* as they passed from an M2-like (macrophages anti-inflammatory) phenotype to a M1-like phenotype (macrophages pro-inflammatory) characterised by high production of TNF-α and reactive oxygen species (ROS), and low production of Transforming Growth Factor Beta (TGF-β), resulting in the control and elimination of the intracellular mycobacteria (López-García et al. 2015). In another study, a clinical trial was performed with adolescents that ingested olive oil enriched with oleanolic acid to obtain postprandial triglyceride-rich lipoproteins that were further incubated with THP-1 (monocyte-like cell line) derived macrophages, showing that this compound has a high potential to prevent metabolic syndrome due to its anti-inflammatory activity (Fernández-Aparicio et al. 2021).

Ginsenosides are a group of glycosylated triterpenes constituents of ginseng that show anti-inflammatory activity both in vitro and animal models. Its mechanism of action is related to the inhibition of the NF-κB signalling pathway, suppressing the

production of pro-inflammatory mediators and enzymes such as TNF-α, inducible Nitric Oxide Synthase (iNOS), and cyclooxygenase-2 (COX2) (Kim et al. 2017).

Studies have revealed that ginsenosides can play a relevant role in treating diabetes and ameliorate insulin resistance in the liver. Experimental and clinical data emerge to support the antidiabetic efficacy of ginsenosides attributed to their anti-inflammatory, antioxidant, and antihyperglycaemic activities, therefore, supporting the use of *Panax ginseng* in traditional medicine as an adjuvant to treat diabetes mellitus (Shao et al. 2020). In addition to their anti-inflammatory effects, ginsenosides also have an antioxidant effect. They can protect cardiomyocytes from oxidative damage caused by internal and external oxidants (Sarhene et al. 2021) and act on other cardiovascular diseases by regulating vascular function, inhibiting cardiomyocyte hypertrophy, and inhibiting thrombosis (Lee and Kim 2014).

Anti-inflammatory activity has also been ascribed to α-amarin, a pentacyclic terpene that exhibited this activity in rat models by reducing neutrophil infiltration and pro-inflammatory cytokine production of acute periodontitis (Holanda Pinto et al. 2008).

Other pentacyclic triterpenoids, such as boswellic acids isolated from the gum resin of *Boswellia* sp. (in particular cetyl-11-keto-ß-boswellic acid and 11-keto-ß-boswellic acid), have also evidence the same activity (Ammon 2016). Among other mechanisms of action, boswellic acids can act by inhibiting leukotriene synthesis and, to a less extent, prostaglandin synthesis. Various preclinical and clinical studies have established that these compounds exhibit substantial potential in managing inflammatory such as diminishing inflammation in osteoarthritis and colitis and helping control the brain oedema associated with radiotherapy of cerebral tumours (Takahashi et al. 2012; Hamidpour et al. 2013). In addition, it has been demonstrated that boswellic acids have an inhibitory and apoptotic effect in vitro against the cellular growth of leukaemia HL-60 cells, inhibiting the synthesis of DNA and RNA in a dose-dependent manner (Shao et al. 1998).

Different triterpenes also play a role in microorganism growth inhibition or elimination. Oleanolic acid and its derivatives present a specific inhibitory effect on *Staphylococcus aureus*, including methicillin-resistant strains, *Streptococcus mutans, Listeria monocytogenes, Enterococcus faecium*, and *Enterococcus faecalis* by destroying the cell membranes of the bacteria (Blanco-Cabra et al. 2019; Kim et al. 2015b). They were also active against *species of mycoplasma*, and Gram-positive and Gram-negative bacteria also have anti-human immunodeficiency virus (anti-HIV) activity (Khwaza et al. 2018).

Likewise, many studies have shown that glycyrrhizin, a saponine from liquorice (*Glycyrrhiza glabra*) has potent effects in inhibiting Gram-positive and Gram-negative bacteria, such as *Staphylococcus aureus*, *Escherichia coli, Pseudomonas aeruginosa,* and *Bacillus subtilis* as well as the yeast *Candida albicans* (Karahan et al. 2016; Wang et al. 2015; Martins et al. 2016b). Glycyrrhizin has also demonstrated antiviral activity, with the possible mechanism of action being the decrease of the expression of genes of virulence. Glycyrrhizin has anti-HSV-1 and anti-HSV-2 activities in vivo and in vitro*;* therefore, can significantly inhibit virus replication and reduce the number of infectious virus particles (Huan et al. 2021). It also effectively

inhibited the Severe Acute Respiratory Syndrome-Associated Coronavirus (SARS-CoV) virus replication through cellular signalling pathways such as protein kinase C (Cinatl et al. 2003). Moreover, it is proposed that the long-term administration of glycyrrhizin protects patients with chronic hepatitis C from developing hepatocellular cancer. It improves the outcomes of interferon administration due to a synergistic effect (Ashfaq et al. 2011).

Antiviral activity has also been evidenced by moronic acid and betulonic acid, two compounds extracted from the plant *Rhus javanica* that showed a potent in vitro inhibitory effect on Herpes Simplex Virus-1 (HSV-1). Betulinic acid and its derivatives also have anti-human immunodeficiency virus activity (Huang et al. 2018) being considered the earliest discovered pentacyclic triterpenoid compound with anti-HIV activity (Kumar et al. 2018; Aiken and Chen 2005). Additionally, they have been shown to inhibit the replication of vesicular stomatitis virus and encephalomyocarditis virus (Cavalcante et al. 2020; Meira et al. 2019).

Notoginsenoside ST-4i, isolated from the Chinese herb *Panax notoginseng*, demonstrated in vitro inhibitory activities against HSV-1 and Herpes Simplex Virus-2 (HSV-2) (Pei et al. 2011).

Lupenone and its saponin showed in vitro viral plaque inhibitory effects against HSV-1 and HSV-2 (Xu et al. 2018b). Lupenone isolated of rhizoma *musae* (the root of *Musa basjoo* Sied. et Zucc.) also has antidiabetic activity by inhibition of α-amylase and α-glucosidase in diabetic mice (Xu et al. 2021). Table 5.5. describes the activities and examples of plant sources for relevant triterpenes.

5.5 Terpene Applications, Challenges, and Future Perspectives

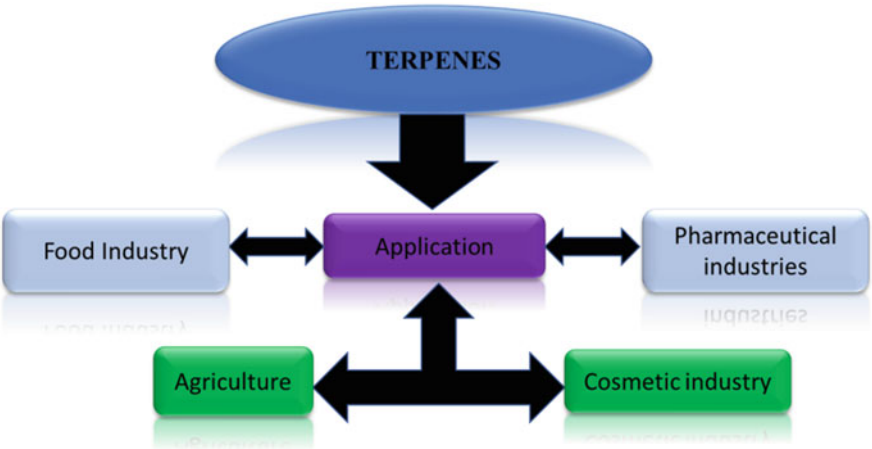

Table 5.5 Triterpenes and their bioactivities

Terpene/ terpenoids	Examples of plant sources	Families	Activity	References
Ursolic acid	*Malus* spp., *Ocimum* spp., *Vaccinium* spp., *Olea* spp., *Origanum* spp., *Rosmarinus* spp., *Salvia* spp., *and Thymus* spp.	Rosaceae, Lamiaceae, Ericaceae, Oleaceae	Antitumour Antimicrobial	Yang et al. (2020), Paduch et al. (2007)
Betulinic acid	*Betula* spp., *Diospyros* spp.	Betulaceae, Ebenaceae	Antitumour Anti-inflammatory Antiparasitic Antiviral	Yang et al. (2020), Singh and Sharma (2015)
Oleanolic acid	*Rosmarinus* spp., *Akebia* spp.	Lamiaceae, Lardizabalaceae	Antimicrobial	Park et al. (2018)
Tripterine	*Tripterygium* spp.	Celastraceae	Anti-inflammatory	Zhu et al. (2021)
Ginsenoside	*Panax* spp.	Araliaceae	Anti-inflammatory	Yang et al. (2020)
Glycyrrhizin	*Glycyrrhiza* spp.	Fabaceae	Antiviral	Yang et al. (2020), Fiore et al. (2008)
Dammarelonic acid	*Cowania* spp., *Gyrocarpus* spp., and *Canarium* spp.	Rosaceae	Antiviral	Poehland et al. (1987)
Moronic acid	*Rhus* spp.	Anacardiaceae	Antiviral	Yang et al. (2020), Paduch et al. (2007)

5.5.1 Application

Besides playing a fundamental role in plants, terpenes and their derivatives find diverse applications in several industries, such as food, pharmaceutical, flavours, fragrances, and biofuels (Bicas et al. 2009; Tetali 2019; Harman-Ware 2020b). Herbs and spices have been used since ancient times to preserve food due to their antimicrobial and insecticidal properties ascribed to their composition in terpenes (Gottardi et al. 2016). These properties were probably the basis of their initial use in ancient medicine and supported their continued use of mouthwashes, cough medicines, disinfectants, and insect repellents. More complex structural terpenes frequently show other properties of interest that have been taken into an advantage for their health benefits (Guimarães et al. 2019; Papada et al. 2018; Bhavaniramya et al. 2019). In this regard, taxol and vinblastine are used as drugs in cancer treatment. In contrast, others mimic animal hormones such as diosgenin, a sterol synthesised from terpenoid precursors and present in the Mexican yam (*Dioscorea*

mexicana), has been used to produce progesterone for birth control pills and other medicinal steroids (Demain and Vaishnav 2011; Jesus et al. 2016). The most important use of terpenes by humans is for consumption since they are required to produce essential molecules, such as vitamin A synthesised from β-carotene, an abundant plant terpene (Wagner and Elmadfa 2003; Grassmann 2005). More commonly, terpenes in foods affect our eating experience. Terpenoid pigments, such as astaxanthin and lycopene, are heavily used in the food industry as natural colours, for they have a large influence on the acceptability of many foods (Mata-Gómez et al. 2014). Volatile terpenoid compounds impart flavour to foods via their detection by the olfactory system (Schieber and Wüst 2020). For example, the ginger flavour is caused by nootkatone (Srinivasan 2017), and zingiberene imparts a grapefruit flavour (El Hadi et al. 2013). Many herbs used as fresh plant material contain volatile organic compounds (VOCs) that are used in the preparation of flavours in beverages. For example, a soda contains more than one citrus oil (e.g., lemon, lime, orange, neroli) (Ameh and Obodozie-Ofoegbu 2016), already in the preparation of cola flavours are used significant amounts of spice oils such as cinnamon (Buglass and Caven-Quantrill 2012). In terms of alcoholic spirits, specifically for gin, the aroma stems from the use of botanicals during the distillation process, whereby individual aroma notes are imparted by constituent odour-active VOCs predominant compounds, whereby monoterpenes were the most frequently identified compounds, assigned mainly to juniper and coriander oil (Buck et al. 2020). More recently, studies have reported that some terpenes have adequate properties, such as viscosity, flash point, high energy density, among others, that make them good candidates for speciality biofuels, capable of supplementing or even replacing current petroleum-derived fuels (Mewalal et al. 2017; Donoso et al. 2022; Donoso et al. 2021). In the last years, the most promising examples include the monoterpenes limonene and β-phellandrene (Lei et al. 2021), the sesquiterpenes farnesene and bisabolene (Gupta and Phulara 2015) and the diterpenes phytene and cambrene (Scown et al. 2021). Although plant terpenes represent a potential source of alternative and sustainable energy, their implementation as commercial biofuels is compromised by the low yield of specific terpenes in plants (Jiang et al. 2016). Nevertheless, commercial-scale recovery of plant terpenes from some plants such as pine, citrus, and eucalyptus is already taking place and together with the recent advances of biotechnological tools such as genome editing, can pave the way for effective use of terpenes as a feedstock for alternative biofuels (Beller et al. 2015).

5.5.2 *Challenges and Future Perspectives in the Terpenes Industry*

Terpenes are compounds of great industrial interest with a wide range of applications. However, there is still a long way between the characterisation and isolation of terpenes and their extraction and application on an industrial scale (Jiang et al. 2016).

There are resources throughout nature that could be discovered with applications of human interest. Secondary metabolites from plant matrices, especially terpenes, are still an underexplored source of compounds, with only a small proportion being investigated. Diverse genetic and climatic factors influence the synthesis of secondary plant metabolites (Pang et al. 2021). Biotyping factors such as plant competition, pollinators, viruses, bacteria and predators, and abiotic conditions such as soil type, hydric or climatic stress, high light intensity, among others, alter the production of terpenes in plants. Besides climatic and biotyping factors, there are also difficulties related to botanical species' slow growth, low yield, and incomplete knowledge of biosynthesis mechanisms (Dudareva et al. 2013). Therefore, developing a model that can standardise the production of terpenes in plants under different environmental conditions is still a challenge for the industry. However, the biosynthesis of many natural products is still poorly understood, and this hampers the ability to engineer microbial hosts to produce these compounds (Beutler 2009). The anticancer agent paclitaxel (taxol) biosynthesis is an excellent example of this problem. The compound is derived from the bark of the slow-growing evergreen Pacific yew tree (*Taxus brevifolia*), and the bark is the only part of the plant that is used commercially. The bark is harvested sustainably, but it is still a limited resource. In addition, the complex biosynthesis of paclitaxel makes it challenging to produce, resorting to recombination techniques and microbial fermentation engineering. A potential solution to this problem may be genetically modified plants (Croteau et al. 2006; Zhu and Chen 2019). This technology would involve constructing a metabolic pathway to produce the natural product in the microbial frame and incorporate the necessary genes into the organism's genome (Heskes et al. 2018). From a future perspective, a better understanding between the action of molecules, the synergy or antagonism of compounds, and the proof of the biological activity in vivo is of paramount importance, with the synthetic production of terpenes being a potentially viable solution in the future for terpenes supply and the development of industries that have these compounds as raw material.

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Chapter 6
Plant Alkaloids: Production, Extraction, and Potential Therapeutic Properties

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Abstract Alkaloids are a type of secondary metabolites that can be found in different parts of plants. This group of compounds is diverse and can be divided into nine subgroups: pyridine, tropane, isoquinoline, phenanthrene, phenylethylamine, indole, purine, imidazole, and terpenoids. Most of these compounds are recognized for their anti-inflammatory, antitumor, antibacterial, antifungal, and antiviral activities, among others. Although more than 27,000 alkaloids have been described up to date, the search for novel compounds with promising therapeutic properties is a hot topic among researchers worldwide. In this line, the production of the currently marketed plant alkaloids including extraction methods, isolation, and purification is reviewed in this chapter. In addition, a deep description of different groups of alkaloids in terms of their chemical structure, plant source, and uses is also

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presented. Recent advances in the therapeutic potential and biological activities of this vast group of phytochemicals are also included.

Abbreviations

Techniques of extraction

AE	Acid extraction
DE	Decoction
DES	Eutectic solvents
HP	Hot water under pressure
HPPE	High-pressure propane extraction
MA	Maceration
MAE	Microwave-assisted extraction
ME	Methylation
PHWE	Pressurized hot water extraction
SFE	Supercritical fluid extraction
SO	Soxhlet
UAE	Ultrasound-assisted extraction
WE	Water extraction

Techniques of identification and quantification

GC-MS	Gas chromatography coupled to mass spectrometry
HPLC	High-performance liquid chromatography
HPLC-Q-TOF-MS	High-performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry
HPLC–UV–ESI MS/MS	HPLC with UV detection coupled with electrospray ionization MS/MS
HPTLC	High-performance thin-layer chromatographic
HSCCC	High-speed counter-current chromatography
NMR	Nuclear magnetic resonance
TLC	Thin-layer chromatography
UHPLC–Q-TOF-MS	Ultra-high performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry

Types of alkaloids

IA	Isoquinoline alkaloids
InA	Indole alkaloids
IzA	Imidazole alkaloids
PA	Pyridine alkaloids
PeA	Phenylethylamine alkaloids
PhA	Phenanthrene alkaloids
PuA	Purine alkaloids
TA	Tropane alkaloids
TeA	Terpenoid alkaloids

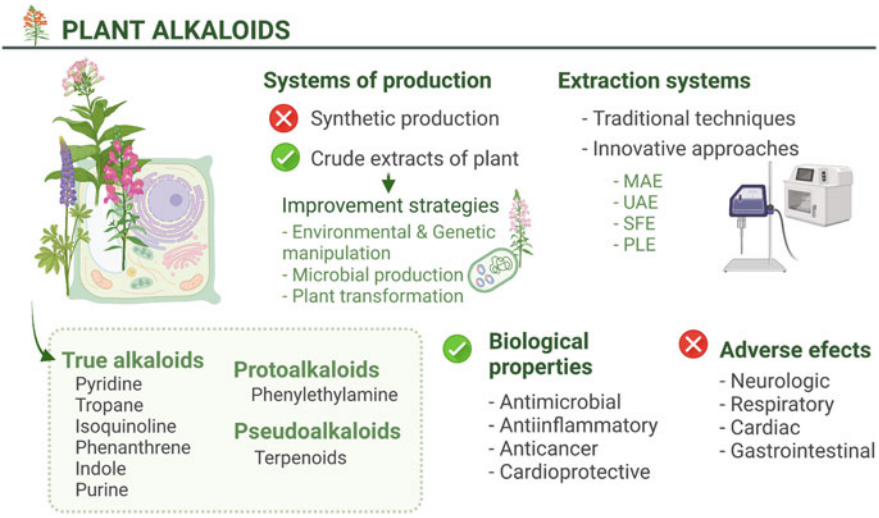

Fig. 6.1 Schematic representation of plant alkaloids identification. From accumulation and extraction to potential therapeutic properties

Others

FDA	Food and Drug Administration
HR	Hairy root culture
MEP	Methyl-erythritol phosphate
Nd	Not determined

6.1 Introduction

Plants have essential compounds for their physiological functions and their development. Among them, approximately 12,000 are alkaloids (Brugnerotto et al. 2021), secondary metabolites, which contain at least one nitrogen molecule in their chemical structure generally forming a ring (Kohnen-Johannsen and Kayser 2019). Alkaloids are present as salts of organic acid (oxalic, acetic, malic, lactic, tartaric, tannic, and aconitic acids) in different parts of plants, such as leaves (*i.e.,* nicotine), bark (*i.e.,* quinine and cinchonine), seeds (*i.e.,* nibidine and strychnine), and roots (*i.e.,* rawelfinine, glycyrrhizin, and punarnavine) (Muthaura et al. 2007). They are extremely abundant in flowering plants (Angiospermae); nevertheless, their presence and distribution depend on plant species and the stage of the plant life cycle (Chiocchio et al. 2021). In plants, alkaloids are involved in roles such as seed dispersal and pollinator attraction. In addition, they can protect plants from pathogens and prevent non-specialist herbivore grazing, showing the purpose of defense (Griffiths et al. 2021). Besides plants, alkaloids can be found in some fungi, like psilocybin in the fungus of the genus *Psilocybe*, and in certain animals, like bufotenin in the skin of some toads and different insects (Awuchi 2019). Many

marine organisms like cyanobacteria also contain alkaloids (Rodriguez et al. 2014; Sanchez et al. 2014). Biological precursors of most alkaloids are amino acids like ornithine, leucine, lysine, tyrosine, tryptophan, histidine, and phenylalanine (Chiocchio et al. 2021). Several biosynthesis pathways of alkaloids have been proposed, including synthesis of Schiff bases and Mannich reaction (Roberts et al. 2018). The biosynthesis and accumulation of alkaloids are related to several kinds of plant cells such as endodermis, epidermis, phloem parenchyma, phloem sieve elements and companion cells, pericycle, specialized mesophyll, and laticifers (Ziegler and Facchini 2008). Despite the difficulty of studying where alkaloids are formed, many studies seem to indicate that the endoplasmic reticulum is the preferred site for alkaloid formation. Moreover, biosynthetic enzymes have been associated with chloroplast thylakoid membranes, vacuoles, mitochondria, and the cytosol (Ziegler and Facchini 2008).

Alkaloids constitute a large diverse group of compounds with different origins and structures. In this context, they can be classified from several points of view. According to their chemical structure, alkaloids derived from amino acids are classified as true alkaloids (containing nitrogen atom in their heterocyclic ring) and protoalkaloids (nitrogen atom is not part of heterocyclic ring). By contrast, those alkaloids that are not derived from amino acids are called pseudoalkaloids (containing nitrogen atom in their heterocyclic ring) (Fig. 6.1) (Rios et al. 1989). Another widely established classification is based on the presence of a basic heterocyclic nucleus in their structure, for example, pyridine, tropane, isoquinoline, phenanthrene, phenylethylamine, indole, purine, imidazole, or terpenoid groups (Swamy 2020).

In general terms, all alkaloids are basic, crystalline, odorless, bitter, and colorless and the alkaloid salts are water soluble and the free bases of alkaloids are soluble in organic non-polar solvents (Cinelli and Jones 2021). These compounds can differ in terms of solubility. For example, pseudoalkaloids and protoalkaloids have shown higher solubility in water than true alkaloids. This is the case of colchicine soluble in alkaline water while the free base of caffeine and quinine hydrochloride is quite soluble, and quinine hydrochloride is highly soluble in water (1000 times more than caffeine) (Browning 2014). The solubility of alkaloids and their salts has been employed in the pharmaceutical industry for the extraction and formulation of therapeutic products and drug development (Das et al. 2020).

Alkaloid-containing plants have shown diverse biological activities including anti-inflammatory, antitumor, antibacterial, antifungal, and antiviral activities (Fig. 6.1). These compounds have been used as medications, recreational drugs, or in entheogenic rituals. The use of alkaloids for treating certain diseases has a long history. In 1888, Kossel isolated theophylline from tea leaves (Reiss and Wyshusek 2021), and in the 1920s Di Macht and S Hirsch described its bronchodilator effect (Barnes 2006), giving application in clinical practice. Another example is the codeine which was isolated by Pierre-Jean Robiquet from opium in 1832 (Wisniak 2013). Today, both alkaloids are found in some medicines as active agents. Other examples of alkaloids with pharmacological potential used in traditional and modern medicine are quinine (antimalarial), ephedrine (antiasthma), homoharringtonine

(anticancer), galantamine (cholinomimetic), vincamine (vasodilatory, quinidine (anti-arrhythmic), morphine (analgesic) chelerythrine (antibacterial), and piperine (antihyperglycemic) (Rios et al. 1989). In the last years, numerous studies have addressed the development of drugs from alkaloids testing new bioactivities (Garg and Roy 2020; Kloc et al. 2020; Borquaye et al. 2020). For example, a recent study has revealed that talimonine and sofalin D obtained from the plants *Thalictrum simplex* and *Sophora alopecuroides*, respectively, could prevent the viral replication of SARS-CoV2 (Garg and Roy 2020). Similarly, cryptosminrin, cryptospirolepine, biscriptolepine from *Cryptolepis sanguinolenta* could be also potential inhibitors of SARS-CoV2 virus proteins (Borquaye et al. 2020). However, many alkaloids are toxic to humans (Griffiths et al. 2021). For example, psilicin possesses psychotropic activity, while cocaine, caffeine, nicotine, and theobromine present stimulant activity. Atropine and tubocurarine are also toxic alkaloids (Chiocchio et al. 2021). Nevertheless, most toxic effects are due to overuse or misuse of such drugs. The increase of side effects is related to the production and sale of these drugs in an uncontrolled or unregulated way or their incorrect use due to the consumer unknowledge (Upadhyay et al. 2012).

In this context, the production of the currently marketed plant alkaloids including extraction methods, isolation, and purification is reviewed in this chapter (Fig. 6.1). In addition, a deep description of different groups of alkaloids in terms of their chemical structure, plant source, and uses is also presented. Recent advances in the therapeutic potential and biological activities of this vast group of phytochemicals are also included.

6.2 Production of Plant Alkaloids

6.2.1 Systems of Production

In recent years, there have been significant advances in elucidating the biosynthetic pathways of plant-derived alkaloids (Lichman 2021). In general, all complex alkaloids are synthesized following some common steps: (i) accumulation of precursor amines, (ii) accumulation of an aldehyde precursor, (iii) formation of an iminium cation, and (iv) a Mannich-type reaction (Lichman 2021). The elucidation of these biosynthetic pathways has allowed the use of enzymes as biocatalysts for the in vitro formation of alkaloids (Roddan et al. 2020). This advance was possible due to the new insights in genomics and advances in sequencing technologies (Lichman 2021). This knowledge allowed the development of different strategies to increase the concentration of alkaloids in plants as alternatives to traditional production methods, also presenting the advantage of having a certain degree of stereoselectivity (Roddan et al. 2020). Thus, the desired isomer could be produced in considerable quantities, increasing the process's yield and purity (Li et al. 2018).

The conventional strategy for improving the alkaloids accumulation in plants consists of manipulating physical aspects and nutritional elements of the crop (*e.g.*,

supplementation with mannitol and sucrose for the production of quinoline alkaloids or nitrate, ammonium, phosphate ions, and sucrose concentration for the production of galanthamine in *Leucojum aestivum*) (Ahmad et al. 2013). Another method for increasing alkaloids accumulation consists of the development of cell plant cultures (Ratnadewi 2017). This method is based on culturing plant seeds, organs, explants, tissues, cells, or protoplasts on a chemically defined synthetic nutrient media under sterile and controlled conditions of light, temperature, and humidity (Bhatia 2015). Thus, it allows improving production yields through environmental and genetic manipulations. In cell cultures it is necessary to optimize culture conditions, select high metabolite producing strains, and select precursors feeding and growth regulators (Ahmad et al. 2013). However, a major limitation of plant tissues and cell lines is the inability to produce certain alkaloids due to a lack of specialized cells (Ratnadewi 2017; St-Pierre et al. 1999; Furusaki and Takeda 2019). More research on the metabolism involved in alkaloid synthesis and the specific cell type necessary for each alkaloid is still necessary (Ratnadewi 2017). Recent advances in the development of genetic tools can overcome problems of slow growth, low yields, and instability in routine cell cultures (Srivastava and Srivastava 2013). In this regard, the most promising genetic modifications are those that simultaneously affect several metabolic points, such as transcription factors (Gantet and Memelink 2002). Another alternative is the use of microbial cells, including bacteria and yeasts (Schläger and Dräger 2016). Microbes are even more scalable than plant tissues and cell cultures. The degree of complexity of microorganisms is significantly lower, in terms of lack of preexistence, branching pathways, and transcription factors, which simplifies metabolic engineering design (Srivastava and Srivastava 2013).

Another alternative to increase production is the use of hairy root culture (HR). HR is obtained after infection of a wide variety of plants by *Rhizobium rhizogenes* (Sharma et al. 2013). This method allows expressing the natural biosynthetic pathways of plants due to rol gene effect. Briefly, the bacterium transfers a gene sequence (rolA, rolB, or rolC) that induces the biosynthesis of various secondary metabolites. The transfer of one or more of these combinations of genes gives different results, being in the case of the combination of all genes (rolABC constructs) comparable to the results obtained in nature (Sharma et al. 2013). Thus, HR produces the same biochemical products as its wild counterparts (Gutierrez-Valdes et al. 2020; Gantait and Mukherjee 2021). Among the advantages of this production system, the high growth rate and the genetic and biochemical stability of the HR over a prolonged period are some of the most significant (Supriya et al. 2020). In addition, alkaloid production by HR can be increased with genetic engineering to regulate secondary metabolism production (Gutierrez-Valdes et al. 2020; Gantait and Mukherjee 2021) or by selecting high productivity root lines based on the somaclonal variation (Sevón and Oksman-Caldentey 2002; Sharma and Agnihotri 2021). In recent years, several bioactive compounds, including tropane and nicotine alkaloids have been produced by large-scale continuous HR cultivation in bioreactors (Supriya et al. 2020; Huang et al. 2018; Häkkinen and Oksman-Caldentey 2018). In fact, it is possible to find products in the market produced in bioreactors (*e.g.*, paclitaxel, morphine). For instance, bioreactor technology has been applied for

the production of ginsenine and ginsenosides, alkaloids from *Panax ginseng* and for artemisinin alkaloid production from *Artemisia annua* (Huang et al. 2018; Häkkinen and Oksman-Caldentey 2018).

6.2.2 *Extraction, Purification, and Isolation of Plant Alkaloids*

Chemical synthesis of alkaloids is expensive due to the existence of numerous chiral centers. The use of alternative systems (for example, microorganisms) is not feasible, due to the incomplete characterization of the pathways required for their synthesis. Thus, plants are still the best source of many of these compounds (Hughes and Shanks 2002). However, in most cases, alkaloids are not the end-product of a metabolic pathway, but they are by-products or parts of diverse metabolic routes. Therefore, these compounds can be metabolized to obtain other alkaloids through synthesis or degradation processes. The final product depends on the physiological needs and signaling of each plant. Thus, it is difficult to predict the concentration or presence of each alkaloid (Aniszewski 2015).

Traditionally, acid, base, or organic solvent extractions have been used for obtaining alkaloids extracts from plants (Hughes and Shanks 2002). As alkaloids are mostly found in plants as salts of organic and inorganic acids bound to complex mixtures of water-soluble compounds, acidic and basic solutions are required to remove those non-alkaloid compounds (Petruczynik 2012). Base extractions consist of mixing the grounded plant material with alkaline solvents (*e.g.*, 1,2-dichloroethane, chloroform, diethyl ether, benzene). This results in the formation of base alkaloids that can be converted to salts by adding weak acids and washed with water to crystallize. Then, appropriate purification methods are applied to obtain the desired level of purity (Madani et al. 2021). On the other hand, acidic extractions consist of mixing the grounded plant material with weak acid solutions (*e.g.*, acetic acid in water, methanol, or ethanol). A basic solution (*e.g.*, ethyl acetate, bicarbonate) is added to the previous solution to convert the alkaloids into basic forms and thus, be able to be extracted with organic solvents (Edagha et al. 2014).

Other extraction approaches have been proposed for obtaining alkaloids from plants such as maceration, percolation, and Soxhlet, although the latter has shown the lowest extraction yield. This may be attributed to the high extraction temperature applied in the process (Zhang et al. 2018). In addition, emerging and sustainable extraction approaches have been applied for such purposes (Klein-Júnior et al. 2016), including microwave-assisted extraction (MAE) (Zhang et al. 2018; Jones et al. 2001; Xiong et al. 2016), ultrasound-assisted extraction (UAE) (Jones et al. 2001; Ashihara et al. 2020; Hien et al. 2021), supercritical fluid extraction (SFE) (Verma et al. 2008), pressurized liquid extraction (PLE), high-pressure propane extraction (HPPE), or pressurized hot water extraction (PHWE) (Kopp et al. 2020). These advanced technologies are preferred, because they are less

Table 6.1 Occurrence of some groups of alkaloids (pyridine, tropane, isoquinoline, and phenanthrene) in plants, extraction conditions to exert certain activities and side effects

Compound	Chemical structure	Source	Activities	Adverse effects	Extraction	Yield	References
Pyridine							
Nicotine	$C_{10}H_{14}N_2$	*Nicotiana tabacum*	Smoking cessation	Nasal mucosa irritation, arthralgia, nausea, vomiting, mild headache	Leaves// SFE	21.3–23.0 mg/g	Ashihara et al. (2020), Fischer and Jefferies (1996), Debnath et al. (2018)
Cytisine	$C_{11}H_{14}N_2O$	*Laburnum anagyroides, Argyrolobium uniflorum, Templetonia incana, Petteria ramentacea*	Smoking cessation	Gastrointestinal complaints: dry mouth, stomach ache, nausea, and gastric disturbances	Seeds// MA	15 mg/g	Pérez et al. (2012), Rouden et al. (2014)
Ricinine	$C_8H_8N_2O_2$	*Ricinus communis*	Insecticide	Elicit hyperreactivity	Leaves// WE, MAE, UAE	3.78 mg/g	Ashihara et al. (2020), Hien et al. (2021)
Anabasine	$C_{10}H_{14}N_2$	*Nicotiana glauca*	Insecticide	Depolarizing block of nerve transmission, teratogenic	Leaves// MA	9.25 mg/g	Ashihara et al. (2020), Kuete (2014)
Nornicotine	$C_9H_{12}N_2$	*Nicotiana glutinosa*	Release of dopamine, analgesic	Loss of attention, dependence on tobacco	Leaves// MA	6.97 mg/g	(Ashihara et al. (2020), Lin et al. (2020)
Arecoline	$C_8H_{13}NO_2$	*Areca catechu*	Beneficial effects on the digestive system	Oral submucosal fibrosis, carcinogenesis	Seeds// MA	1.15 mg/g	Ashihara et al. (2020), Peng et al. (2017), Dutta et al. (2017)

Tropane							
Atropine	$C_{17}H_{23}NO_3$	*Atropa belladonna*	Anticholinergic/ parasympatholytic, antimuscarinic, muscle relaxation	Tachycardia	Leaves// MA	0.06 mg/mL	(Robenshtok et al. (2002), Koetz et al. (2017)
Scopolamine	$C_{17}H_{21}NO_4$	*Hyoscyamus niger*	Peripheral antimuscarinic, sedative, antiemetic, and amnestic effects	Drowsiness, dizziness, dry/itchy eyes, feeling restlessness, memory problems	Leaves, roots//AE	0.85 mg/g	Renner et al. (2005), Zhao et al. (2019)
Cocaine	$C_{17}H_{21}NO_4$	*Erythroxylaceae* spp.	Limited to nasal or lacrimal surgeries, as an anesthetic	CNS stimulant agent	Leaves// MA	0.38–3.24 mg/g	Kukula-Koch and Widelski (2017), Balick et al. (1982)
Catuabine	$C_{19}H_{23}N_3O_4$	*Trichilia catigua*	Memory stimulant, antinociceptive and antidepressant effects	Headaches, dizziness, excessive sweating	Bark//DE	nd	Bernardo et al. (2018)
Isoquinoline							
Berberine	$C_{20}H_{18}NO_4^+$	*Hydrastis canadensis, Rhizoma coptidis, Berberis vulgaris*	Inflammatory disorders, skin diseases, wound healing, reducing fevers, affections of eyes, treatment of tumors, digestive and respiratory diseases, and microbial pathologies	Uterine contractions and miscarriages when overdosed	Leaves, bark, root, stem//MA, SO	40 mg/g	Kukula-Koch and Widelski (2017), Sravanthi and Sampath Kumar (2017), Neag et al. (2018)
Morphine	$C_{17}H_{19}NO_3$	*Papaver somniferum*	Opioid analgesic, antitussive, antidiarrheal drug	Addiction, constipation, dysphoria, depression, fetal poisoning, blood	Seeds// UAE	1.69 mg/g	Kukula-Koch and Widelski (2017), Bulduk et al. (2015)

(continued)

Table 6.1 (continued)

Compound	Chemical structure	Source	Activities	Adverse effects	Extraction	Yield	References
				changes, increase in breathing frequency, tachycardia, hypertension, gastrointestinal afflictions			
Codeine	$C_{18}H_{21}NO_3$	*Papaver somniferum*	Analgesic, sedative, hypnotic, antinociceptive, antiperistaltic, insomnia	Addiction, cannot be used as a pain relief drug among children under 18 years (respiratory depression), drug interactions, apathy, drowsiness, obstipation	Seeds// ME	2.7–3.6 mg/g	Kukula-Koch and Widelski (2017), Bhandari et al. (2011), Dittbrenner et al. (2009)
Papaverine	$C_{20}H_{21}NO_4$	*Papaver somniferum*	Spasmolytic, antispasmodic drug, coronary vasodilator, prophylactic in migraine headaches	Tachycardia, constipation, increased transaminase and phosphatase levels, hyperhidrosis, tachycardia, allergic reactions	Seeds// MA	0–400 ng/mL	Kukula-Koch and Widelski (2017), Kleinmeier et al. (2021)
Palmatine	$C_{21}H_{24}NO_4^+$	*Berberis* spp.	Treatment of jaundice, liver-related diseases, hypertension, inflammation, dysentery, central nervous system-related problems	Metabolism of enzymes in the liver, DNA toxicity.	Roots// MAE	20.54 mg/g	Tarabasz and Kukula-Koch (2020), Long et al. (2019), Belwal et al. (2020)

Jatrorrhizine	$C_{20}H_{20}NO_4^{+1}$	*Enantia chlorantha*	Antiprotozoal, treatment of metabolic disorders, gastritis, stomachache	Neurotoxicity	Bark//MA	408.2 mg/g	Bourdat-Deschamps et al. (2004), Rolle et al. (2021)
Phenanthrene							
11 derivatives (1 new and 10 known compounds)	$C_{19}H_{14}N_2O_6SNa$ (for the new identified compound)	*Asarum heterotropoides*	Antibacterial (*X. oryzae, R. solanacearum, X. axonopodis, P. syringae, E. carotovora*)	nd	All//MA	0.006–0.05 mg/g	Fang et al. (2021)
6 derivatives (1 new and 5 known compounds)	7-(4-hydroxybenzyl)-8-methoxy-9,10-dihydro-phenanthrene-2,5-diol (for the new identified compound)	*Cymbidium faberi* Rolfe	Antioxidant activity (DPPH assay); some compounds cytotoxic and anti-inflammatory activities (not including the new one)	nd	Root//SO	0.033–0.433 mg/g	Lv et al. (2020)
17 derivatives (2 new and 15 known compounds)	$C_{18}H_{18}O_2$ and $C_{18}H_{18}O_3$ (for the new two identified compounds)	*Juncus effusus*	Cytotoxic against five human cancer cell lines (including one of the two new compounds), anti-inflammatory	nd	Medullae//MA	0.011–18.33 mg/g	Ma et al. (2016)
6 derivatives (4 new and 2 known compounds)	$C_{15}H_{12}O_4$, $C_{15}H_{12}O_5$, $C_{21}H_{22}O_{10}$ and $C_{15}H_{10}O_4$ (for the 1-4 new compounds, respectively)	*Bulbophyllum retusiusculum*	New compounds 1 and 2 strong cytotoxic activities; compound 4 moderate cytotoxic activity; compound 3 no cytotoxic effect	nd	Tubers//MA	0.120–0.840 mg/g	Fang et al. (2021)

SFE: supercritical fluid extraction; MA: maceration; WE: water extraction; MAE: microwave-assisted extraction; UAE: ultrasound-assisted extraction; AE: acid extraction; DE: decoction; SO: Soxhlet; ME: methylation; nd: not studied

Table 6.2 Occurrence of some groups of alkaloids (phenylethylamine, indole, purine, imidazole, and terpenoid) in plants, extraction conditions to exert certain activities and side effects

Compound	Chemical structure	Source	Activities	Adverse effects	Extraction	Yield	References
Phenylethylamine							
Mescaline	$C_{11}H_{17}NO_3$	*Echinopsis* spp.	Release of neurotransmitters and hormones	Hallucinogens, anxiety, tachycardia, dizziness, diarrhea, vomiting, headache	Stems//SO	0.053–4.7% dry weight	Debnath et al. (2018), Ogunbodede et al. (2010)
Ephedrine	$C_{10}H_{15}NO$	*Ephedra* spp.	Sympathetic stimulation, prevents low blood pressure, bronchodilator, allergic condition, analgesic, anticancer, anti-influenza	Palpitations, hypertension, insomnia, dysuria	Aerial parts//HP	0.88 mg/g	Awuchi (2019), Hyuga et al. (2016), Pellati and Benvenuti (2008)
Synephrine	$C_9H_{13}NO_2$	*Citrus* spp.	Increases resting metabolic rate, energy expenditure, and modest increases in weight loss	None	Fruit//UAE	11.17 mg/g	Stohs et al. (2012), Yan et al. (2021)
Hordenine	$C_{10}H_{15}NO$	*Hordeum vulgare, Sceletium tortuosum*	Promotes greater levels of cognitive energy, improved serious motivation rush	Hypertension, tachycardia	Stems//MA	0.027–1.071 mg/g	Awuchi (2019), Appley et al. (2021)
Indole							
Reserpine	$C_{33}H_{40}N_2O_9$	*Rauwolfia* spp.	Antipsychotic, antihypertensive	Dizziness, loss of appetite, diarrhea	Roots//MA	40.26–57.64 mg/g	Awuchi (2019), Panwar and Guru (2015)
Ergotamine	$C_{33}H_{35}N_5O_5$	*Claviceps purpurea*	Vasoconstrictor, alpha adrenoreceptor antagonist	Weakness, headache, confusion	Seeds//MA	nd	Awuchi (2019)
Yohimbine	$C_{21}H_{26}N_2O_3$	*Pausinystalia yohimbe*	Pre-synaptic alpha-2-adrenergic blocking agent,	Headache, excessive sweating	Bark//MA	0.56 mg/g	

			increases parasympathetic activity and decreases sympathetic activity, antidiuretic				Awuchi (2019), Singh and Alvi (2011)
Vinblastine	$C_{46}H_{58}N_4O_9$	*Catharanthus roseus*	Antineoplastic, immunosuppressant	Cough, fever, painful urination	Cell culture//UAE	0.81 mg/g	Awuchi (2019), Guo et al. (2013)
Vincristine	$C_{46}H_{56}N_4O_{10}$	*Catharanthus roseus*	Antitumor	Double vision, constipation, difficulty in walking, drooping eyelids, headache, jaw pain, joint pain, lower back or side pain, and stomach cramps	In vitro// MA	5.12 mg/g	Awuchi (2019), Fatima et al. (2015)
Purine							
Caffeine	$C_8H_{10}N_4O_2$	*Camellia sinensis*	Analgesics, anorectants, CNS stimulant	Caffeinism syndrome	Seed, leaves, flowers// MA	3–8 mg/g	Awuchi (2019), Kukula-Koch and Widelski (2017), Lin et al. (2003)
Theobromine	$C_7H_8N_4O_2$	*Camellia sinensis*	Strong diuretic, vasodilatory, blood pressure decreasing, antiedematous properties, tranquilizing effect	Loss of appetite, nausea, vomiting, withdrawal headaches, sleeplessness, tremors, restlessness, anxiety, addiction to chocolate.	Leaves// DES	4.87 mg/g	Kukula-Koch and Widelski (2017), Li et al. (2017)
Theophylline	$C_7H_8N_4O_2$	*Camellia sinensis*	Diuretic, β1 and β2 receptors stimulator, spasmolytic, anti-asthmatic, acid and pepsin secretion enhancer	Hypokalemia, hyperglycemia, hypercalcemia, hypophosphatemia, acidosis when overdosed	Leaves// DES	5.07 mg/g	Kukula-Koch and Widelski (2017), Li et al. (2017)
Imidazole							
Pilocarpine	$C_{11}H_{16}N_2O_2$	*Pilocarpus microphyllus*	Atropine antagonist, diaphoretic and pyretic agent,	Diarrhea, irregular heartbeat, headache	Leaves// nd	nd	

(continued)

Table 6.2 (continued)

Compound	Chemical structure	Source	Activities	Adverse effects	Extraction	Yield	References
			inducing the production of sweat saliva and digestive juices; muscarinic receptor agonist				Awuchi (2019), Kukula-Koch and Widelski (2017)
Terpenoid							
Paclitaxel	$C_{47}H_{51}NO_{14}$	*Taxus* sp., *Corylus avellana*	Antineoplastic	Edema, vomiting, nausea, anemia, hypotension	Aerial parts// MA, MAE, UAE	0.2-0.8 mg/g	Kukula-Koch and Widelski (2017), Ghaffar et al. (2021), Ottaggio et al. (2008)
Aconitine	$C_{34}H_{47}NO_{11}$	*Aconitum* sp.	Anesthetic, anti-inflammatory	Vomiting, diarrhea, hypotension, bradycardia, asystole	Roots// MA, UAE	50-154 mg/g	Chan (2009), Csupor et al. (2009)
Lappaconitine	$C_{32}H_{44}N_2O_8$	*Aconitum* sp., *Delphinium* sp.	Analgesic, anti-inflammatory, anti-arrhythmic	Vomiting, diarrhea, hypotension, bradycardia, asystole	Roots// MA	0.052 mg/g	Chan (2009), Xu et al. (2021)
Hetisine	$C_{20}H_{27}NO_3$	*Aconitum* sp., *Delphinium* sp., *Consolida* sp. *Thalictrum* sp.	Antineoplastic, anti-arrhythmic, insecticide	Vomiting, nausea, anemia, hypotension	Roots, leaves// MA	0.08 mg/g	Yin et al. (2021), Qu et al. (2011), Suzgec et al. (2009)

SO: Soxhlet; HP: hot water under pressure; UAE: ultrasound-assisted extraction; MA: maceration; DES: eutectic solvents; MAE: microwave-assisted extraction. Nd: not determined

time-consuming, less solvent required, and energy-efficient while obtaining high extraction yields in a more sustainable way (Cassani and Gomez-Zavaglia 2022). However, at an industrial level, traditional techniques (*e.g.*, maceration) are still the most used for their low investment cost (Tables 6.1 and 6.2). The suitability of each technique will depend on the target alkaloid due to physical, chemical, and stability parameters (Dey et al. 2020). Therefore, there are as many extraction methods as the diversity of alkaloid structures (Daly 2003). Most of these extraction methods are based on the use of solvents with different polarities (methanol, ethanol, ethyl acetate, hexane) (Kurek 2019). For example, indole alkaloids are usually extracted using acidified methanol, isoquinoline alkaloids with aqueous extractants or alcohols, and quinoline alkaloids with toluene, benzene, or methanol (Petruczynik 2012).

Crude extracts obtained with the above-mentioned techniques include a mix of secondary metabolites (phenolics, carotenes, glycosides, alkaloids, and terpenes), leading to low recovery of alkaloids. Therefore, the alkaloids should be purified and isolated. Main purification methods consist of chromatographic methods, derivatization, differences in solubility, crystallization (Gonçalves Paterson Fox et al. 2013), adsorption properties (e.g., silica gel, alumina), or ionic strengths (*e.g.*, cationic ion exchange resins) (Zhang et al. 2018). Crystallization or formation of precipitates can be achieved using solvents (*e.g.*, sodium bicarbonate, ammonia, or tartaric acid) that affect pH values of suspensions and lead to alkaloids precipitation. This is because most alkaloids are water insoluble but soluble in organic solvents, while their salts are water soluble or soluble with dilute acids, thus, remaining in the supernatant fraction (Kukula-Koch and Widelski 2017). Recent techniques such as pH-zone-refining counter-current chromatography have also been developed for such purposes (Leitão et al. 2021).

The extracts obtained by these techniques are then subjected to identification and quantification processes. Alkaloids have been identified using thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), or nuclear magnetic resonance (NMR). For alkaloids derived from known compounds, the identification can be easily accomplished by HPLC with UV detection coupled with electrospray ionization MS/MS (HPLC–UV–ESI MS/MS). Other available techniques used in alkaloids identification include ultra-high performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (UHPLC–Q-TOF-MS) (Sai et al. 2020), high-speed counter-current chromatography (HSCCC) (Zhao et al. 2012), or high-performance thin-layer chromatographic (HPTLC) (Takla et al. 2018). For example, monoterpene indole alkaloids from *Vinca minor* L. were identified through TLC and gas chromatography coupled to mass spectrometry (GC-MS) analysis (Vrabec et al. 2022); tropane alkaloids from *Pellacalyx saccardianus* were elucidated by NMR (Chan et al. 2021); and indole alkaloids from the roots of *Isatidis radix* were identified with high performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (HPLC-Q-TOF-MS) (Wang et al. 2021).

6.3 Currently Marketed Plant Alkaloids

Although more than 27,000 alkaloids have been described up to date, the search for novel compounds with promising therapeutic properties is a hot topic among researchers worldwide. In this section, alkaloids were classified in nine structural groups such as pyridine, tropane, isoquinoline, phenanthrene, phenylethylamine, indole, purine, imidazole, and terpenoid. In addition, a deep description of different groups of alkaloids in terms of their chemical structure, plant source, and uses is also presented.

6.3.1 Pyridine Group

Among nitrogen-containing compounds, pyridine alkaloids (PA) constitute one of the most important groups (Table 6.1) (Lião 2003). PA are characterized by having a pyridine ring in their structure and being derived from L-lysine (Kukula-Koch and Widelski 2017). Several of these alkaloids have chiral structures, thus, the different conformations of the molecule (R or S) may have different bioactivities (Lin et al. 2020). PA have shown therapeutic properties as they are used against central nervous system disorders, in treatments to stop smoking or for the treatment of dementia and neurodegenerative diseases (sclerosis, Parkinson's) (Lin et al. 2020). These molecules considered the active principles of several medicinal plants (Lião 2003), being mainly found in angiosperm plants such as Aizoaceae, Annonaceae, Apocynaceae, Araceae, Berberidaceae, Bignoniaceae, Cannabaceae, Celastraceae, Chenopodiaceae, Crassulaceae, Compositae, Dipsacaceae, Dioscoreaceae, Euphorbiaceae, Goodeniaceae, Gramineae, Icacinaceae, Labiatae, Leguminosae, Loganiaceae, Menyanthaceae, Molluginaceae, Orobanchaceae, Piperaceae, Plantaginaceae, Punicaceae, Palmae, Rubiaceae, Solanaceae, Umbelliferae, and Valerianaceae (Fig. 6.2) (Silva Teles et al. 2019). In particular, Celastraceae and Hippocrateaceae are rich sources of sesquiterpene PA, being these compounds considered chemotaxonomic markers (Lião 2003). The most common examples of pyridine in plants are nicotine, anabasine, ricinine, and nornicotine (Yates 1984; Debnath et al. 2018). Other pyridine alkaloids are arecoline in betel and ricinine in castor oil. Some PA are currently used for the prevention and treatment of neurodegenerative disorders, as well as for the treatment of mood disorders, like militarinone A, artpyrone C, (2S)-N-hydroxybenzyl anabasine, casuarinine H, paecilomide, coprismycin A/B, trigonelline, daminin, euphorbialoids, aspernigrin B, and cantleyine (Lin et al. 2020). However, the exact mechanisms of action are still poorly understood (Lin et al. 2020).

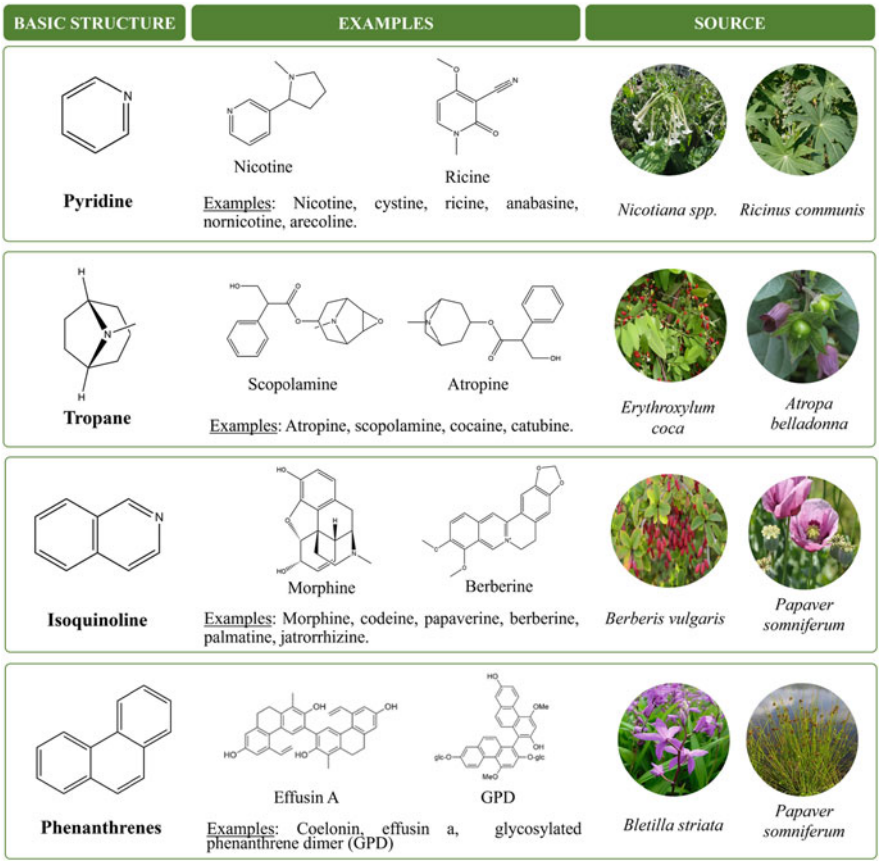

Fig. 6.2 Basic chemical structure and some examples of pyridine, tropane, isoquinoline, and phenanthrenes

6.3.2 Tropane Group

Tropane alkaloids (TA) are characterized by having an 8-azabicyclo[3.2.1]octane group in their structure (Afewerki et al. 2019) and comprise mono-, di-, and tri-esters, tropane carboxylated, and benzoylated (Table 6.1 and Fig. 6.2) (Gadzikowska and Grynkiewicz 2002). Several of these alkaloids have chiral structures due to the presence of a tropic acid residue attached to the ecgonine nucleus as an ester (Kukula-Koch and Widelski 2017). Even though all TA have a tropane bicyclic ring system exclusive of this family of compounds, significant differences in chemical structure can be found among TA. These differences led to classify TA into six large groups: tropine, pseudotropine, calystegines, ecgonine, 4α-benzyltropane, pyranotropane and their derivatives (Kohnen-Johannsen and Kayser 2019; Huang et al. 2021). Each of the mentioned subgroups differs in its biological, chemical, and

pharmacological properties (Kohnen-Johannsen and Kayser 2019; Huang et al. 2021). For example, cocaine manifests its effects at the synaptic cleft by inhibiting the reuptake of dopamine, norepinephrine, and serotonin. In contrast, scopolamine acts as a competitive antagonist at muscarinic receptors. Ingestion of both substances can cause hallucinations and psychoactive effects or death (Langmead et al. 2008). Calystegines are not absorbed in the central nervous system because they are hydrophilic, thus they do not exert psychoactive effects in humans (Dräger 1995). Most of these compounds are used as medicines due to their pharmacological properties (Afewerki et al. 2019). In fact, TA are considered as ancient medicinal plant compounds due to their ethnopharmacological properties analgesics, hallucinogens, antidotes, and poisons (Gadzikowska and Grynkiewicz 2002).

TA are secondary metabolites of Erythroxylaceae, Convolvulaceae, Brassicaceae, and Euphorbiaceae plant families (Kohnen-Johannsen and Kayser 2019; Gadzikowska and Grynkiewicz 2002). Among plants with higher alkaloid content are coca (Erythroxylaceae) and mandrake, henbane, belladonna, datura, potato, and tomato (Solanaceae) (Adamse and Van Egmond 2010). Despite tropane's abundance in nature, TA are a group of compounds that have been historically produced through chemical synthesis. For example, Willstätter's first cocaine synthesis and Robinson's efficient total tropane synthesis a hundred years ago (Afewerki et al. 2019). Despite the exact biosynthetic pathway of hyoscyamine and scopolamine has not been elucidated so far, their *de novo* production (production for the first time) in yeast was reported (Huang et al. 2021). Currently, more than 300 TA are known, being the most common atropine, hyoscyamine, and scopolamine (Huang et al. 2021; Adamse and Van Egmond 2010).

6.3.3 Isoquinoline

Isoquinoline alkaloids (IA) are one of the largest groups of alkaloids. These compounds are characterized by being derivatives of phenylalanine and tyrosine. The common structure of IA is an isoquinoline nucleus or a tetrahydroisoquinoline ring (Table 6.1) (Kukula-Koch and Widelski 2017; Grycová et al. 2007). From this molecular skeleton, a group of structurally heterogeneous compounds is derived. The main differences are due to different degrees of oxygenation, intramolecular rearrangements, and the distribution and presence of additional rings connected to the main system (Kukula-Koch and Widelski 2017). IA can be divided into two main categories: simple isoquinolines (*e.g.*, salsoline, mimosamycin), which are composed of a benzene ring fused to a pyridine ring, and benzylisoquinolines, which contain a second aromatic ring (Khan and Suresh 2015). Benzylisoquinolines are divided into benzylisoquinoline alkaloids (*e.g.*, reticulin, imbricatin), bisbenzylisoquinoline alkaloids (*e.g.*, fumaricin), manzamine alkaloids (*e.g.*, manzamine a), pseudobenzylisoquinoline alkaloids (*e.g.*, polycarpine, ledecorin), secobisbenzylisoquinoline alkaloids (*e.g.*, baluchistanamine), bisbenzylisoquinoline alkaloids containing one ether bond (*e.g.*, dauricine), bisbenzylisoquinoline

alkaloids containing two ether bonds (*e.g.*, berbamine), bisbenzylisoquinoline alkaloids containing only aryl bonds (*e.g.*, pisopowetine), bisbenzylisoquinoline alkaloids containing one aromatic bond and one or two ether bonds (*e.g.*, rhodiasine) (Dey et al. 2020). Many other groups of alkaloids such as protoberberines, protopines, pavines, and aporphines are structurally related to the benzylisoquinolines (Khan and Suresh 2015).

IA are a large family of natural compounds which have shown a wide variety of biological activities, including anti-inflammatory, antimicrobial, antileukemic, antitumor (Zein et al. 2012), antiviral, antifungal, antioxidant, antispasmodic, and enzyme inhibitors (Dey et al. 2020; Qing et al. 2020). For example, morphine and codeine are two of the most important painkillers used in medicine, with plants still being the main commercial source (O'Connor 2010). Both compounds are made from a dopamine precursor (3,4-dihydroxytryptamine) associated with a ketone or aldehyde (Dey et al. 2020). Other IA of interest are berberine, palmatine, jatrorrhizine, papaverine, corydaline, emetine, sanguinarine, and chelerythrine (Khan and Suresh 2015). IA have been found in diverse plant families such as Papaveraceae (mainly in the form of tetrahydro bases), Berberidaceae, Fumariaceae, Menispermaceae, Ranunculaceae, Rutaceae, Annonaceae (dihydro), Monimiaceae, Magnoliaceae, Convolvulaceae (Kukula-Koch and Widelski 2017; Misra et al. 1999), Alangiaceae, Fabaceae, and Lauraceae (Fig. 6.2) (Qing et al. 2020). To date, more than 4000 IA have been identified. In addition to these detected compounds, several IA which may have potential biological activities are still unidentified and thus require more research (Qing et al. 2020).

6.3.4 Phenanthrene Group

Phenanthrene alkaloids (PhA) are aromatic metabolites present in numerous plants. From a chemical point of view, PhA are three-ring polycyclic aromatic hydrocarbons ($C_{14}H_{10}$) that can be produced via oxidative coupling of the stilbene's aromatic rings (precursor) (Table 6.1) (Ahammed et al. 2013). In addition, other biosynthetic pathways for forming phenanthrenes have been proposed, such as from diterpenoid precursors and morphine alkaloids (Sut et al. 2017; Tóth et al. 2018). The type of substituent groups bound to three-ring polycyclic aromatic hydrocarbons is dependent on the plant family. For example, phenanthrenes from Juncaceae species are characterized by having vinyl groups, while prenylated derivatives are typically found in Euphorbiaceae species and methoxy groups in Orchidaceae species (Tóth et al. 2018). In this sense, PhA are considered chemotaxonomic markers due to their limited presence in plants. It is hypothesized that the enzymes needed for the PhA' biosynthesis are plant-specific (Tóth et al. 2018). In addition, PhA can be divided into three groups, namely, monophenanthrenes, diphenanthrenes, and triphenanthrenes according to the monomers linked to the molecule (Kovács et al. 2008). Most of the naturally occurring phenanthrenes are present in the monomeric form and they can also be subdivided according to the number and type of the

structural moieties (Kovács et al. 2008). More than 450 of these compounds have been identified from different plant families such as Annonaceae, Aristolochiaceae, Cannabaceae, Combretaceae, Dioscoreaceae, Euphorbiaceae, Juncaceae, Lauraceae, Malpighiaceae, Orchidaceae, and Stemonaceae (Fig. 6.2) (Tóth et al. 2018). However, the most studied phenanthrenes were mainly isolated from species of the Orchidaceae and Juncaceae species (Tóth et al. 2018). These compounds are naturally found in different parts of plant such as roots, stem, leaves, branch, rhizome, stem bark, wood, medulla, aerial part, pith, and tuber (Tóth et al. 2018). Traditionally, phenanthrene-containing plants were used to treat some diseases, for example, *Dendrobium* Sw. (Orchidaceae) plants were extensively used in Chinese medicine to stimulate gastric motility and thus, improve gastric acid secretion, among other health benefits (Lin et al. 2013). This way, from the biological perspective, phenanthrenes have shown promising bioactivities such as antioxidant, anti-inflammatory, anti-proliferative, antimicrobial, spasmolytic, anti-platelet aggregative, and cytotoxic activities (Nanjala et al. 2022). In this context, PhA are a growing group of bioactive compounds with great potential to be further explored by the nutraceutical, pharmacological, and food industries.

6.3.5 Phenylethylamine Group

Phenylethylamine alkaloids (PeA) are aromatic metabolites constituted by an aromatic amine (phenethylamine) attached to a benzene ring through an ethyl group (Table 6.2 and Fig. 6.3). This group of compounds is precursor to many natural and synthetic chemicals and thus, they can be used as potential replacements for active pharmaceutical substances. The structure of PeA allows substitutions on the aromatic ring, the α and β carbons and terminal amino group. PeA can be classified into different groups according to their biological properties (Güven et al. 2010): One of these mentioned groups is catecholamines (hydroxylated phenethylamines), which include the hormone adrenaline (epinephrine) and neurotransmitters (noradrenaline, dopamine). Catecholamines allow organisms to fine-tune the response to stress conditions and work as antidepressants (Kulma and Szopa 2007). It has been reported that dopamine having two hydroxyl groups in positions 3 and 4 of the phenyl ring showed important physiological activities as neurotransmitters (da Silveira 2020) and it can be used to treat cardiovascular disease and kidney disorders (Güven et al. 2010). N-methyltyramine, a hydroxylated compound found in some cacti such as *Cereus jamacaru*, has shown beneficial effect in treating gastrointestinal disorders by mediating epinephrine synthesis (da Silveira 2020). Mescaline is a powerful hallucinogen found in several cacti. Among the adverse effects experienced by most users include hallucinations, alteration of consciousness and perception, accelerated migraine, physical reactions such as respiratory pressure and muscle tension (Gibbons and Arunotayanun 2013). Ephedrine and synephrine are used as a bronchodilator and decongestant in medicine, respectively. Synephrine can be obtained from tangerines in sufficient concentrations to be physiologically

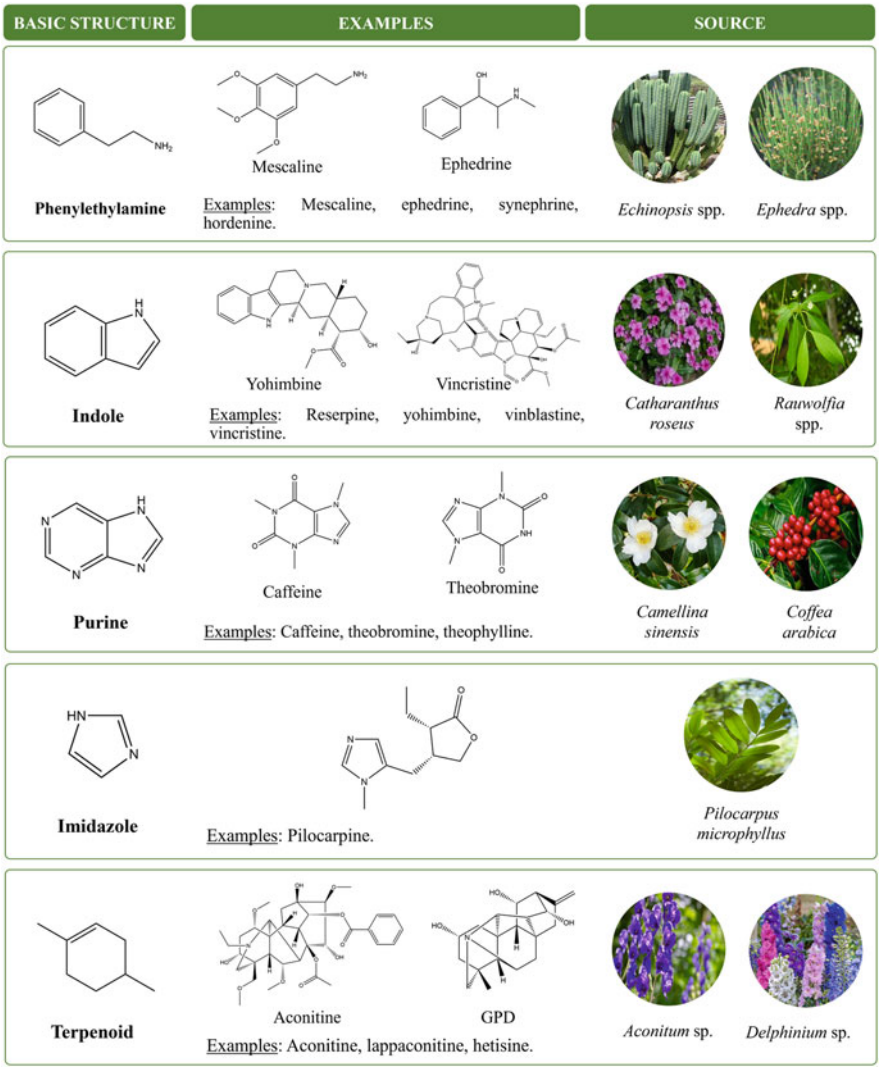

Fig. 6.3 Basic chemical structure and some examples of phenylethylamine, indole, purine, imidazole, and terpenoid

effective (Stohs et al. 2020). Hordenine was first isolated from terrestrial plant *Anhalonium fissuratus* in 1894. It is a diuretic and a remedy for diarrhea and dysentery, probably due to its antiseptic properties. Hordenine is found in the flower, bark, and leaves of *Tamarindus indica* with associated activities such as diuretic, psychostimulant, anti-inflammatory, anti-asthmatic, antidiabetic, and antiobesity properties (Ahmad et al. 2018). Tyramine (precursor of dopamine) is a monoamine derivative of tyrosine's amino acid. Besides acting as a stimulator for the nervous

system, it causes vasoconstriction, increases the heart rate and blood pressure, and is also involved in migraines (Güven et al. 2010).

6.3.6 *Indole Group*

Indole alkaloids (InA) are the most extensive and varied alkaloid groups represented by more than 4,000 known compounds. This extent may be because tryptophan is the main constituent of plant proteins and acts as a precursor to indole alkaloids (Rosales et al. 2020). The bicyclic structure of InA comprises a six-membered benzene ring fused to a nitrogen-containing five-membered pyrrole ring (Table 6.2 and Fig. 6.3) (Kumari and Singh 2019). Based on their biosynthesis, InA can be further divided into two parts, isoprenoids, and non-isoprenoids. Isoprenoid InA consists of monoterpenoid indole alkaloids, bisindole alkaloids, and ergot alkaloids (Cheang 2018). Monoterpenoid InA can be classified into three groups depending on their chemical structures: corynanthe, iboga, and aspidosperma and represent the most significant number of compounds. The difference between these molecules is the presence of carbonyl, methoxyl, and hydroxyl groups at different positions (Rosales et al. 2020). Until now, 1,800 different monoterpene-derived InA have been characterized and classified according to their biosynthesis pathway (Marinho et al. 2016). Monoterpenoids are a significant source of pharmacologically active compounds (Marinho et al. 2016). Bisindole alkaloids, including three important compounds vinblastine, vincristine, and vindesine, have been authorized for clinical use by the Food and Drug Administration (FDA, USA) for many types of cancer. In addition, they are used to treat diabetes (Kumar et al. 2022). The anticancer alkaloids, vinblastine and vincristine, are derived from the stem and leaf of *Catharanthus roseus* which belong to the dogbane family Apocynaceae (Das et al. 2017). Vindesine is also called desacetyl-vinblastine-amide and it exhibits similar effects to those of vinblastine (Pandrangi et al. 2022). Ergot alkaloids were originally found in *Claviceps purpurea*, but they were also found in various fungal species and plants (Yao et al. 2022). Their molecular structure comprises a complex tetracyclic ergoline skeleton with several chiral centers, which has shown to be important for the treatment of Parkinson's disease, uterine hemorrhage, migraines, affecting cardiovascular function in various ways (Yao et al. 2022; Florea et al. 2017). On the other hand, they are incredibly poisonous, causing nausea, vomiting, poor circulation, a quick and weak pulse, and even coma (Pandrangi et al. 2022). Generally, InA are found in Apocynaceae, Rubiaceae, Annonaceae, and Loganiaceae families (Rosales et al. 2020). These alkaloids are pharmacologically active in different ways, such as anticancer, antihistaminic, antifungal, antimicrobial, antioxidant, plant growth regulator, anti-HIV, anticonvulsant, anti-inflammatory, antiviral, and analgesic and affect the nervous system centrally and peripherally (Singh and Singh 2017). Compounds of these groups are often expensive due to their time-consuming separation and purification processes (Kumar et al. 2022).

6.3.7 Purine Group

Purine nucleotides are found in various secondary metabolites in plants, known as purine alkaloids (PuA). Almost 100 different plant species produce these alkaloids; most of them are from the genera *Camellia, Coffea, Cola, Ilex, Paullinia,* and *Theobroma* (Table 6.2 and Fig. 6.3) (Weckerle et al. 2003; Ashihara et al. 2008). In this group, methylxanthines (caffeine and theobromine) and methyluric acids play a crucial role (Ashihara et al. 2008). Caffeine (1,3,7-trimethylxanthine) is most abundant in coffee beans and tea leaves. It has been consumed for centuries because of its alluring taste and stimulating effects. The most common caffeine-rich sources are tea leaves, cocoa beverages, soft drinks, and chocolate products (Cui et al. 2020; Anaya et al. 2006). The pharmacological effects of caffeine in different animal systems, such as central nervous system, immune system, digestive system, respiratory system, and urinary tract have been extensively specified (Rodak et al. 2021; Ashihara and Crozier 1999). Depending on age, sex, source, and dose, caffeine can have different effects. It has been claimed that caffeine positively impacts cognitive performance, memory, and brain function at low doses, but it may also cause nervousness and anxiety at high doses (Rodak et al. 2021). It is known that caffeine has both positive and negative effects on a myriad of diseases (Alzheimer's disease, Parkinson's disease, asthma, cirrhosis, fibrogenesis, kidney stones, some cancers, etc.) as well as adverse effects (Huntington's disease, arrhythmia, tachycardia, and lung cancer). Besides relieving pain, caffeine contains anti-inflammatory and antioxidant properties that make it helpful in pharmacology and cosmetics. Caffeine appears to have a multi-directional effect on motor and respiratory functions, a function that appears to be crucial in sports (Rodak et al. 2021). In different types of tea, theobromine, theophylline, and theacrine are also found as PuA. Theacrine is structurally similar to caffeine, but it has no side effects on sleep quality (Jhuo et al. 2021). Theacrine (1,3,7,9-tetramethyluric acid) is associated with beneficial effects such as antidepressant activity, sedation, hypnosis,, regulation of lipid metabolism (Chen et al. 2021), and also anti-inflammatory properties (Lu et al. 2022).

6.3.8 Imidazole Group

Imidazole (IzA) is a five-membered heterocyclic ring ($C_3H_4N_2$) containing three carbon atoms, two nitrogen atoms, and two double bonds. It is an essential constituent of several biologically and industrially applicable natural products, including purine, histamine, histidine, and nucleic acid. One of the nitrogen atoms is pyrrole-type nitrogen, and another is pyridine-type nitrogen, thereby forming the imidazole ring, which belongs to the π electron-rich aromatic ring, capable of receiving a lot of suction from the electronic group (Table 6.2 and Fig. 6.3). Further, these metal-organic frameworks are formed by the coordination of nitrogen atoms of the imidazole ring (Siwach and Verma 2021; Brown 2008). Arthur Rudolf Hantzsch

first described IzA in 1887 (Mishra et al. 2020). It is a white or colorless solid. Water and other polar solvents are excellent solubilizers of the imidazole ring (Gupta 2015). Due to its amphoteric nature, IzA can act as both an acid and a base. The hydrogen atom can be positioned on either nitrogen atom, which results in having two equivalent tautomeric forms. There are many applications of IzA drugs in the clinical area, including their potential biological activities such as anticancer (dacarbazine, zoledronic acid, azathioprine, and tipifarnib), antifungal (clotrimazole, miconazole, ketoconazole, and oxiconazole), antiparasitic (metronidazole, benznidazole, ornidazole, and secnidazole), antihistaminic (cimetidine, imetit, immepip, and thioperamide), antineuropathic (nafimidone, fipamezole, and dexmedetomidine), and antihypertensive (losartan, eprosartan, and olmesartan) that are widely used for treating a wide range of diseases with high therapeutic potency, showing that they are valuable compounds for future research (Siwach and Verma 2021). Imidazole-based supramolecular drugs are gaining attention due to their ease of preparation, affordability, high safety, low toxicity, less adverse effect, high bioavailability, fewer drug resistances, good biocompatibility, and beneficial effects, etc. (Brown 2008; Mishra et al. 2020; Ouakki et al. 2022). Pilocarpine is one of the most well-known compounds of this group, which Hardy and Gerrard first isolated in 1875. It is extracted from *Pilocarpus jaborandi* and is a drug used in glaucoma treatment preparations in ophthalmology (Davies et al. 2009; Bufo and Karaman 2019).

6.3.9 Terpenoid Group

Terpenoid alkaloids (TeA) are considered with steroidal alkaloids as pseudoalkaloids. They are constituted by a terpenoid, and an aminated group not derived from amino acids but from methyl/ethylamine or β-aminoethanol (Han et al. 2021; Cherney 2011). TeA have shown biosynthesis and structural differences compared to other alkaloids and thus, they have been classified as pseudoalkaloids, also called aminated terpenes (Table 6.2 and Fig. 6.3) (Cherney 2011). The terpenoid moiety is derived from isoprene by the methyl-erythritol phosphate (MEP) pathway and, depending on their structure, may be classified as monoterpene, sesquiterpene, and diterpene alkaloids (Kukula-Koch and Widelski 2017; Shen et al. 2020). They have been found in Asteraceae, Ranunculaceae, Taxaceae, and Cornaceae families. The most significant group among the TeA are taxanes (paclitaxel, Taxol®, is the most relevant) and they are currently used for chemotherapy due to their antineoplastic properties (Garcia-Oliveira et al. 2021). Taxol was first described in barks of Pacific yew (*Taxus brevifolia*) in 1967, and later in other species of yew such as *T. baccata* or *T. chinensis* (Li et al. 2015). Since it is present in scarce quantities in these barks, currently fungal synthesis or semi-synthesis from 10-deacetylbaccatin isolated from the leaves of *T. baccata* are the major sources of this compound (Li et al. 2015). Other relevant diterpene alkaloids are aconitine and aconitine-type alkaloids, obtained from aconite species (*Aconitum* sp.) and are the first described

terpenoid alkaloid (Shen et al. 2020). Roots of aconite have a long history of being traditional remedies for its antipyretic, analgesic, or anti-inflammatory properties, especially in traditional Chinese and Indian medicine (Chan 2009). However, these alkaloids have shown to be extremely toxic and thus, they have been historically used both as medicinal remedies and poison. It has been reported that aconitine may be lethal at doses as low as 0.3 mg/kg bw (Csupor et al. 2009). Lappaconitine is a closely related alkaloid but with an additional methyl and amine group, described to exert anti-arrhythmic effects and with traditional use as an analgesic (Yin et al. 2021). Besides being described in *Aconitum* sp., it has also been reported in *Delphinium* sp. (Xu et al. 2021). Hetisine-type alkaloids are also a relevant class of diterpenoid alkaloids and differ from the previous groups in that they are structured in complex fused polycyclic skeletons (Yin et al. 2021). They have been reported in both *Aconitum* sp., *Delphinium* sp., but also in *Consolida* sp. or *Thalictrum* sp. (Ranunculaceae). These alkaloids have shown to exert highly diverse biological properties, from anti-arrhythmic to antineoplastic or even insecticidal against coleopters (Yin et al. 2021; Qu et al. 2011).

6.4 Biological Activities

Given their widely diverse structure, plant alkaloids have shown a great extent of beneficial therapeutical properties such as anti-inflammatory, anticancer, cardioprotective, sedative, or analgesic (Table 6.1). For this reason, these natural compounds have been employed in medicine. However, additional properties of alkaloids have been described, such as antioxidant or antimicrobial. In fact, as mentioned before, many alkaloids such as quinine, cocaine, or colchicine have been identified and used in clinical practice for more than a century, but as medical methods advanced, their use has been limited or restricted due to hazardous side effects (Kukula-Koch and Widelski 2017). Nonetheless, most research in this area is focused on identifying biological properties in currently used alkaloids, as well as in newly discovered ones. Thus, in this section, recent advances in therapeutic and biological activities of alkaloids of the addressed groups are reviewed.

One of the most extended and studied properties of alkaloids are their anticancer properties. Many alkaloids have a long history of use as chemotherapy agents since some of these compounds are capable of blocking α/β-tubulin, thus inhibiting microtubule formation and subsequent mitosis (Garcia-Oliveira et al. 2021). Some examples of alkaloids acting through this mechanism are InA like dacarbazine, vinblastine, or vincristine, but also TeA such as paclitaxel (Table 6.3). InA with anticancer properties are mostly referred to as vinca-alkaloids, as they are derived from *Vinca rosea*. Vinblastine or vincristine are used as chemotherapy agents, especially for tumors with difficult surgery such as pancreatic (Tazi et al. 2017) or brain cancers (Bouffet et al. 2012). Their use is mostly as a first-line cancer treatment since they are generally well-tolerated and do not exert excessive toxicity. Similarly, paclitaxel has been long used as a chemotherapy agent (Garcia-Oliveira et al. 2021).

Table 6.3 Biological and therapeutical properties of some of the most relevant alkaloids

Alkaloid	Bioactivity	Type of study	Main findings	Commercial status	References
Pyridine					
Ricinine	Anticancer	In vitro & in vivo	Migration and invasion inhibition in MCF-7 & MDA-MB-231 (human breast cancer) cells by induction of proapoptotic proteins. 4 doses of 0.5 mg/kg bw reduced up to 88% tumor volume in treated mice	Not available	Majumder et al. (2019)
Nornicotine	Stimulant	In vivo	Reduced food intake (25%) & increased physical activity in rats	Not available	Grebenstein et al. (2022)
Tropane					
Atropine	Ameliorate myopia	Clinical trial	Daily 0.01% atropine eye drops slowed myopia progression (av. −1.38 diopters) during a 5-year trial in children ($N = 400$)	In clinical use	Chia et al. (2016)
	Sedative	In vivo	Anticonvulsant effects in induced seizures by single injection (2 mg/kg bw)	In clinical use	Miller et al. (2015)
Calystegines	Antioxidant & anti-inflammatory	In vitro & in vivo	In vitro antioxidant activity, antihemolytic activity in human erythrocytes and reduction in rat paw edema (58%) by 200 mg/kg bw of calystegines-rich extracts	Not available	Bourebaba et al. (2016)
Isoquinoline					
Colchicine	Anti-inflammatory & cardioprotective	Clinical trial	Reduced incidence of acute coronary syndromes by 0.5 mg/day of colchicine ($N = 532$)	In clinical use	Nidorf et al. (2013)
	Anti-inflammatory		1.8 mg/1 h of colchicine achieved ≥50% pain reduction in gout patients ($N = 184$)		Terkeltaub et al. (2010)
Berberine	Antidiabetic	In vivo	Daily 380 mg/kg bw berberine significantly reduced food intake, body fat and plasma glucose and triglyceride levels in rats and *db/db* mice	Not available	Lee et al. (2006)
		Clinical trial	Reduction in glycated hemoglobin (av. −2%), fasting glycemia (av. −3.3 mmol/L) by 1.5 g/day of berberine in type 2 diabetic patients ($N = 84$)		Yin et al. (2008)

Palmatine	Antimicrobial	In vitro	Antimicrobial activity on *Helicobacter pylori* (MIC = 75 μg/mL) and its urease (IC_{50} = 0.53 mM)	Not available	Zhou et al. (2017)
	Antiobesity	In vivo	Lower levels of total cholesterol, plasma lipids and improved gut microbiota by 140 mg/kg bw		He et al. (2016)
Phenanthrene					
Antofine	Antiviral	In vitro & in vivo	53% infection inhibition of tobacco mosaic virus in *Nicotiana tabacum* by 0.5 mg/mL after 12 days	Not available	Yu et al. (2016)
	Antitumor	In vitro	Growth inhibition of A549 paclitaxel-resistant alveolar cancer cells (IC_{50} = 18 nM)		Kim et al. (2012)
Coelonin	Anti-inflammatory	In vitro	>10-fold reduction of IL-1β, IL-6, TNF-α expression levels by 20 μg/mL treatment of LPS-stimulated murine macrophages	Not available	Jiang et al. (2019)
			~2-fold reduction of IL-1β, IL-6, TNF-α, and COX-2 by 20 μg/mL treatment of A549 alveolar cancer cells		Cheng et al. (2021)
Neonthrene	Antimalarial	In vitro	Inhibition of erythrocyte infection by *Plasmodium falciparum* IC_{50} = 9.8 μg/mL	Not available	Namukobe et al. (2014)
Phenylethylamine					
Ephedrine	Hypertensive	Clinical trial	Used to treat hypotension during birth in women with anesthesia by 4 mg (N = 166)	In clinical use	Wang et al. (2019)
Indole					
Vinblastine	Anticancer	Clinical trial	Complete tumor disappearance by 0.2 mg/kg bw weekly vinblastine in 31 glioma-affected children (N = 51)	In clinical use	Bouffet et al. (2012)
		Clinical trial	Retrospective study of oncologic patients treated with vinblastine, finding that those treated with vinblastine had higher survival rate and lower reincidence (N = 35)	In clinical use	Tazi et al. (2017)
Vincamine	Antioxidant & anti-inflammatory	In vivo	Reduction of IFNγ by blocking TLR4 and restoration of antioxidant proteins (Nrf2, MDA, MPO) to levels of control group by 40 mg/kg bw	In clinical use	El-Sayed et al. (2021)

(continued)

Table 6.3 (continued)

Alkaloid	Bioactivity	Type of study	Main findings	Commercial status	References
Purine					
Theobromine	Cardioprotective	Clinical trial	Systolic blood pressure reduction (av. ~4 mmHg) by oral intake of theobromine-rich cocoa (~1 g/day) for 4 weeks ($N = 42$)	Not available	Van Den Bogaard et al. (2010)
	Anticancer	In vivo	Reduced incidence of colon tumors (80.1%) and tumor volume (72.19%) in dimethylhydrazine cancer induced rats	Not available	Shojaei-Zarghani et al. (2021)
Allantoin	Anti-inflammatory	In vivo	Gastroprotective properties in mice by 60 mg/kg bw through reduced inflammation and protection of gastric mucosa	Commercialized	da Silva et al. (2018)
		In vivo	Reduction of asthmatic symptoms in mice, lowering mucus secretion and neutrophil infiltration in alveolar tissue by 25 mg/kg bw		Lee et al. (2010)
Theacrine	Anticancer	In vitro & in vivo	Slower tumor progression and reduced volume, increased expression of caspase 3, inducing apoptosis by 100 mg/kg bw	Not available	Jhuo et al. (2021)
Caffeine	Antioxidant & antidiabetic	In vivo	Increased levels of reduced glutathione and glutathione reductase, and lower production of carbonylated protein in rat pancreas by 5 mg/kg bw	Commercialized	Fernandez-Gomez et al. (2016)
	Anticancer	In vivo	Slower tumor progression, volume, number and increased expression of anti-inflammatory interleukins in induced melanoma rats by 0.1 w/v caffeine in water ad libitum		Eini et al. (2015)
Imidazole					
Losartan	Cardioprotective	Clinical trial	Blood pressure reduction (av. 145/89 to 128/79 mmHg) and reduced insulin resistance (7%) by 100 mg/day in hypertensive patients ($N = 47$)	In clinical use	Kwang et al. (2004)
Dacarbazine	Anticancer	Clinical trial	Treatment of melanoma by 1 g/m^2 for 2 years ($N = 91$)	In clinical use	Robert et al. (2013)
			Treatment of liposarcoma & leiomyosarcoma by 1 g/m^2 for 2 years ($N = 518$)	In clinical use	Demetri et al. (2016)

Terpenoid					
Paclitaxel	Anticancer	Clinical trial	Slower tumor progression and tumor volume reduction in breast cancer patients with 175 mg/m^2 of paclitaxel ($N = 229$)	In clinical use	Gradishar et al. (2005)
	Anticancer	Clinical trial	Successful treatment of melanoma by 90 mg/m^2 of paclitaxel ($N = 52$)	In clinical use	Bedikian et al. (2004)
	Anticancer	Clinical trial	Intravenous paclitaxel (80 mg/m^2) combined with curcumin (300 mg) resulted in increased survivability and significant reduction of cancer antigens in blood ($N = 238$)	In clinical use	Saghatelyan et al. (2020)
Lappaconitine	Anti-arrhythmic	In vivo	Arrhythmia inhibition in guinea pigs by 0.35 μg/mL	In clinical use	Heubach and Schüle 1998)
	Analgesic	In vivo	Inhibition of nociceptive behaviors by 4 mg/mk bw in rats	In clinical use	Ou et al. (2011)
Guan-fu base A	Anti-arrhythmic	*Ex vivo*	Inhibition of sodium ionic channels in atrial cardiac guinea pig myocytes	In clinical use (China)	Jin et al. (2015)

Nonetheless, its poor solubility and inducement of inflammatory responses by increasing pro-inflammatory factors expression hinder its effective treatment application. For this reason, research on anticancer applications for paclitaxel has focused on combining with other carrier molecules or combinate its use with other chemicals (Gradishar 2012). Paclitaxel combined with curcumin has demonstrated to significantly increase its effectiveness and patient survival while decreasing carcinogenic antigens in plasma (Saghatelyan et al. 2020). The IzA dacarbazine is also used as a chemotherapy agent and has been reported to be effective in treating cancers when other chemotherapies do not (Demetri et al. 2016). Despite its long use, its mechanism of action is still unknown. In addition to these examples, other alkaloids have been recently explored for their potential anticancer properties. Some of these are the PhA antofine, which has shown in vitro tumor growth inhibition at concentrations as low as 18 nM (Kim et al. 2012), or the PA ricinine, which achieved as much as 88% tumor volume in mice by inducing apoptosis related to increased caspase-9 expression (Majumder et al. 2019). Moreover, PuA like theobromine, theacrine, and caffeine have been investigated as anticancer agents in in vivo studies. Theobromine, in particular, achieved as much as 80% tumor incidence reduction when administered orally in mice (Shojaei-Zarghani et al. 2021). Caffeine, besides its usage as a psychostimulant compound, has also been investigated for potential anticancer properties. Due to its interaction with the adenosine A2 receptors, which are intrinsic to the development of many types of breast cancer, caffeine was able to achieve slower tumor progression and reduced tumor incidence and volume in vivo (Eini et al. 2015). Theacrine has been investigated for its mechanism of action similar to caffeine, showing that was able to block the adenosine 2A receptor, and reduce tumor incidence and volume in mice in a dose-dependent manner (Jhuo et al. 2021).

Some alkaloids can also act as anti-inflammatory agents by diverse pathways. For example, calystegine-related TA are reported to possess both antioxidant and anti-inflammatory properties, being able to inhibit erythrocyte lysis and edema in rats (Bourebaba et al. 2016). In a similar way, coelonin (PhA) has been shown to greatly inhibit the expression of pro-inflammatory cytokines such as interleukins 1 and 6 (Jiang et al. 2019; Cheng et al. 2021). In this regard, the PuA allantoin is the most extensively used plant-derived alkaloid as a cosmetic anti-inflammatory agent. This alkaloid has been reported to alleviate gastric inflammation and improvement of gastric function by improving mucus secretion (da Silva et al. 2018). Allantoin has also been described to inhibit asthma-related symptoms in animal models, alleviating mucus secretion and dilating alveoli, as a result of decreased expression of pro-inflammatory interleukins (Lee et al. 2010). The IA colchicine is also one of the most long-used alkaloids and is still the reference treatment for gout and Mediterranean fever since it can prevent infection and exert anti-inflammatory and antipyretic effects (Terkeltaub et al. 2010). This anti-inflammatory effect also results in analgesic properties, albeit not related to nociceptors (Terkeltaub et al. 2010). The IzA vincamine has been reported to be able to stop induced inflammatory cascades in vivo by hindering toll-like receptor 4 mediated release of interferon gamma, which is one of the primary endogenous inducers of the inflammatory response (El-Sayed et al. 2021). Nevertheless, the mechanism of action of many alkaloids to

deter inflammation is still poorly understood, although their anti-inflammatory effects are supported by reduced expression of pro-inflammatory mediators (Bourebaba et al. 2016; El-Sayed et al. 2021).

Considering antimicrobial and antiprotozoal properties of alkaloids, one of the major representatives is quinine. Quinine has been the recommended treatment for malaria. Despite its long use for over a century, its mechanism of action has remained elusive. Recently, it has been proposed that quinine is able to inhibit *Plasmodium* sp. infection by blocking its purine nucleoside phosphorylase, and thus inhibiting its replication (Dziekan et al. 2019). PhA are the main group of alkaloids that reported antimicrobial activities. For instance, neonthrene has been reported to display antimalarial effects on *P. falciparum*, inhibiting the in vitro infection of erythrocytes (Namukobe et al. 2014). Other PhA like antofine have also been reported with antiviral activities, *i.e.* against the tobacco mosaic virus, achieving a 56% inhibition in a treated tobacco plant (Yu et al. 2016). Novel PhA isolated from *Asarum heterotropoides* (Aristolochiaceae) exhibited potent antimicrobial activity against phytopathogenic bacteria like *Ralstonia solanacearum, Xanthomonas oryzae, X. axonopodis, Erwinia carolovora,* or *Pseudomonas syringae*, with minimum inhibitory concentrations (MIC) below 6.25 μg/mL. This antibacterial effect was attributed to cell wall lysis, which suggests potential field applications (Fan et al. 2021). While PhA are the main alkaloid group displaying antimicrobial properties, other alkaloids like the IA palmatine have also been described as such. For instance, palmatine was reported to inhibit *Helicobacter pylori* growth (MIC = 75 μg/mL) while also inhibiting its urease, which relates to the survival ability of this bacteria in the stomach mucosa (Zhou et al. 2017). This may suggest that palmatine could be used in treatments against ulcers

Concerning cardioprotective activity of alkaloids, TeA such as lappaconitine and mainly Guan-fu base A are employed for treating arrhythmias as they were able to block sodium and potassium ionic channels. In fact, these alkaloids are approved for clinical use in China and are the main component of traditional herbal preparations of *Aconitum* sp. (Yin et al. 2021). For instance, Guan-fu base A has shown specific inhibition of sodium ionic channels in guinea pig cardiac myocytes (Jin et al. 2015). Lappaconitine, besides inhibiting induced arrhythmias in vivo, has also shown analgesic properties (Ou et al. 2011). IzA like losartan is widely used in medicine for the treatment of hypertension since it decreases blood pressure without lowering heart beating rate by blocking the binding of angiotensin II to the angiotensin-transient receptor (Kwang et al. 2004). The PuA theobromine has been described to be able to lower blood pressure when supplemented as part of cocoa, albeit its mechanism of action remains elusive (Van Den Bogaard et al. 2010). Colchicine has also been reported to exert cardioprotective effects due to its anti-inflammatory properties, as was demonstrated by (Nidorf et al. 2013) assessing the effects of colchicine in in vivo cardiac muscle. Caffeine is considered a cardioprotective alkaloid as well, but with an antagonistic effect, as it moderately increases blood pressure and heart beating rate due to its activation of the adenosine receptors (Haller et al. 2004). Similar to caffeine, the PeA ephedrine is used by intravenous

administration to treat hypotension, due to its stimulation of α/β adrenergic receptors (Wang et al. 2019).

Alkaloids have also been studied as potential treatments for diabetes and obesity. In this sense, the IA berberine has been described to be an effective antidiabetic drug, both in vivo by decreasing insulin resistance in liver tissue (Lee et al. 2006) and evidencing lower glycemia and glycated hemoglobin levels in clinical trials (Yin et al. 2008). Similarly, the IA palmatine has been suggested together with berberine as a potential treatment against obesity, diabetes, and its related symptoms (Tarabasz and Kukula-Koch 2020). Both IA were reported to lower total cholesterol, plasma triacylglycerides as well as improve gut microbiota in mice with significant results (He et al. 2016). The PuA caffeine has also shown to improve pancreatic function in vivo by reducing inflammation of this organ, which led to reduce glycemia (Fernandez-Gomez et al. 2016). The PA nornicotine has also been studied as an alternative to treat obesity and elevated glycaemia. It has been reported to reduce body fat and stimulate physical activity, acting in a different manner to berberine or palmatine. This is due to its activation of dopamine receptors, which causes an increase in the satiety sensation (Grebenstein et al. 2022).

The antioxidant properties of alkaloids are also a recent matter of interest since an adequate oxidative balance is related to stable inflammatory status and may prevent the incidence of related diseases, such as cardiovascular pathologies and even cancer (Garcia-Oliveira et al. 2021). This property has been highlighted in caffeine, which was shown to increase levels of glutathione reductase and reduced glutathione in diabetes-induced rat pancreas, hinting at an improvement in its oxidative status and function by increasing the level of these endogenous antioxidants (Fernandez-Gomez et al. 2016). Likewise, the IzA vincamine has demonstrated to exert antioxidant effects in vivo by restoring the levels of the endogenous antioxidants myeloperoxidase, malondialdehyde, and hemeoxygenase-1 levels (El-Sayed et al. 2021). These restorations of endogenous antioxidants are related to an amelioration of other symptoms, whether related to metabolism and/or inflammation (El-Sayed et al. 2021).

Nevertheless, other applications for alkaloids have been explored. For example, the TA atropine is used for diverse treatments. It is commonly used for pupil dilatation in ophthalmic inspections, but also as an immediate treatment against seizures. This was demonstrated in in vivo studies showing an immediate anticonvulsant effect (Miller et al. 2015). Atropine has been recently described to significantly ameliorate myopia progression in a recent 5-year long clinical trial involving 400 children. Daily administrations of low concentration (0.01%) droplets of atropine resulted in a reduced myopia progression on an average of 1.38 diopters compared with non-treated children (Chia et al. 2016).

To sum up this section, alkaloids share some common functional groups in their chemical structure that affect their potential biological and therapeutical properties. In addition, their vast chemical diversity is translated into an immense range of biological properties that are continuously explored. Therefore, depending on dose, target condition, and other factors, many alkaloids can display many biological properties. Thus, deep research and re-evaluation of known plant alkaloids can

lead to the discovery of a great number of potential therapeutical applications of these compounds.

6.5 Conclusions

In this chapter, an overview of the current production systems and biological activities of marketed alkaloids has been presented. Regarding the obtention of these compounds, the extraction from plants is the best option, due to the drawbacks of chemical and microbial synthesis. Different strategies to enhance the accumulation of alkaloids in plants (based on genetic or environmental manipulation) have been proposed, but it is still necessary to fully elucidate several metabolic pathways in order to design more efficient production systems. Therefore, more advances in the field of alkaloid metabolism are expected in the coming years. During the extraction of alkaloids from plants other compounds can also be co-extracted and thus, purification and isolation steps are necessary to obtain alkaloids with the desired purity which can increase the production costs. This fact may also justify their commercialization by the pharmaceutical and nutraceutical industries. The main limitation of producing alkaloids-rich extracts is associated with the effort of producing them at an industrial scale at reasonable costs, and the lack of deep knowledge of their biosynthesis and chemical characterization. In this sense, technological progress in the field of extraction, purification, and isolation of alkaloids could contribute to facilitate the scalability of these compounds and thus, allow the commercialization of a greater number of alkaloids. Currently, there are many alkaloids with extremely diverse chemical structures that are marketed, such as caffeine, codeine, paclitaxel, or morphine due to their beneficial properties, like antioxidant, anti-inflammatory, anticancer, etc. In this sense, the evaluation of the biological properties of currently commercialized alkaloids and those more recently discovered open different markets for their commercialization and extend the scope of applications.

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Chapter 7
Polyketides

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Abstract During the evolution of the plant species, several biochemical mechanisms have been raised as adaptation pathways to external abiotic and biotic factors by generating chained biosynthetic reactions of resistance. The molecular response to the surrounding environment conditions was consistently translated by plants through synthesizing chemical mediators otherwise named "*secondary metabolites*" with multiple biological functions. Thus, the structural diversity of these metabolites makes the plant kingdom the widest source of biomolecules with various applications. The synthetic routes of those bio-compounds differ in relation to several influenceable elements and respecting the genetic coding of the plant species. The presence of functionally distinct multiple enzymes such as the enzymatic group of polyketide synthase (PKS) that catalyzes the initial key reactions in the biosynthesis of the plant polyketides (PKs) is at the origin of structurally assorted bioactive compounds or plant drugs. Hence, careful observations of the chemical structures of these complex molecules will substantially inform about the extraordinary biological abilities that may result from their variability, explaining the tropism and the different effects of the potential developed drugs on the organism's entities. For the benefit to the humanity, these molecules have been able to serve as natural remedies many times during human evolution resolving serious health issues or even saving generations from waves of infections and pandemics. Nowadays, scientists are aiming to study the bio-compounds that have important industrial applications in the food and pharmaceutical industries. Scientific works were then consolidated to classify plant secondary metabolites and also to explain their biosynthesis pathways, their molecular reactivity, and biological interactions. More specifically, the scientific community focuses research on molecules with a large broad spectrum of applications with fewer side effects on the human organism such as the plant

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polyketides and derivatives. Those groups of molecules are very diversified and contain a variety of chemical functions such as carbonyl, hydroxyl, ester groups, etc. with complex structures containing one or more aromatic rings. Their study is, however, of great importance because it opens up substantial opportunities for the development of new undeniable therapeutic avenues.

Abbreviations

2AQ	2-Alkylquinolone
2-PS	2-pyrone synthase
4CL	4-coumarate-CoA ligase
ACC	Acetyl-CoA carboxylases
AcCoA	Acetoacetyl-CoA
Ac-CoA	Acetyl-coenzyme A
ACP	Acyl carrier protein domain
ACS	Acridone synthase
ADCS	Aminodeoxychorismate synthase
ADS	Alkyldiketide-CoA synthase
Ala	Alanine
ALS	Aloesone synthase
ANS	Anthocyanidin synthase
AQS	Alkylquinolone synthase
Arg	Arginine
ARO	Aromatases
AS	Aureusidin synthase
ASA	Anthranilate synthase
ASCL	Anther-specific chalcone synthase-like
Asn	Asparagine
Asp	Aspartate
AT	Acyltransferase domain
ATM	Ataxia telangiectasia mutated
BAS	Benzalacetone synthase
BBS	Bibenzyl synthase
Bcl-2	B-cell lymphoma-2
BIS	Biphenyl synthase
BL	Biotin ligase
BNY	Bis-noryangonin
BPS	Benzophenone synthase
BUS	Isobutyrophenone synthase
C	Carbon
C=O	Carbonyl oxygen
C4H	Cinnamate-4-hydroxylase
cdc25C	Hepatocellular carcinoma cell division cycle 25C

CHI	Chalcone flavanone isomerase
Chk1/2	kinase-1/2 checkpoints
CHS	Chalcone synthase
CHSs	Chalcone synthases
CM	Chorismate mutase
CO_2	Carbon dioxide
CoA	Coenzyme A
CS	Chorismate synthase
CTAL	4-Coumaroyltriacetic acid lactone
CTAS	4-Coumaroyltriacetic acid synthase
CTAS	Coumaroyl triacetic acid synthase
CURS	Curcumin synthase
Cys	Cysteine
DCS	Diketide-CoA synthase
DFR	Dihydroflavonol 4-reductase
DH	Dehydratase domain
DKS	β-diketone synthase
DOXP	1-Deoxy-D-xylulose 5-phosphate
DXR	Deoxyxylulose 5-phosphate reductoisomerase
ER	Enoyl reductase
F3′5′H	Flavonoid 3′5′ hydroxylase
F3′H	Flavonoid 3′ hydroxylase
F3H	Flavanone 3-hydroxylase
FLS	Flavonol synthase
FS1	Flavone synthase 1
FS2	Flavone synthase 2
Glu	Glutamate
Gly	Glycine
HCC	Hepatocellular carcinoma
His	Histidine
HKS	Hexaketide synthase
HvCHS	Homoeriodictyol/eriodictyol synthase
ICM	Iso-chorismate synthase
Ile	Isoleucine
iPKSs	Iterative type I polyketide synthase
ISPdf	Methylerythritol 4-phosphate cytidylyltransferase methylerythritol 2,4-cyclodiphosphate synthase
ISPe	4-Diphosphocytidyl-2-C-methylerythritol kinase
ISPg	4-Hydroxy-3-methylbut-2-en-1-yl diphosphate synthase
ISPh	1-Hydroxy-2-methyl-butenyl 4-diphosphate reductase
KAS	Ketoacyl synthase
kDa	Kilodaltons
KR	Ketoreductase domain
KS	Ketosynthase domain

Leu	Leucine
Lys	Lysine
Man-CoA	Malonyl-coenzyme A
MDcx	Mevalonate-5-pyrophosphate decarboxylase
MEP	2-C-methyl-D-erythritol 4-phosphate
Met	Methionine
mPKSs	Modular type I polyketide synthase
MVA	Mevalonate
NO	Nitrogen oxide
OH	Hydroxyl
OKS	Octaketide synthase
PAL	Phenylalanine ammonium lyase
PCS	Pentaketide chromone synthase
PDH	Pyruvate dehydrogenase
Phe	Phenylalanine
pHPB	4-Hydroxybenzalacetone 4-(4-hydroxyphenyl)-but -3-in-2-one
PKS I	Type I polyketide synthase
PKS II	Type II polyketide synthase
PKS III	Type III polyketide synthase
PKS	Polyketide synthase
PKs	Polyketides
Pro	Proline
PSMs	The plant secondary metabolites
PstrCHS2	C-methylchalcone synthase
PYKS	Pyrrolidine ketide synthase
QNS	Quinolone synthase
RESV	Resveratrol
SEK4a/SEK4b	Octaketide
Ser	Serine
Sk	Shikimate Kinase
SMs	Secondary metabolites
STCS	Stilbenecarboxylate synthase
STS	Stilbene synthase
STSs	Stilbene synthases
TAL	Triacetic acid lactone
TE	Thioesterase domain
Thr	Threonine
Trp	Tryptophan
Try	Tryptophan
UDP	Uridine diphosphate
UFGT	UDP-glucose:flavonoid 3-O-glucosyltransferase
UV	Ultraviolet
Val	Valine
VPS	Phlorisovalerophenone synthase

ZoCURS	(*Zingiber officinale*) curcumin synthase
α-KS	a-keto acyl synthase
β-KS	β-keto acyl synthase

7.1 Introduction

One of the most important challenges medicinal chemists facing today is to design new drugs with improved properties and reduced side effects (Zhou and Zhong 2017; Kiriiri et al. 2020). Chemists typically start the process by taking one main structure and then finding analogs of it with the preferred biological activities (Zhou and Zhong 2017; Ganellin 2020). Then experimentation helps them to eventually choose an analog designed for further development (Zhou and Zhong 2017; Kiriiri et al. 2020; Ganellin 2020). This process is difficult, expensive, and time-consuming. The molecular design is usually inspired from molecules coming from living entities which have a character/property of marked resistance to the various surrounding aggressions (Zhou and Zhong 2017; Ganellin 2020; Atanasov et al. 2021). However, once a natural molecule proves its effectiveness by biological tests, chemists try to mimic it chemically and use it to develop unnatural compounds for a specific usage (Atanasov et al. 2021). Actually, one of the groups of compounds which has attracted so much attention from biochemists and biologists is the polyketides (PKs) which occur in nature and have aroused particular importance for targeting treatment pathways in relation to their bioactive effects already noted in the producing organisms (Eckermann et al. 1998; Bisht et al. 2021; Yu and Jez 2008; Richardson and Khosla 1999). They are produced by several living entities in the form of secondary metabolites (SMs) or intermediate products with more or less branched structures (Bisht et al. 2021; Abe 2020). Polyketides regroup a wide variety of chemical building blocks pending from SM pathways and present several biological activities (Gokulan et al. 2014; Ridley and Khosla 2009; Tsai and Ames 2009), they designate natural compounds containing ketone groups and/or hydroxy, separated by a carbon atom (Richardson and Khosla 1999; Caldara-Festin et al. 2015; Tan et al. 2020). The metabolic pathway of plant polyketide biosynthesis is shown to resemble that of fatty acids (Schaub et al. 2019; Kim et al. 2013; Austin and Noel 2003; Ferrer et al. 1999) with a significant distinction in the formation of fatty acids is the reduction of the β carbon in the aliphatic chain containing a carboxylic acid also the plant-PKs synthases uses CoA thioesters for shuttling substrates and intermediate PKs instead of the acyl carrier proteins (ACP) used by the fatty acid synthases (ß-ketoacyl synthase) (Austin and Noel 2003; Xie et al. 2016). Both classes of compounds derive from the condensation of carboxylic acids in the form of acetyl-coenzyme A (acetyl-CoA) and acyl-coenzyme A (Acyl-CoA) (Abe 2020; Yu et al. 2012; Jez et al. 2002; Morita et al. 2007, 2010a). The CoA-tethered will be the starter unit while acyl-CoA A (dicarboxyl-CoA), usually malonyl-CoA in plant species (Bisht et al. 2021), will act as an elongation unit (Bisht et al. 2021; Morita et al. 2007; Crawford and Townsend 2010). In plants, the type III polyketide

synthase (PKS III) enzymes biosynthesize the PKs chain through decarboxylative condensation of the starter derived two-carbon units (Bisht et al. 2021; Abe 2020; Shi et al. 2008) with a range of extender-CoA units which are defined according to the enzymatic specificity and the reaction mechanisms occurring at the active site of each enzyme (Bisht et al. 2021; Wakimoto et al. 2012; Healy et al. 2018). Those enzymes are well endowed with chemodiversity (Bisht et al. 2021; Abe 2020; Morita et al. 2010a; Wakimoto et al. 2012) from several precursor molecules (Bisht et al. 2021; Yu et al. 2012; Shi et al. 2008; Wakimoto et al. 2012), depending on their specificity (Wakimoto et al. 2012; Flores-Sanchez and Verpoorte 2009), and can control the condensation number and direct a specific cyclization of the common linear intermediate (Ferrer et al. 1999; Wakimoto et al. 2012; Healy et al. 2018; Lussier et al. 2012). The resultant chemically reactive chain of PKs undergoes tautomerization steps into keto and enol (enolate) substructures that acquire further bio-functionalities (Bisht et al. 2021; Abe 2020; Healy et al. 2018). Hence, specific tautomer dictated by the catalytic environment of specific PKS enzymes follows cyclization and unloads various polyphenolic chemicals (Bisht et al. 2021; Abe 2020; Morita et al. 2010a; Healy et al. 2018; Watts et al. 2006). Thus, the set of plant-PKs enzymatic environment involves various biochemical interactions including reduction (Frisvad et al. 2020; Wang et al. 2020; Zhou et al. 2016; Hu et al. 2020), dehydration (Zhou et al. 2016), decarboxylation (Zhou et al. 2016; Morita et al. 2010b), and cyclization (Bisht et al. 2021; Healy et al. 2018; Wang et al. 2020; Hu et al. 2020) leading to structurally diverse PKs and a variety of biologically and medicinally important natural product classes. Several aromatic polyketides are highly substituted fused-ring polyphenols (Austin et al. 2004a; Abe 2008), containing an aromatic aglycone pending from the poly-β-ketone backbone (Caldara-Festin et al. 2015; Crawford and Townsend 2010). The aglycone can then be rearranged by specific reactions such as methylation (Storm et al. 2018; Skiba et al. 2018), oxidation, and glycosylation (Wang et al. 2020) to give various bioactive natural products.

Scientific works focused on the plant-PKs have lifted the veil on their wide range of adaptive roles in plants, including protection against UV radiation (Bisht et al. 2021; Dao et al. 2011), specific coloring of flowers (Pandith et al. 2016, 2020), the astringent and bitter flavors (Bisht et al. 2021; Zhou et al. 2016; Niaz and Khan 2020), the pollen development (Xie et al. 2016), the root nodulation (Xie et al. 2016; Stewart et al. 2013), plant architecture and chemical defense against infectious agents (Bisht et al. 2021; Abe 2020; Morita et al. 2010a). The scientific community continues to emphasize the diversity of the in-vivo functions of PKs which is reflected in their varied medicinal properties (Bisht et al. 2021; Abe 2020). In the plant system, PKs-derived products majorly induce the formation of flavonoids (Bisht et al. 2021; Ferrer et al. 1999; Dao et al. 2011; Niaz and Khan 2020; Naake et al. 2021), stilbenes (Bisht et al. 2021; Healy et al. 2018; Niaz and Khan 2020), chalcones (Bisht et al. 2021; Wakimoto et al. 2012; Naake et al. 2021), benzalacetones (Bisht et al. 2021; Stewart et al. 2013), curcuminoids (Abe 2020; Niaz and Khan 2020; Zhang et al. 2016), cannabinoids (Bisht et al. 2021; Tahir et al. 2021), chromones (Bisht et al. 2021; Abe 2020; Abe et al. 2005), pyrones (Bisht

et al. 2021; Zhou et al. 2016; Abe et al. 2005), anthrones (Bisht et al. 2021; Niaz and Khan 2020), phloroglucinols (Zhou et al. 2016; Niaz and Khan 2020; Abe et al. 2005), resorcinols (Abe et al. 2005; Miyanaga and Horinouchi 2009), xanthones (Bisht et al. 2021; Niaz and Khan 2020; Franklin et al. 2009), acridones (Bisht et al. 2021; Michael 2017), and quinolones (Abe 2020; Resmi et al. 2013; Shen et al. 2019) that are very representative products of PK synthesis reaction. They have repeatedly shown an antioxidant (Watts et al. 2006; Franklin et al. 2009; Palkina et al. 2021), anti-inflammatory (Palkina et al. 2021; Hook et al. 2014), and protective properties by slowing aging in model organisms (Ping 2016). These biological capacities are in direct correlation with the prevention of cancer (Atanasov et al. 2021; Palkina et al. 2021; Ralston et al. 2005) and new researches on the potential of these molecules continue to emerge for introduction into treatment programs (Shen et al. 2019; Omar 2017) against chronic and infectious diseases (Atanasov et al. 2021; Wang et al. 2020; Bhattarai et al. 2021) in which PKs can be effective and efficient agents. Meanwhile, production of these molecules through chemistry and biocatalysts is restricted in yield and titer (Gao et al. 2010). Biosynthetic engineering technologies are constantly needed to enable more efficient discovery and development of polyketide antibiotics of plant origin (Morita et al. 2007; Wakimoto et al. 2012; Lussier et al. 2012; Lim et al. 2016).

This chapter focuses on the structural diversity of large groups of plant-polyketides, presenting the intrinsic mechanisms of their synthesis, probing their mode of cyclization during the enzymatic biogenesis while approaching their biological significance and by commenting on the potential of biosynthesis, the limitation, and the unique character of each synthetic enzymes. The enzymology, mechanistic aspect, and mode of synthesis of plant polyketides are depicted through this chapter. The various enzymatic structures responsible for the synthetic pathway of PKs and the reaction mechanisms of initiation and condensation are discussed to provide paramount information on the molecular factors governing the selectivity of starting units and the control of chain length. Moreover, the enzymatic structures modifying polyketides and their derivatives exhibit the functionality of enzymes in the reduction and cyclization pathway which further add to the diversity of polyketide products. The biological role as well as the economic interest of these polyketides is also discussed since a complete understanding of the structural and biochemical features controlling polyketide biosynthesis and modification offers a powerful tool to control and rationally design novel polyketides through enzyme engineering leading into useful drug candidates.

7.2 Plant Polyketides as Secondary Metabolites

7.2.1 Outlines of Plant Secondary Metabolites Synthesis

During all the growth phases, plants accumulate valuable metabolites (Jan et al. 2021; Bhattacharya 2019) in specific tissues and structures such as vacuoles,

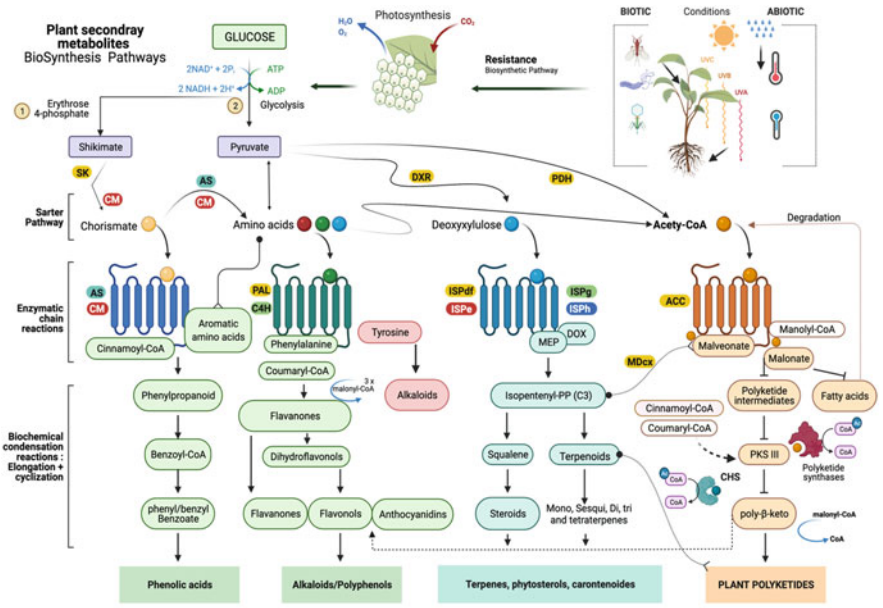

Fig. 7.1 Outline of the PSMs biosynthesis pathways representing shikimate/phenylpropanoid/mevalonate/MEP/DOXP pathways|Biotic and abiotic conditions affect biosynthetic pathways, starting from glucoselcertain classes of PSMs are synthesized in plant cells with complex localization of specific enzymes. *Abbreviations*: **Sk:** Shikimate Kinase, **CM:** Chorismate synthase, **AS:** Anthranilate synthase, **PAL:** phenylalanine ammonium lyase, **C4H:** cinnamate-4-hydroxylase, **DXR:** deoxyxylulose 5-phosphate reductoisomerase, **ISPdf:** methylerythritol 4-phosphate cytidylyltransferase methylerythritol 2,4-cyclodiphosphate synthase, **ISPe:** 4-diphosphocytidyl-2-C-methylerythritol kinase, **PDH:** Pyruvate dehydrogenase, **ISPg:** 4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase, **ISPh:** 1-hydroxy-2-methyl-butenyl 4-diphosphate reductase; **ACC:** Acetyl-CoA Carboxylases|**MDcx:** mevalonate-5-pyrophosphate decarboxylase, **PKS III:** type III polyketide synthase **CHS:** chalcone synthase, **AcCoA:** acetoacetyl-CoA|**DOX:** 1-deoxy-D-xylulose 5-phosphate, **MEP:** 2-C-methyl-D-erythritol 4-phosphate

cytoplasm, endoplasmic reticulum, plastids, chloroplasts, specialized glands, trichomes, and sometimes only at certain stages of development to cover plant needs (Abe 2020; Seemann et al. 2006). The plant secondary metabolites (PSMs) biosynthesis begins from the process of photosynthesis and the production of primary metabolites such as glucose and amino acids, which will follow a series of modifications and stereochemical reactions at the enzymatic level to be transformed into SMs necessary for adaptations to the surrounding biotic and abiotic conditions (Yu and Jez 2008; Jan et al. 2021; Geris et al. 2012). Their synthesis is affected by the genotype (Francisco et al. 2012; Shamloo et al. 2017), plant physiology, climate, environmental conditions (Jan et al. 2021; Li et al. 2020b), and pathogens (Pang et al. 2021), among others. The outline of the PSMs biosynthetic pathway and the involved enzymology is illustrated in Fig. 7.1. The fundamental PSMs biosynthetic pathways in higher plants are conducted within the enzymatic metabolic core

evoking reactions of glycosylation, methylation, hydroxylation, acylation, oxidation, phosphorylation, and prenylation (Wang et al. 2019a; Friso and Van Wijk 2015) as well as fewer chemical alterations due to adaptation of enzymes, causing a wide range of changes in basic structures. In the beginning, the precursors of the PSMs (Fig. 7.1) mainly derived from the Krebs cycle through the pyruvate and the shikimate pathway (Jan et al. 2021). As an essential metabolite of the shikimate pathway (Jan et al. 2021; Tohge et al. 2013), the chorismate is revealed as the precursor of tryptophan, tyrosine, phenylalanine, salicylate, phylloquinone, and folate; it is regulated by enzymes such as chorismate mutase (CM), iso-chorismate synthase (ICM), anthranilate synthase (AS), and aminodeoxychorismate synthase (ADCS). However, deoxyxylulose 5-phosphate reductoisomerase (DXR) (Seemann et al. 2006) and pyruvate dehydrogenase (PDH) (Lussier et al. 2012; Friso and Van Wijk 2015) produce, respectively, deoxyxylulose and acetyl-CoA units to be the precursors for terpenoids, steroids, and polyketides (Yu and Jez 2008). Based on biosynthetic pathways (Fig. 7.1), PSMs can be divided into three main groups: terpenes and terpenoids synthesized through the mevalonate (MVA), 2-C-methyl-D-erythritol 4-phosphate/1-deoxy-D-xylulose 5-phosphate (MEP/DOXP) pathways (Wang et al. 2019c), phenolic compounds, synthesized by the shikimate/phenylpropanoid pathway (Francenia Santos-Sánchez et al. 2019), and nitrogen-containing molecules synthesized via the tricarboxylic acid cycle pathway (Zhang and Fernie 2018). Since PSMs are synthesized mainly from small simple molecules beginning with primary metabolisms, such as acetyl-coenzyme A (acetyl-CoA) or amino acids (Fig. 7.1), these molecules show great chemical and functional divergence once arranged and restructured (Jan et al. 2021; Cummings et al. 2014). The shikimate as a precursor of the shikimate pathway (Jan et al. 2021; Francenia Santos-Sánchez et al. 2019) is produced from the glycolytic pathway (Jan et al. 2021; Bhattacharya 2019; Francenia Santos-Sánchez et al. 2019) by a combination of phosphoenolpyruvate and from the pentose phosphate pathway (erythrose 4-phosphate), it is the initial pathway for biosynthesis of aromatic amino acids (Jan et al. 2021; Tohge et al. 2013); under stress conditions, it is activated to produce tryptophan, tyrosine, and phenylalanine, which further enhance PSMs biosynthesis (Fig. 7.1).

Several main classes of PSMs are distinguished according to their structure and according to their biosynthetic pathway (Yu and Jez 2008); Alkaloids as nitrogen-containing compounds are metabolites mostly of plant origin, derived from amino acids (phenylalanine, tyrosine, and tryptophan), usually cyclic and nitrogen compounds (Abe 2020; Michael 2017), they are produced via the shikimate pathway and constitute the building blocks of protein synthesis and common precursors for plant SMs (Yu and Jez 2008; Tohge et al. 2013). Some molecules of this family attracted medical attention like morphine, codeine, nicotine, and caffeine (Jan et al. 2021; Bhattacharya 2019). The largest group of PSMs also known as terpenoids or isoprenoids are synthesized from five-carbon base units derived from isoprene units (C5), itself resulting from acetyl-CoA (Jan et al. 2021; Seemann et al. 2006; Załuski et al. 2015). For terpenoids, the MVA and MEP pathways are involved in terpene synthesis (Fig. 7.1). Acetyl coenzyme-A (acetyl Co-A) is the starting

material that also involves in the fusion of C5 isoprene units called isoprenoid (Wang et al. 2019c; Załuski et al. 2015). Isopentenyl diphosphate is formed as an intermediate in both MVA and MEP pathways (Kuzuyama and Seto 2012), which ultimately leads to the formation of terpenes (Jan et al. 2021; Seemann et al. 2006). The MEP pathway offers isoprenoid precursors (isopentenyl pyrophosphate-IPP and dimethylallyl pyrophosphate-DMAPP) for the synthesis of mono- (C10 or [C5] 2 units) and diterpenes ([C5]4 units), triterpenes ([C5]6 units), carotenoids ([C5] 8 units), polyterpenoids ([C5]n), phytohormones, gibberellins (C5]4), abscisic acid ([C5]3 units), phytol (florasol, phytosol), side-chain plastoquinones, phylloquinones, tocopherols, chlorophylls, etc. (Jan et al. 2021), while the MVA pathway (Wang et al. 2019c; Kuzuyama and Seto 2012) provides isopentenyl diphosphate (isopentenyl-PP-C3) involved in the synthesis of sesquiterpenes ([C5] 3 units), squalenes ([C5]6 units), sterols (C17), brassinosteroids, polyphenols, and its fraction and is used for prenylated proteins (Wang et al. 2019a). The second class of PSMs is represented by aromatic phenolic compounds (Fig. 7.1) which have a phenyl ring containing one or more acidic hydroxyl groups (Bhattacharya 2019). They constitute a group of heterogeneous molecules comprising several bioactive derivatives essentially formed by the malonate and shikimate pathways (Francenia Santos-Sánchez et al. 2019), which is very common in plant species. Precursors derived from glycolysis and pentose phosphate (phosphoenolpyruvate and erythrose-4-phosphate) are converted into different aromatic amino acids in which the most frequent intermediate is phenylalanine (Jan et al. 2021; Tohge et al. 2013). Phenolic compounds are essentially derived from the catabolism of phenylalanine (Jan et al. 2021; Tohge et al. 2013), they are classified into distinct groups such as coumarins, flavonoids, stilbenes, condensed tannins, lignins, lignans, styrylpyrones, and arylpyrones (Bhattacharya 2019; Francenia Santos-Sánchez et al. 2019). Moreover, simple phenolic compounds otherwise called phenylpropanoids (Yu and Jez 2008; Watts et al. 2006) such as trans-cinnamic acid, p-coumaric acid, and its derivatives as well as the phenylpropanoid/benzenoid volatiles are belonging to the class of phenolic compounds resulting from phenylalanine precursor (Lussier et al. 2012). Tyrosine in turn will generate pigment betalain, alkaloids, isoquinoline, and quinones (e.g., tocochromanols) (Seemann et al. 2006; Tohge et al. 2013). Tryptophan will be transformed mainly into alkaloids, phytoalexins, indole glucosinolates, and the plant hormone auxin (Franklin et al. 2009; Tohge et al. 2013; Alhadi Fatima et al. 2012).

Concomitantly, plant polyketides (PKs) (Fig. 7.1) hold a crucial place in the PSMs synthesis pathways (Yu and Jez 2008; Abe 2020; Wakimoto et al. 2012), taking place in enzyme systems called polyketide synthases III (PKS III) which are diversified and specific to each plant species (Bisht et al. 2021; Lussier et al. 2012; Schröder et al. 1998; Shimizu et al. 2017). Mechanistic enzymatic potentials are engaged to produce a broad array of PKS-derived products (Bisht et al. 2021; Abe 2020; Schaub et al. 2019; Wakimoto et al. 2012; Lim et al. 2016) by utilizing simpler starter and various extender units (Bisht et al. 2021; Abe 2020; Wakimoto et al. 2012). In turn, those polyketides are major precursors evoked by important secondary metabolic pathways. They lead to chemically distinct classes (Bisht et al. 2021;

Abe 2020; Morita et al. 2010a) such as flavonoids, stilbenes, chromones, acridones, pyrones, anthrones, benzalacetones, xanthones, cannabinoids, and aliphatic waxes to be valued in the bioengineering sector (Watts et al. 2006; Wang et al. 2020). To emphasize, PKs and derivatives will be able to behave at the same time as an antimicrobial, an antifungal, an antiparasitic, an antitumor, a cytotoxic, or an immunosuppressant (Bisht et al. 2021; Abe 2020; Xie et al. 2016; Shimizu et al. 2017; Christensen and Christensen 2013). Accordingly, this broad spectrum of activity is responsible for several profits generated by the pharmaceutical industry (Bisht et al. 2021; Gokulan et al. 2014), and as science progresses numerous PK derivative compounds continue to be discovered then commercially exploited providing solutions to human health disorders as well as consequent economic gains.

7.2.2 *Biosynthesis Pattern and Molecular Diversity of Polyketides*

As a broad aspect, polyketides are a large class of secondary metabolites regarded as acetate derived natural products ranging from antibiotics to toxins with clinically important applications (Flores-Sanchez and Verpoorte 2009; Bhattarai et al. 2021; Cummings et al. 2014). They are produced in bacteria (Crawford and Townsend 2010; Tang et al. 2017), fungi (Frisvad et al. 2020; Bhattarai et al. 2021), and plants (Yu et al. 2012; Morita et al. 2010a) or even marine animals (Wakimoto et al. 2012) through distinct biosynthesis pathways. Several examples of polyketides are found in bacterial antibiotics or "*bacterial aromatic polyketides*" (Hu et al. 2020; Tang et al. 2017) such as actinorhodin, tetracycline, erythromycin, and tetracenomycin (Wang et al. 2020). Several of them ensure important pharmaceutical effects such as the antimicrobial potential (rifamycin, erythromycin A, and virginiamycin M) (Wang et al. 2020; Skiba et al. 2018), antifungal (amphotericin) (Ridley and Khosla 2009; Weissman 2009), antiparasitic (avermectin) (Ridley and Khosla 2009; Cummings et al. 2014), antitumor (lankacidin, epothilone B, doxorubicin) (Ayoub et al. 2016; Chou et al. 1998), and immunosuppressant drugs (rapamycin) (Cummings et al. 2014). Polyketides from fungal/actinomycetes species (Frisvad et al. 2020; Bhattarai et al. 2021) are also abundant such as chrysophanol (Bhattarai et al. 2021; Fujii 1999), 9,10-anthraquinone, ophiocordin, ophiosetin, phaeosphenon, rousselianone A, spartinol A-D-C, spartinoxide (Bhattarai et al. 2021) and mycotoxins such as the aflatoxins (Aflatoxin B1, versicolorin, fumonisins, and zearalenone) (Bhattarai et al. 2021; Fujii 1999), ochratoxin A, citrinin, vomitoxin, patulin, nivalenol, and sterigmatocystin (Bhattarai et al. 2021; Geris et al. 2012). Albeit, aflatoxins synthesized by *A. flavus* and *A. parasiticus* (Bhattarai et al. 2021) are well-known examples of fungal polyketides with carcinogenic properties. Equally important, flavonoids, stilbenoids, chalones, alkaloids, benzalacetones, xanthones, cannabinoids, and several other complex molecules are produced from plant polyketides (Bisht et al. 2021; Morita et al. 2007; Wakimoto

et al. 2012; Flores-Sanchez and Verpoorte 2009). Exhibiting high degrees of structural diversity, they have a wide range of pharmacological properties with positive effect on human health and could prevent or reduce the risk of many diseases, including heart diseases (Tauchen et al. 2020), digestive and vascular disorders (Christensen and Christensen 2013; Dryden et al. 2006), diabetes, cancer, and age-related functional decline (Luo et al. 2021). Those bio-functionalities go back to the biosynthetic assembly lines during which the functional groups are added by the specific enzymes that strictly control the selection and incorporation of simple starter/extender units.

7.2.2.1 The Enzymology of Polyketide Synthases

Fundamental enzymological surveys indicate that the synthesis of polyketides is usually governed by a simple or complex multifunctional enzymatic system called "polyketide synthases (PKS)" (Bisht et al. 2021; Shi et al. 2008; Wakimoto et al. 2012; Schröder et al. 1998) which is a group of mono or multi-domain enzymes (Fig. 7.2) responsible for catalyzing the reactions of condensation of a CoA-ester

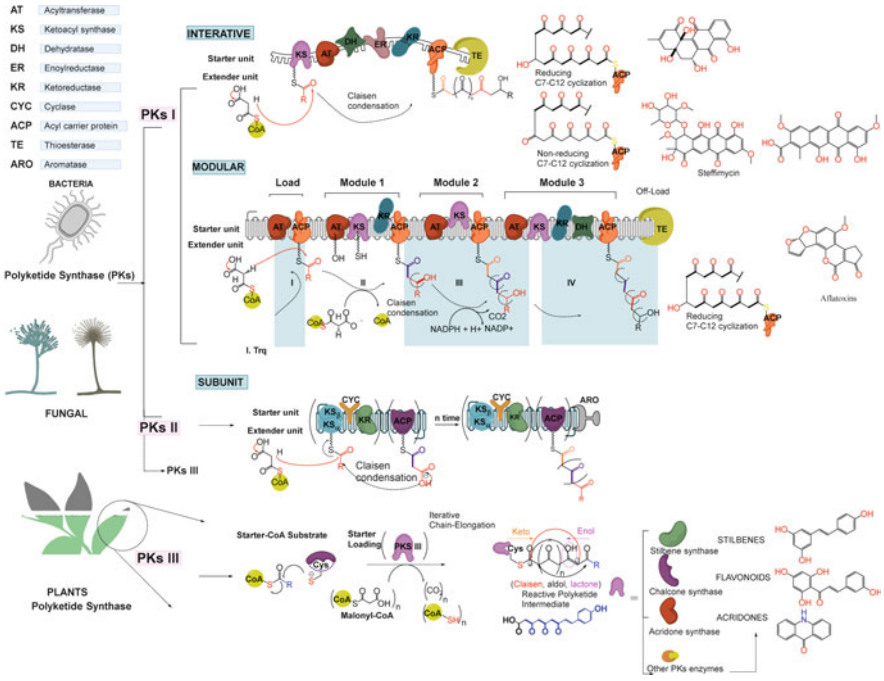

Fig. 7.2 Main types of polyketide synthases and mechanisms of catalytic reaction| Type I PKS **(PKSI)** exhibiting the Iterative and Modular rearrangement domains in bacterial and fungi species| Type II PKS **(PKSII)** clustering pattern on subunits| Type III PKSs **(PKSIII)** in plant system using acyl-CoA as the starter unit and malonyl-CoA as the extender unit

such as acetyl-CoA, with extension CoA esters, such as malonyl-CoA and cyclization of the products thus assembled (Tan et al. 2020; Shi et al. 2008; Austin et al. 2004a; Abe et al. 2004). Whereas, various simple homodimeric PKS control the PKs synthesis in higher plant species (Abe 2020; Morita et al. 2010a; Pandith et al. 2016; Hook et al. 2014). Each living family (from prokaryotes to eukaryotes) is characterized by specific PKSs with distinct chemistry, usually classified according to their structural features and functionality.

PKS present in bacteria and fungi species describes a system of one or more multifunctional protein clusters (Richardson and Khosla 1999; Hu et al. 2020; Tang et al. 2017) that contain independent catalytic sites (Fig. 7.2). There will be as many active sites on all of the subunits as it takes enzymatic steps to synthesize the enzyme product (Tsai and Ames 2009; Caldara-Festin et al. 2015; Hu et al. 2020; Tang et al. 2017). Numerous similarities at the genetic, biosynthetic, and product structural levels (Bisht et al. 2021; Tsai and Ames 2009; Morita et al. 2010a) can be raised in polyketides and the enzymes responsible for their synthesis; conversely, for each living entities, there are some differences allowing their classification into subcategories (Bisht et al. 2021; Yu et al. 2012; Morita et al. 2010a; Shimizu et al. 2017) explained by the slight differences of Pk types, structures, and condensation reactions in the specific enzymes among those living kingdoms, although bacterial strains (such as actinomycetes, streptomyces genus) (Geris et al. 2012; Pandey et al. 2018) and fungal species (aspergillus, penicillium...) (Frisvad et al. 2020) produce a broad range of aromatic-PK, that are a polycyclic bioactive molecule of microbial origin deriving from multiple decarboxylative condensations (Tsai and Ames 2009; Frisvad et al. 2020; Tang et al. 2017) of starter malonyl units to form relatively long-chain (>,14 carbon atoms) polyketide intermediates (Caldara-Festin et al. 2015; Crawford and Townsend 2010; Dunstan et al. 2020). This highly reactive intermediates are then transformed within the enzyme's active sites through a unique set of intramolecular cyclization (Caldara-Festin et al. 2015; Healy et al. 2018; Wang et al. 2020), elimination, oxidoreduction, and group transfer reactions to produce a highly functionalized PK including hydroxyl/carbonyl groups with fused and/or disconnected carbocyclic and/or heterocyclic rings (Richardson and Khosla 1999). For the plant species, the presence of functionally distinct PKS enzymes (Bisht et al. 2021; Xie et al. 2016; Shi et al. 2008) that catalyze the initial key reactions in the biosynthesis of the PK-derived products is at the origin of bioactive polyketides. The enzyme required for the condensation of acetylated Coenzyme A (CoA) derivatives to form a polyketide is called polyketide synthase (PKS) (Eckermann et al. 1998; Shi et al. 2008; Wakimoto et al. 2012; Schröder et al. 1998).

Until recently the PKSs were majorly located in prokaryotes and filamentous fungi (Frisvad et al. 2020; Pandey et al. 2018) where presumably evolved from fatty acid synthases (Abe 2020; Wakimoto et al. 2012; Flores-Sanchez and Verpoorte 2009). They were therefore classified into 3 types (PKS I, PKS II, and PKS III) (Shen 2003) (Fig. 7.2) very early according to their similarity to the two types of fatty acid synthases (Smith and Tsai 2007; Chan et al. 2009). PKS type I bears a strong resemblance to type I fatty acid synthases of vertebrates (Smith and Tsai 2007). For type II, the similarity is stronger to the type II fatty acid synthases of bacteria and

plants (Lussier et al. 2012; Hu et al. 2020; Shen 2003). Polyketide synthases of type I are mainly found in filamentous fungi (Frisvad et al. 2020; Fujii 1999; Taura et al. 2016) and in bacteria (Tsai and Ames 2009; Shen 2003), and more particularly in actinomycetes (Dhakal et al. 2019). Type III PKSs are found mainly in higher plants (Shi et al. 2008; Wakimoto et al. 2012; Flores-Sanchez and Verpoorte 2009; Stewart et al. 2013) and some fungi and bacteria species (Katsuyama and Horinouchi 2010), where they are classified as small homodimers holding the hole PK synthesis in a single site referred to keto synthase enzymatic domain. In a comprehensive approach, the classification of this type of enzymes and their catalytic mechanisms can be considered as follows:

7.2.2.1.1 Type I Polyketide Synthase

Type I polyketide synthase are multifunctional proteins containing linearly arranged and covalently fused domains (Wang et al. 2020; Shen 2003; Cheng et al. 2009). This complex system regroups a series of heteromeric enzymes (Fig. 7.2) each containing a different active site specializing in reaction catalysis throughout the assembly and modification stages of the polyketide carbon chain (Shen 2003; Salas 2004). The type I PKSs can be further classified into iterative type I PKSs (iPKSs) (Shen 2003; Cheng et al. 2009; Salas 2004) and modular type I PKSs (mPKSs) (Schröder et al. 1998; Shen 2003; Smith and Tsai 2007; Morita et al. 2019) (Fig. 7.2) where most research studies have focused on those two types of bacterial PKSs. In fungal or bacterial systems, they are usually present in both iterative or modular subgroups where a distinct domain works cooperatively and/or iteratively to catalyze the carbon chain elongation and functional group regeneration (Cheng et al. 2009). The mPKSs are primarily found in bacteria and have large, multifunctional proteins which use each enzyme once to synthesize macrolide or polyether polyketides (Richardson and Khosla 1999). Then, iPKSs do not contain a thioesterase or cyclase domain (Fujii 1999) that is found in other PKSs for the release of the covalently linked polyketide chain so the domains are used repeatedly to catalyze multiple rounds of elongation and are mainly found in fungi (Frisvad et al. 2020; Bhattarai et al. 2021). Actually, mPKSs are organized in interconnected modules (Wang et al. 2020; Shen 2003; Cheng et al. 2009), ranging from acyltransferase (AT), acyl carrier protein (ACP), and b-keto acyl synthase (b-KS) (Tsai and Ames 2009; Wang et al. 2020; Cheng et al. 2009). Figure 7.2 illustrates the reaction chain mechanism of mPKS I multimodular complex that consists of acyltransferase (AT), ketosynthase (KS), thioesterase (TE), and optional domains. These domains make it possible to initiate the synthesis of PKs by loading an acyl-coenzyme A (CoA) starter unit on the active site of the acyl carrier protein (ACP) which is catalyzed by the AT domain (Shen 2003). While the KS domain (Cummings et al. 2014) will lengthen the carbon chain by maintaining decarboxylative condensations of the Claisen-type and during condensation (Shen 2003; Chan et al. 2009; Cheng et al. 2009), acetyl-CoA continuously attached to malonyl-CoA leaves behind the acid group. The β-keto chain produced will undergo further modifications generating different polyketide

structures by additional domains, notably the ketoreductase (KR), dehydratase (DH), and enoyl reductase (ER) domains (Tsai and Ames 2009; Shen 2003; Smith and Tsai 2007; Dhakal et al. 2019). The TE domain intervening at the end of the chain will allow hydrolysis or cyclization of the complete polyketide chain of the ACP domain to complete the elongation (Shen 2003; Cheng et al. 2009). Fungal PK biosynthesis mainly involves two particular classes of polyketide synthase PKS I and III (Yu et al. 2012; Frisvad et al. 2020). Thus, fungal PKS I synthesis is similar to that of eukaryotic fatty acids, evoking a multimodular complex allowing the production of the majority of PKs of fungal origin (Crawford and Townsend 2010; Bhattarai et al. 2021).

7.2.2.1.2 Type II Polyketide Synthases

Type II polyketide synthase are a multi-enzyme complex (Lussier et al. 2012; Shen 2003) aggregated from subunit monofunctional proteins (Fig. 7.2) responsible for the production of various aromatic-PKs with chain length of C-10 to C-30 in bacteria (Tang et al. 2017; Salas 2004). Generally using acetate as the starting unit, this enzymatic complex produces the aromatic-PKs following an iterative Claisen condensation (Weissman 2009; Shen 2003; Cheng et al. 2009). Their production is, however, governed by a sequence of the following reactions (Wang et al. 2020; Shen 2003; Hertweck et al. 2007): (a) Loading of α-carboxylated precursor (acetate) on ACP to form acyl-ACP. (b) iterative chain extension is continued with ketone reduction, during this step acyl-ACP is transferred to the KS subunit and iteratively extended with malonyl-CoA forming the poly-β-keto chain (c) under catalysis of aromatases (ARO) and oxygenases, cyclization and/or aromatization conversions occurs on the poly-β-keto chain to generate the aromatic polyketide nucleus (d) oxygenases, methyltransferases and glycosyltransferases concentrate to transform the aromatic-PK core into bioactive derivatives. As shown in Fig. 7.2, the biosynthesis of aromatic-PKs by type II is carried out iteratively. A set of enzymes called minimal type II PKS contains a ketoacyl synthase KSα-CLF factor (Wang et al. 2020) that can determine the carbon chain length of aromatic-PK by controlling the number of Claisen condensations, and chain length factor (or ketoacyl synthase KSβ-CLF) and ACP subunits with surrounding cyclase and KS units (Wang et al. 2020; Tang et al. 2017). The sequences of the KSα and KSβ components are similar, with a difference in the active site of KSα containing cysteine involved in the assembly of aromatic-PKs (Wang et al. 2020; Hertweck et al. 2007). The bacterial PKs-derived from PKS II are aromatic and contain diversified chemical groups directed by the employment of different starter units (such as propionyl-CoA, acetyl-CoA, methylmalonyl-CoA) that could provide additional structurally diverse products (Ridley and Khosla 2009; Wang et al. 2020). For instance, kinamycins, contains a diazo group, resulting from the intermediate dehydrorabelomycin by condensation of 1 acetyl-CoA and 9 malonyl-CoA molecules (Wang et al. 2020; Hu et al. 2020). Lomaiviticins, as the main class of diazo-containing aromatic-PKs with antibiotic and antitumor activities, were reported to be synthesized after the

conversion of methylmalonyl-CoA to propionyl-CoA as starter unit within a bifunctional enzyme (Lom62 in *Salinispora pacifica*) that catalyzes acyltransferation and decarboxylation reactions (Wang et al. 2020). In addition, the oxytetracycline (broad-spectrum antibiotic belonging to the tetracycline class) is biosynthesized by *S. rimosus* PKS II enzymatic clusters from malonamate (3-amino-3-oxopropanoate) starter unit derived from malonyl-CoA by aminotransferase OxyD and thiolase OxyP (Wang et al. 2020; Yin et al. 2015).

7.2.2.1.3 Microbial Type III Polyketide Synthase

As regards the second fungal PK synthesis pathway, the type III PKS pathway consists of a single keto synthase enzymatic domain (KS domain) (Fig. 7.2) and is responsible for a limited number (a dozen) of PKs already characterized in some species of fungi (Flores-Sanchez and Verpoorte 2009; Lussier et al. 2012; Austin et al. 2004a; Shen 2003). According to Bhattarai et al. (2021), type III PKSs have been characterized from the fungi, including 10 from Ascomycota and one from Basidiomycota (Bhattarai et al. 2021; Navarro-Muñoz and Collemare 2020). For example, pentaketide resorcylic acid was the first type III fungal PKS isolated from *Neurospora crassa* (Funa et al. 2007). As long as plants and fungi share common synthetic pathways with respect to the PKS III pathway through three-dimensional overall fold with a conserved Cys-His-Asn catalytic triad (Abe 2020; Wakimoto et al. 2012; Abe et al. 2004), the produced PK derivatives are frequently present in both kingdoms (Shi et al. 2008; Flores-Sanchez and Verpoorte 2009; Lussier et al. 2012). In particular, anthraquinones or anthraquinonoids (alizarin, purpurin, munjistin, emodin, chrysophanol, phaeosphenone, aloe-emodin, physcion, rhein, etc.) (Geris et al. 2012; Diaz-Muñoz et al. 2018) are a class of natural phenolic compounds based on the 9,10-anthraquinone skeleton (Geris et al. 2012; Diaz-Muñoz et al. 2018; Martínez and Bermejo 2005). They widely occur in plants (Aloe latex, senna, rhubarb, and cascara buckthorn) (Abe et al. 2005; Diaz-Muñoz et al. 2018) and fungi (*Aspergillus* spp., *Eurotium* spp., and *Penicillium* spp.). Emodin (6-methyl-1,3,8-trihydroxyanthraquinone) was isolated from *Aspergillus* spp. and *Penicillium* spp. (Fujii 1999) and also produced in coffee weed (*Semen cassia*), in rom rhubarb, buckthorn, and Japanese knotweed (*Reynoutria japonica*) (Abe et al. 2004). The Chrysophanol which is a C-3 methyl substituted chrysazin (1,8-dihydroxyanthraquinone) (Martínez and Bermejo 2005) occurs in several plant species belonging to liliaceae, meliaceae, asphodelaceae, and fabaceae (Diaz-Muñoz et al. 2018) and fungi (*Trichoderma* spp.) (Crawford and Townsend 2010; Fujii 1999).

7.2.2.1.4 Plant Type III Polyketide Synthase (PPKS III)

Different from type I and type II PKSs, plant-PKSs are part of the iterative type III PKS, which structurally form simple homodimeric proteins (Fig. 7.2) with two

functionally independent active-site cavities (Shi et al. 2008; Wakimoto et al. 2012; Abe et al. 2004; Pothiraj et al. 2021) where they use CoA rather than ACP as an anchor for chain extension (Flores-Sanchez and Verpoorte 2009). As a protein, ubiquitous plant-PKS III are reported to have a 40–45 kDa polypeptide chain containing approximately 400 amino acids forming a homodimeric ketoacyl synthase (KAS) domain (Morita et al. 2019). Around 20 functionally different type III plant-PKSs (Wakimoto et al. 2012; Morita et al. 2019) have been isolated and approximately 38 to 95% of their amino acid sequence homology has been revealed where they all share the same independent active site comprising a catalytic triad Cys-His-Asn (Morita et al. 2007, 2010a; Shi et al. 2008; Wakimoto et al. 2012; Abe et al. 2004). Their mechanism is generally based on a chemical strategy of priming CoA-tethered substrates to carry out thioester exchange reactions, polyketide chain elongation, and select cyclization paths in a single active site (Bisht et al. 2021; Abe 2020; Yu et al. 2012; Morita et al. 2007; Shi et al. 2008). Thus, within the active site, specific starter substrates (monocarboxyl-CoA) undergo repetitive decarboxylative condensations with a simple dicarboxyl-CoA extension substrate to generate a poly-β-keto intermediate undergoing specific condensation then cyclized or released in the form of a linear product forming several chemically assorted structures (Bisht et al. 2021; Wakimoto et al. 2012; Abe et al. 2005). Indeed, the architectural simplicity of type III PKSs as well as their catalytic power of initiation and combination of different starting units makes these enzymes attractive manipulable targets for the engineering of biocatalysts (Stewart et al. 2013). Structurally, for each dimer, the end of the CoA binding tunnel has a large catalytic internal cavity where the catalytic triad resides and the reactions are initiated by binding acyl-CoA and loading the acyl group on an active-site cysteine (Morita et al. 2007; Stewart et al. 2013) and iterative cycles of decarboxylative condensation then proceed with two-carbon units derived from malonyl-CoA (Fig. 7.2). A wide variety of CoA-related starter substrates are known to be used by plant type III PKSs (Bisht et al. 2021; Morita et al. 2007, 2010a; Shi et al. 2008; Wakimoto et al. 2012), including intermediates of the phenylpropanoid pathway (Bisht et al. 2021; Schro 1999), CoA/N-acetyl cysteamine and activated aliphatic/aromatic mono- and di-carboxylic acids. To name only some, acetyl-CoA, malonyl-CoA, methylmalonyl-CoA, p-coumaroyl-CoA, cinnamoyl-CoA, N-methylanthraniloyl-CoA, n-hexanoyl-CoA, isobutyryl-CoA, isovaleryl-CoA, and 3-hydroxybenzoyl-CoA (Bisht et al. 2021; Morita et al. 2010a; Wakimoto et al. 2012; Schröder et al. 1998; Weissman 2009). These type III PKSs generally catalyze iterative decarboxylated condensations of malonyl units with the aforementioned molecules (Table 7.1 and Fig. 7.3): Chalcone synthase (CHS), 2-pyrone synthase (2-PS), stilbene synthase (STS), bibenzyl synthase (BBS), homoeriodictyol/eriodictyol synthase (HEDS or HvCHS), acridone synthase (ACS), benzophenone synthase (BPS), phlorisovalerophenone synthase (VPS), isobutyrophenone synthase (BUS), coumaroyl triacetic acid synthase (CTAS), C-methylchalcone synthase (PstrCHS2). CHS and STS are massively studied enzymes due to their extraordinary functional diversification. Often referred to as the CHS/STS-like superfamily where they share approximately 46–95% amino acid sequence identity. They differ in their starting

Table 7.1 Plant type III PKSs classification according to the number/type of intramolecular cyclization, the preferred substrates, the main intermediates, reaction products and derivatives, and their occurrence of the plant species

Number of cyclization	PKS superfamily_ intramolecular cyclization	Starter-CoA)/extender-CoA (xn)	Polyketide intermediate	Main products and derivatives	Occurrence in the main plant species	References
One cyclization reaction	**CHS-type PKS** Cyclization (C1-C5 Claisen)_ring (aromatic)					
	Chalcone synthase (CHS)	(p-Coumaroyl-CoA)/ Malonyl-CoA (3x)	Tetraketide p-coumaroyl triacetyl thioester	**Main product:** Naringenin chalcone, **Derivatives:** Chalcones, trihydroxychlacone, 2′,4′- -dihydroxychalcone, flavokawains, aurones, flavonoids anthocyanes	*M. sativa, M.domestica, F. hybrida, P. methysticum, R. verniciflua, D. pinnata, B. monosperma, A. keiskei, Alpinia species, Acacia genus*	Bisht et al. (2021), Austin and Noel (2003), Ferrer et al. (1999), Dao et al. (2011), Pandith et al. (2016), Pandith et al. (2020), Niaz and Khan (2020), Abe et al. (2005), Ralston et al. (2005), Gao et al. (2010), Jan et al. (2021), Bhattacharya (2019), Seemann et al. (2006), Li et al. (2020b), Tohge et al. (2013), Shimizu et al. (2017), Tauchen et al. (2020), Schro (1999), Rammohan et al. (2020), Kontturi and Type (2017), Kumar and Pandey (2013), Rencoret et al. (2022), Abu et al. (2013), Jasim et al. (2021), Yoshioka et al. (2020), Gonçalves et al. (2014), Ghribi et al. (2021), Christensen (2018), Huang et al. (2020), Dixon (1999), Forkmann and Heller (1999), Liu et al. (2021), Mazziotti et al. (2022), Lee et al. (2012)

	Acridone synthase (ACS)	(N-methylanthraniloyl-CoA)/Malonyl-CoA (3x)	Tetraketide intermediate	**Main product:** 1,3-Dihydroxy-Nmethylacridone **Derivatives:** Alkaloids acridones, anthranilate-derived alkaloids, 4-hydroxy-N-methylquinolone, 1,3-dihydroxy-N-methylacridone, N-methylanthranilriacetic, C-prenyl acridone alkaloids, furoacridone alkaloids, dimeric acridone alkaloids, miscellaneous alkaloids, azacridone-A, acrimarines	*Ruta graveolens, Huperzia serrata, B. albiflora, Citrus microcarpa, C. maxima, S. buxifolia, C. sinensis, C. medica.* Genus Ruta, Citropsis, Glycosmis, Severinia, Swinglea, Vepris, Conchocarpus, Zanthoxylum	Bisht et al. (2021), Austin and Noel (2003), Ferrer et al. (1999), Xie et al. (2016), Jez et al. (2002), Morita et al. (2010a), Shi et al. (2008), Flores-Sanchez and Verpoorte (2009), Zhou et al. (2016), Abe et al. (2005), Michael (2017), Resmi et al. (2013), Shimizu et al. (2017), Taura et al. (2016), Schro (1999), Matsui et al. (2017)
	Valerophenone synthase (VPS) • Phlorisovalerophenone synthase (VPS) • Isobutyrophenone synthase (BUS).	• (Isovaleryl-CoA)/ Malonyl-CoA (3x) • (Isobutyryl-CoA)/ Malonyl-CoA (3x)	Tetraketide Isovaleroyl triacetyl thioester Isobutyryl triacetyl thioester	**Main products:** Phlorisovalerophenone, Phlorisobutyrophenone, 4-hydroxy-6-methyl-2-pyrone **Derivatives:** Prenylflavonoids (xanthohumol, isoxanthohumol, phloroglucinol), humulone, cohumulone, adhumulone, lupulone, colupulone and adlupulone	*H. lupulus, H. calycinum, G. hybrida, Eucalyptus sp., M. spicatum*	Bisht et al. (2021), Austin and Noel (2003), Morita et al. (2010a), Flores-Sanchez and Verpoorte (2009), Zhou et al. (2016), Austin et al. (2004a), Niaz and Khan (2020), Schröder et al. (1998), Shimizu et al. (2017), Schro (1999), Rammohan et al. (2020), Gonçalves et al. (2014), Ramawat and Mérillon (2013), Clark et al. (2013), Kráľová and Šeršeň (2012)
	Homoeriodictyol/ eriodictyol synthase (HvCHS)	• (Feruloyl-CoA)/ Malonyl-CoA (3x) • (Caffeoyl-CoA)/ Malonyl-CoA (3x)	Tetraketide Feruloyl triacetyl thioester Caffeoyl triacetyl thioester	**Main products:** Homoeriodictyol, Eriodictyol **Derivatives:** Chalcones, sterubin (7-methoxy-3′,4′,5-trihydroxyflavanone) Eriodictyol-glycosides (saponarin and lutonarin)	*Ho. vulgare,* *E. californicum*	Bisht et al. (2021), Austin and Noel (2003), Morita et al. (2010a), Shimizu et al. (2017), Dunstan et al. (2020), Ley et al. (2005), Kamiyama and Shibamoto (2012)

(continued)

Table 7.1 (continued)

Number of cyclization	PKS superfamily_ intramolecular cyclization	Starter-CoA)/extender-CoA (xn)	Polyketide intermediate	Main products and derivatives	Occurrence in the main plant species	References
	STS-type PKS Cyclization (C2-C7 aldol) + decarboxylation _ ring (aromatic)					
	Stilbene synthases (STSs)	p-Coumaroyl-CoA/ Malonyl-CoA (3x)	Tetraketide p-coumaroyl triacetyl thioester	**Main product:** Resveratrol **Derivatives:** Phytoalexins, pterostilbene, rhapontigenin, piceatannol (astringenin), astringin, gnetol, piceid (polydatin), pinosylvin, 2-isopentenyl-resveratrol, viniferin, oxyresveratrol, RESV trimethylether, pinosylvin monomethylether, resveratroloside, desoxyrhapontigenin, isorhapontigenin, isorhapontin, pinostilbenoside, rhaponticin (rhapontin), rhapontigenin	*Arachis hypogaea, Pinus sylvestris, P. strobe, Vitis vinifera*	Bisht et al. (2021), Shi et al. (2008), Flores-Sanchez and Verpoorte (2009), Watts et al. (2006), Niaz and Khan (2020), Resmi et al. (2013), Gao et al. (2010), Jan et al. (2021), Shimizu et al. (2017), Tauchen et al. (2020), Luo et al. (2021), Hertweck (2009), Liu et al. (2021), Zuiter and Zarqa (2014), Yang et al. (2018)
	Biphenyl synthase (BPS)	(benzoyl-CoA)/Malonyl-CoA (3x)	Tetraketide 3, 5-dihydroxybiphenyl	**Main product:** 3, 5-dihydroxybiphenyl **Derivatives:** Phytoalexins biphenyl (dibenzofuran and biphenyl aucuparin), dibenzofurans	*S. aucuparia* Rosaceae species	Bisht et al. (2021), Austin and Noel (2003), Ferrer et al. (1999), Flores-Sanchez and Verpoorte (2009), Abe (2008), Pandith et al. (2016), Abe et al. (2004), Songsiriritthigul et al. (2020), Stewart et al. (2017), Liu et al. (2011)
	Bibenzyl synthase (BBS),	(3-hydroxyphenylpropionyl-CoA)/Malonyl-CoA (3x)	Tetraketide intermediate 3, 3′5-trihydroxybibenzyl	**Main product:** 9,10-dihydrophenanthrene **Derivatives:** Dihydrophenanthrenes (batatasins), tricyclic phenanthrenoid 9, 10-dihydrophenanthrene derivatives (hircinol, Orchinol)	*Phalaenopsis sp., B. striata, D. thyrsiflorum O. militaris*	
	Hexaketide synthase (HKS)	(Acetyl-CoA)/Malonyl-CoA (5x)	Hexaketide: Pyrone, 6-(2,4-dihydroxy-6-methylphenyl)-4-hydroxy-2-pyron	**Main product:** Naphthoquinone hexaketide **Derivatives:** Naphthoquinones and naphthalenes, plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone), 6-hydroxy	*A. arborescens* Genus Plumbago	Atanasov et al. (2021), Bisht et al. (2021), Yu et al. (2012), Morita et al. (2010a), Shi et al. (2008), Wakimoto et al. (2012), Flores-Sanchez and

				plumbagin, isoshinanolone, isoplumbagolone, chitranone, maritinone, chitranane, resorcinol hexaketide		Verpoorte (2009), Abe (2008), Pandith et al. (2020), Stewart et al. (2013), Abe et al. (2005), Hook et al. (2014), Jan et al. (2021), Geris et al. (2012), Załuski et al. (2015), Shimizu et al. (2017), Taura et al. (2016), Morita et al. (2019), Diaz-Muñoz et al. (2018), Martínez and Bermejo (2005), Lajis and Ahmad (2006), Kontturi and Type (2017), Abe and Morita (2010), Mazziotti et al. (2022)
	Octaketide synthase (OKS)	(acetyl-CoA)/Malonyl-CoA (7x)	Linear octaketide	**Main product:** Anthraquinone Octaketide **Derivatives:** Anthranoid, octaketides SEK4 and SEK4b, phloroglucinol heptaketide, anthrones, anthraquinones, dianthrones, emodin anthrone, anthrone glycosides (barbaloin), sennoside A and B	*A. arborescens, A. nyeriensis, and A. cheranganiensis, Cassia angustifolia*	
	Cyclization (C2-C7 aldol) without decarboxylation _ ring (aromatic)					
	Stilbenecarboxylate synthase (STCS)	(4-coumaroyl-CoA or dihydro-4-coumaroyl-CoA)/ Malonyl-CoA (3x)	Tetraketide p-coumaroyl triacetyl thioester	**Main products:** Hydrangeic acid and lunularic acid **Derivatives:** 5-Hydroxyhydrangeic acid, 5-hydroxylunularic acid, phenyldihydroisocoumarin, hydrangenol.	*H. macrophylla, L. cruciata*	Bisht et al. (2021), Morita et al. (2010a), Flores-Sanchez and Verpoorte (2009), Austin et al. (2004b), Eckermann et al. (2003)
	Benzophenone synthase	(benzoyl-CoA)/Malonyl-CoA (3x)	Tetraketide 2,4,6-trihydroxybenzophenone	**Main product:** 2,4,6-trihydroxybenzophenone **Derivatives:** Benzophenones, xanthones/ hyperxanthone (sampsoniones, 7-epi-clusianone, peroxysampsone A, sampsonione A and I, hypersampsone D and E), prenylated xanthones (α-mangostin, 1,5,8-trihydroxy-3-methoxy-2-(3-methylbut-2-enyl) 1,6-dihydroxy-3-methoxy-2-(3-methyl-2-buthenyl)-xanthone)	*H. androsaemum, C. erythraea H. sampsoniisuch*	Bisht et al. (2021), Austin and Noel (2003), Morita et al. (2010a), Flores-Sanchez and Verpoorte (2009), Abe et al. (2005), Franklin et al. (2009), Abe et al. (2004), Schro (1999), Songsiriritthigul et al. (2020), Stewart et al. (2017), Crockett et al. (2011), Chen et al. (2014), Zhu et al. (2014)

(continued)

Table 7.1 (continued)

Number of cyclization	PKS superfamily_ intramolecular cyclization	Starter-CoA)/extender-CoA (xn)	Polyketide intermediate	Main products and derivatives	Occurrence in the main plant species	References
More than 2 cyclization reactions	STS-type PKS Cyclization (C2-C7 aldol) _ ring (aromatic/heterocyclic)					
	Pentaketide chromone synthase (PCS)	Malonyl-CoA (5x)	Tetraketide α-pyrones	**Main product:** 5,7-dihydroxy-2-methylchromone **Derivatives:** Chromones, 2-phenoxychromone, pyranochromones (allopteroxylin, 3,3-dimethylallylspatheliachromene, spatheliabischromene, 5-*O*-methylcneorumchromone), furanochromones (visnagin and khellin)	*P. aculeata,* *A. arborescens,* *A. rupestris, E. koreanum,* *S. aromaticum,* *D. vandellianum,* *A. visnaga*	Bisht et al. (2021), Yu et al. (2012), Morita et al. (2010a), Morita et al. (2007), Wakimoto et al. (2012), Flores-Sanchez and Verpoorte (2009), Abe et al. (2005), Shimizu et al. (2017), Abe et al. (2004), Diaz-Muñoz et al. (2018), Lajis and Ahmad (2006), Aisa et al. (2006), Jin et al. (2014), Youssef et al. (2022), Lee et al. (2016), Alves et al. (2017), Khalil et al. (2020)
	Aloesone synthase (ALS)	Malonyl-CoA (6x, 7x, 8x)	Hexaketides Heptaketides Octaketides	**Main products**: Hexaketide/ heptaketide/octaketide chromone aloesone **Derivatives:** Heptaketide 6-(2-acetyl-3,5-dihydroxybenzyl)-4-hydroxy-2-pyrone, heptaketide 6-(2-(2,4-dihydroxy-6-methylphenyl)-2-oxoethyl)-4-hydroxy-2-pyrone, octoketides (SEK4/SEK4b), aloesone O-glucoside (7-O-β-D-glucopyranoside)	*R. palmatum,* *A. arborescens*	Bisht et al. (2021), Yu and Jez (2008), Yu et al. (2012), Morita et al. (2007), Wakimoto et al. (2012), Flores-Sanchez and Verpoorte (2009), Abe (2008), Shimizu et al. (2017), Abe et al. (2004), Mizuuchi et al. (2009), Jindaprasert et al. (2008)
	CTAS -type PKS C1-O-C5 Lactonization, heterocyclic)					
	4-Coumaroyltriacetic acid synthase (CTAS)	(p-Coumaroyl-CoA)/ Malonyl-CoA (3x)	Coumaroyl triacetyl thioester	**Main product:** P Coumaroyl triacetic **Derivatives:** Acid lactones,	*Hydrangea macrophylla*	Yu and Jez (2008), Austin and Noel (2003), Jez et al. (2002), Dao et al. (2011), Eckermann et al. (2003),

				dihydroxymethylphenyl methylpyrone		Hertweck (2009), Akiyama et al. (1999), Klundt et al. (2009)
	C-Methylchalcone synthase (PstrCHS2)	(2RS)-methylmalonyl-CoA/ cinnamoyl diketide (1X)	Triketide 3-methyl-2-pyrone	**Main product:** Methylstyrylpyrone, Dimethylated chalcones, C-methylated flavonoids	*Pinus strobes, P. methysticum*	Xie et al. (2016), Jez et al. (2002), Shi et al. (2008), Wakimoto et al. (2012), Flores-Sanchez and Verpoorte (2009), Morita et al. (2010b), Pandith et al. (2020), Abe et al. (2005), Jan et al. (2021), Schröder et al. (1998), Shimizu et al. (2017), Abe et al. (2004), Sun et al. (2015)
	2-Pyrone synthase (2-PS)	(Acetyl-CoA)/malonyl-CoA (2X)	Triketide 3,5-diketohexanoate thioester	**Main product:** Triacetic acid lactone (TAL), 6-methyl-4-hydroxyl-2-pyrone **Derivatives:** α-pyrones, phenylpyrones, oxygenated polyketide lactones, monobenzo α-pyrones (esculetin, scopoletin), umbelliferone, Djalonensone, kavalactones, dihydrokavain, kavain	*G. hybrida, R. verniciflua, G. biloba C. intybus, A. djalonensis, C. novoguineensis, P. methysticum Umbelliferae family, genus Scopolia*	Kim et al. (2013),Austin and Noel (2003), Shi et al. (2008), Schro (1999), Eckermann et al. (2003), Klundt et al. (2009), Anyanwu et al. (2018), Hegazy et al. (2019), Gurley et al. (2008), Chunyan et al. (2009), Cheong et al. (2010), Li et al. (2014), Schäberle (2016), Nwafor et al. (2017)
None cyclization reaction	**PKS/**assembly without cyclization					
	Diketide-CoA synthase (DCS)	(Feruloyl-CoA)/Malonyl-CoA (1x)	Feruloyl diketide-CoA	**Main product:** Feruloyldiketide	*C. longa, O. sativa*	Abe (2020), Shi et al. (2008), Flores-Sanchez and Verpoorte (2009), Zhang et al. (2016), Morita et al. (2019), Matsui et al. (2017), Christensen (2018)
	Curcumin synthase (CURS)	(Feruloyldiketide-CoA)/ Feruloyl-CoA (1x)		**Main product:** Curcumin **Derivatives:** Bisdemethoxycurcumin, demethoxycurcumin	*C. longa, O. sativa Z. officinale*	
	β-Diketone synthase	(Fatty acyl-CoA (C12-C16))/Malonyl-CoA (1x)	Tetraketide intermediate	**Main product:** 1,3-Diketones (-β-diketones) **Derivatives:** Hydroxy-β-diketones, β-diketones/	*Poaceae species (Hordeum vulgare, Triticum sp, A. sativa), Orchidaceae (V. fragrans, V. tahitensis)*	Bisht et al. (2021), Hen-Avivi et al. (2016), Ramaroson-Raonizafinimanana et al. (2000)

(continued)

Table 7.1 (continued)

Number of cyclization	PKS superfamily_ intramolecular cyclization	Starter-CoA)/extender-CoA (xn)	Polyketide intermediate	Main products and derivatives	Occurrence in the main plant species	References
				epicuticular wax, hentriacontane-14,16-dione		
	Benzalacetone synthase (BAS)	4-Coumaroyl-CoA/ Malonyl-CoA (1x) N-methylanthraniloyl-CoA/ Methyl-malonyl-CoA) (1X)	Diketide benzalacetone	**Main product:** 4-hydroxybenzalacetone 4-(4-hydroxyphenyl)-but -3-in-2-one **Derivatives:** Phenylbutanoids 4-Hydroxy-2(1H) quinolones, lindleyin	*R. palmatum R. idaeus, Rutaceae species*	Bisht et al. (2021), Austin and Noel (2003), Morita et al. (2010a), Flores-Sanchez and Verpoorte (2009), Morita et al. (2010b), Zhang et al. (2016), Resmi et al. (2013), Matsui et al. (2017), Shimokawa et al. (2012)
	Alkyldiketide-CoA synthase (ADS)	(Acyl -fatty CoA (C8-C12))/ Malonyl-CoA (1x)	Alkyldiketide-CoA	**Main product:** Diketidic acid	*E. rutaecarpa, T. ruticarpum*	Bisht et al. (2021), Abe (2020), Zhang et al. (2016), Morita et al. (2019), Matsui et al. (2017), Rebhun et al. (2015), Li et al. (2020a)
	Alkylquinolone synthase (AQS)	(Diketidic acid)/N -methylanthraniloyl-CoA		2-Alkylquinolone (2AQ) alkaloids '(evocarpine) Quinazolinocarboline alkaloids (rutecarpine, evodiamine)	*E. rutaecarpa, T. ruticarpum*	
	Quinolone synthase (QNS)	(N-methylanthraniloyl-CoA) Malonyl-CoA (2x)	Diketide 4-hydroxy-1-methyl-2H-quinolone	**Main product:** 4-hydroxy2 (1H) -quinolone **Derivatives:** Quinolone alkaloids, quinine, 2(1H)-quinolone, 4(1H)-quinolone, meflo-quine, and casimiroine	*A. marmelos, S. japonica, A. baueri, M. fareana Rutaceae species*	Bisht et al. (2021), Abe (2020), Shi et al. (2008), Wakimoto et al. (2012), Resmi et al. (2013), Shen et al. (2019), Ping (2016), Matsui et al. (2017), Seneca (2007)
	Pyrrolidine ketide synthase (PYKS)	(Malonyl-CoA)/Malonyl-CoA (1x)	Diketide 4-carboxy-3-oxobutanoyl	**Main product:** Racemic 4-(1-methyl-2-pyrrolidinyl)-3-oxobutanoic acid **Derivatives:** Tropinone and tropane alkaloids, Pseudotropine, 2-Carbomethoxytropinone, ecgonine, ecgonidine, atropine and scopolamine	*E. coca, H. niger, A. belladonna*	Abe (2020), Tsai and Ames (2009), Morita et al. (2010a), Morita et al. (2019), Seneca (2007), Huang et al. (2019), Bedewitz et al. (2018), Shah et al. (2020), Passos and Mironidou-Tzouveleki (2016)

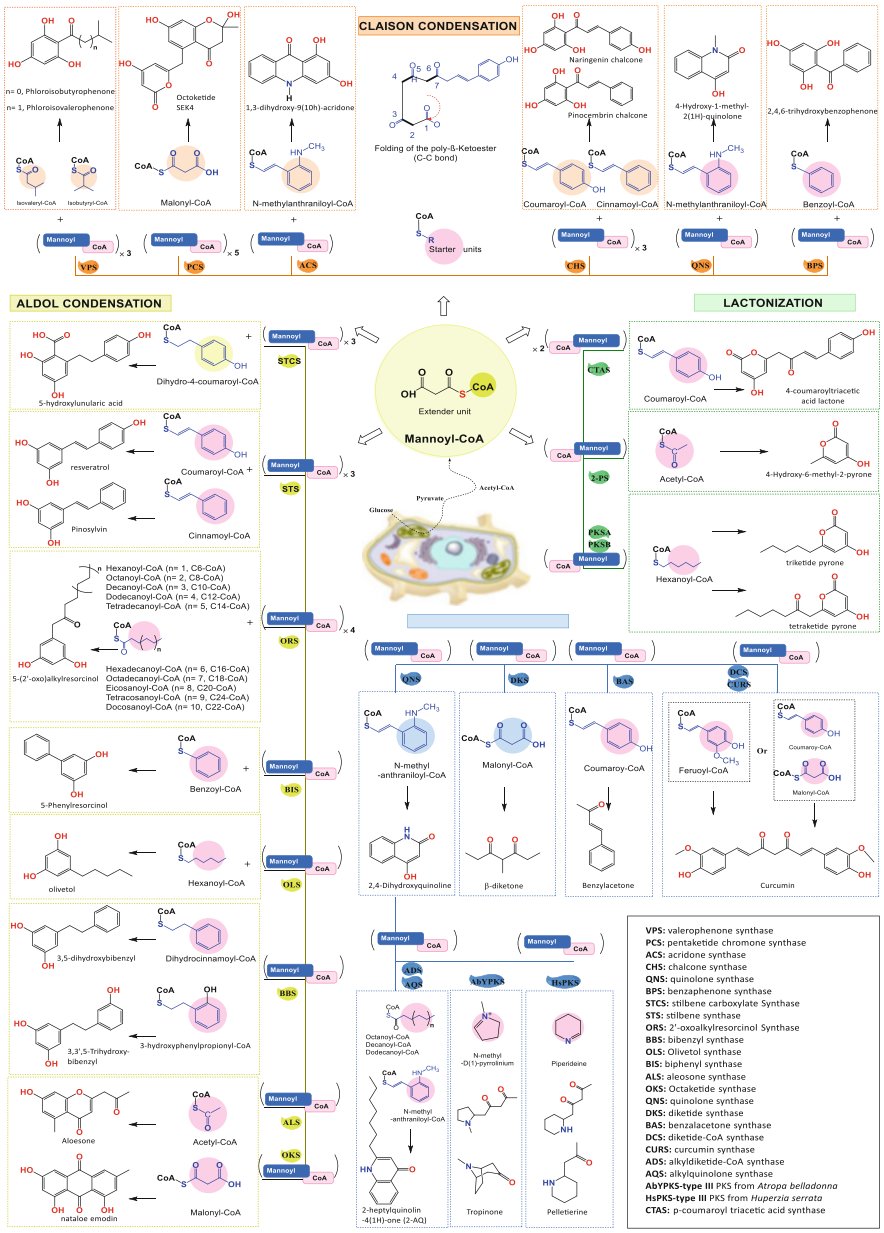

Fig. 7.3 Main biosynthesized PKs cyclization pattern conducted on plant-PKSs III using a common malonyl-CoA extender unit and the preferred starter molecules, The CoA-starter units are represented in relation to the specific enzymes. At the top, the C6-O-C1 Claisen condensation is represented in orange color, at left the aldol condensation (C7-O-C2) is depicted in yellow| At the right| Lactonization condensation (C5-O-C1) is shown in green and the non-cyclized polyketides are presented in blue (bottom)

substrates ranging from small substrates such as acetyl-CoA to bulky substrates (p-coumaroyl-CoA), also from aliphatic-CoA substrates to aromatic-CoA substrates, likewise, polar substrates (malonyl-CoA) to nonpolar substrates (isovaleroylCoA) (Bisht et al. 2021; Morita et al. 2007; Shi et al. 2008; Stewart et al. 2013; Abe et al. 2005).

7.2.2.2 Insights of the Plant-PKS III Reactional Mechanistic Aspect

Plant-derived PKs are grouped together on purely biosynthetic grounds where they can have a succession of alternating carbonyl/hydroxyl and methylene groups (-CO-CH_2-) or they are derived from molecules thus arranged (Fig. 7.3). Their assorted structures can be explained as being derived from poly-β-keto chains, resulting from starter acetate (C2) and extender-CoA esters units coupling (i.e., $nCH_3CO_2H \rightarrow -[CH_2CO]n$) via a series of enzymatic Claisen (Tan et al. 2020; Shi et al. 2008; Healy et al. 2018; Rammohan et al. 2020), Aldol (Bisht et al. 2021; Kim et al. 2013; Morita et al. 2007; Healy et al. 2018; Funa et al. 2007; Austin et al. 2004b), or Lactone condensations (Tan et al. 2020; Jez et al. 2002; Alexander 2016). In a global way, the formation of this poly-β-keto chain is determined in the catalytic site of each particular enzyme where specific catalysis/condensation reactions could be repeated and controlled to generate a poly-β-ketoester of appropriate chain length (Morita et al. 2007; Abe 2008; Abe et al. 2005). Differences in the structural and functional properties of each specific enzyme, such as the width of the catalytic cavity, the amino acid residues involved in the active site, and the length of the CoA linkage tunnel (Bisht et al. 2021; Morita et al. 2010a; Wakimoto et al. 2012; Abe et al. 2005) as well as the possible mutation occurring in peptide sequences at the catalytic zone (Jez et al. 2002; Pandith et al. 2016, 2020) that may determine acquired properties of the promiscuity of the substrate (Bisht et al. 2021; Austin and Noel 2003; Wakimoto et al. 2012; Pandith et al. 2020; Stewart et al. 2013; Pandey et al. 2018) and cyclization reactions (Shimizu et al. 2017; Weissman 2009; Abe et al. 2004; Chan et al. 2009; Morita et al. 2019) offer a panoply of PK with extraordinarily varied chemical structures. All those factors may however provide insight into the enzymatic reaction mechanisms.

Scientific works (Bisht et al. 2021; Kim et al. 2013; Yu et al. 2012; Morita et al. 2010a; Flores-Sanchez and Verpoorte 2009; Pandith et al. 2020; Stewart et al. 2013; Shimizu et al. 2017; Shen 2003; Chan et al. 2009; Salas 2004) were tied to explain the high intrinsic reactivity of the active site of PKS III. Those studies have identified several key amino acid residues affecting the starter specificity, cavity volume, rounds of chain elongation, and cyclization. Thus, the differences in the number of condensation or polyketidic chain elongation reactions within the PKS III catalytic site and the type of cyclization reaction allow the classification of the plant PK derivative bioproducts. The study of PKS III structural enzymology and the structure–function relationship at the crucial residue positions may however clarify the reaction specificity of its catalytic pocket(s) which tames and redirects the poly-ß-ketides to PK-derived products (Tsai and Ames 2009). It should also be

noted that recent studies (Bisht et al. 2021; Austin and Noel 2003; Wakimoto et al. 2012; Songsiriritthigul et al. 2020) have indicated that the volume and shape of the initiation/elongation/cyclization cavity govern the selectivity of the starter molecule, the length of the polyketide chain, and the folding and cyclization pathways of the PKS type III.

7.2.2.2.1 Global Mechanism/Specificity of Plant-PKs Biogenesis Reactions

(a) Plant-PKs Biogenesis Based on Mode of Condensation

The entire plant-PKs synthesis pathway comprises various steps of biochemical interactions carried out in PKS III. To recall, each enzyme shows differences in the specificity of the starter, the number of extensions, and the cyclization of the final product (Table 7.1 and Fig. 7.3). The steric, dynamic, and electronic factors (Flores-Sanchez and Verpoorte 2009; Stewart et al. 2013) within the active site concentrate the multiple points of chemical reactivity intervening to modulate the length of the chain and the bonds formation during the regiospecific cyclization of enzyme-bound intermediate at the end of the reaction, then, further post-synthesis modifications evolve the polyketide core into distinct bio-functionalities.

Besides, the chemical reactions that take place during cyclization are regioselective, and this dictates the structure of the final aglycone of the PKs (Fig. 7.4) originating from the poly-B-Keto chain is characterized structurally by the number of cycles determined by the total length of the carbon skeleton (Morita et al. 2007; Abe et al. 2005); the cyclization patterns as dictated by the regioselectivities of the individual carbon–carbon bonds merging the rings (Austin and Noel 2003; Flores-Sanchez and Verpoorte 2009; Pandith et al. 2016; Shimizu et al. 2017) and by the ring topology resulting from different oxidative rearrangement reactions. Distinct ring-folding interactions directly determine the chemical class of PKs derivatives (Bisht et al. 2021; Austin and Noel 2003; Tsai and Ames 2009; Eckermann et al. 2003); the majority of literature documents specify three main chemistries (Table 7.1); C6-C1 Claisen condensation catalyzed by enzymes such as chalcone synthases (CHSs)-type subfamily (Bisht et al. 2021; Austin and Noel 2003; Morita et al. 2010a; Austin et al. 2004a), including the chalcone synthase (CHS), acridone synthase (ACS), quinolone synthase (QNS), valerophenone synthase (VPS), homoeriodictyol/eriodictyol synthase (HvCHS), and benzophenone synthase (BPS) where a C-C bond forms between the C6 position carbon and C1 position carbon of the catalytic cysteine-bound intermediate. Moreover, a C2-C7 aldol (aromatic and or heterocyclic) type condensation (Austin and Noel 2003; Pandith et al. 2020; Stewart et al. 2013; Shimizu et al. 2017; Austin et al. 2004b) is unique to the stilbene synthases (STSs), stilbenecarboxylate synthase (STCS), biphenyl synthase (BIS), bibenzyl synthase (BBS), aloesone synthase (ALS), pentaketide chromone synthase (PCS), and octaketide synthase (OKS), where the cyclization pattern is based on a C-C bond formation from C2 position carbon to C7 position carbon with an additional decarboxylative loss of the C1 as CO_2 (Bisht et al. 2021; Morita et al. 2010a; Abe et al. 2005; Schröder and Schröder

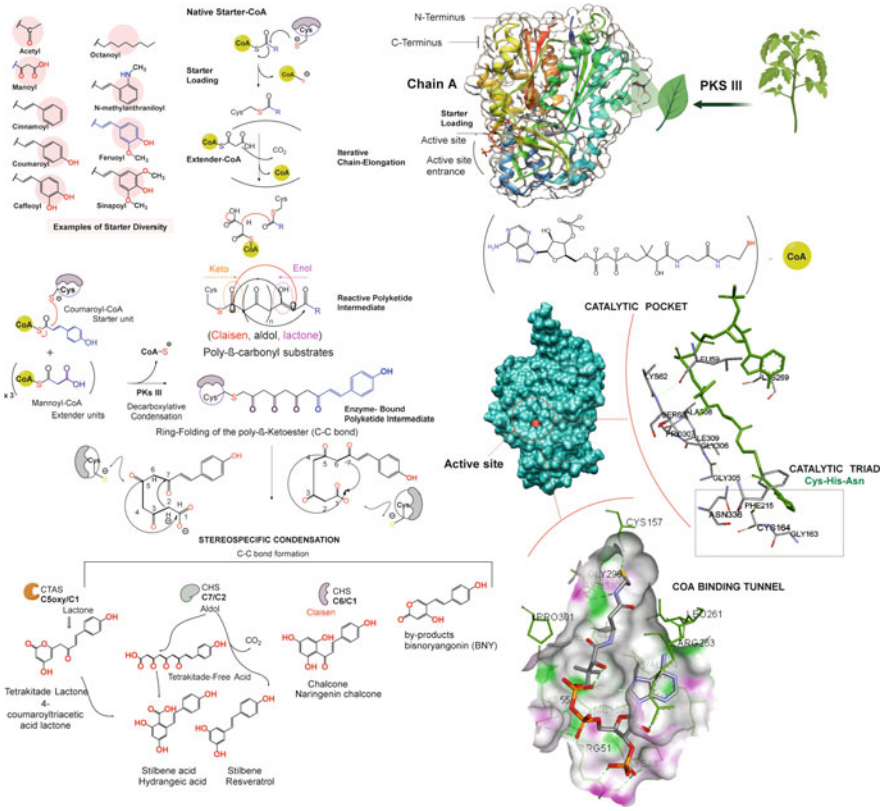

Fig. 7.4 Global mechanism/specificity of plant-PKS biogenesis reactions based on mode of condensation/cyclization. Representation of the overall reaction sequence, the possible starter substrates, the 3D enzymatic structure of CHS (represented in cyan color), the catalytic domain, and the residues evoked in the production of polyketides

1999). Concomitantly, for the same cyclization type a C-C bounding may occur between carbonyl carbon of the starter substrate and methylene carbon of the third extender without a decarboxylation such as conducted in ALS and OKS enzymes (Bisht et al. 2021; Morita et al. 2010a). Ultimately, a C5-O-C1 lactonization condensation results in derailment products with pyrone rings (such as the p-coumaroyl triacetic acid) observed in certain cases being theoretically suggested enzymatically carried out or not (Bisht et al. 2021). Certain enzymes such as the synthase (CTAS) and pyrone synthase (2-PS) are a typical example of enzymes involved in pyrone ring formation reactions. To those condensation reactions, other plant-PKS III such as quinolone synthase QNS, diketide synthase (DKS), benzalacetone synthase (BAS), diketide-CoA synthase (DCS), curcumin synthase (CURS), alkyldiketide-CoA synthase (ADS), and alkylquinolone synthase (AQS) may adapt condensation reactions without a cyclization (Bisht et al. 2021; Morita et al. 2010a; Matsui et al. 2017). A number of plant-PKS related to CHS by sequence

identity, including stilbene synthase (STS), bibenzyl synthase (BBS), and acridone synthase (ACS), share a common chemical mechanism, but differ from CHS in their substrate specificity and/or in the stereochemistry of the polyketide cyclization reaction (Ferrer et al. 1999).

- *Mechanistic of the intramolecular reaction/cyclization:* Chemically, several aromatic structures may result from polyketides cyclization, which is rationalized in terms of Claisen, Aldol, and Lactonization reactions (Bisht et al. 2021; Morita et al. 2010a, 2019; Abe et al. 2005). Thus, specific enzymatic reactions involving alkylation, phenolic oxidative coupling, oxidative cleavage of aromatic rings, and employing starter groups other than acetate result complex structures (Hertweck 2009). The *C6-C1* Claisen reaction/cyclization is carried out following an organic coupling to form a C-C bond (Fig. 7.4) commonly between a single ester and a carbonyl compound or even between two esters (Lim et al. 2016), thus, the reaction product is often a β-keto ester or a β-diketone. The mechanism behind this reaction proceeds in the elimination of an α-H proton of the methylene CH2 situated between the two carbonyl functions of the dicarboxyl-CoA to result in the formation of an enolate ion (Flores-Sanchez and Verpoorte 2009; Morita et al. 2010b; Chan et al. 2009), and a nucleophilic attack on the carbonyl carbon belonging to the starter unit follow. This leads to the elimination of the CoA group each time and the regeneration of the conjugate base (Ferrer et al. 1999). This enolate anion is relatively stable due to the delocalization of the negative charge (electrons) (Ferrer et al. 1999). The alkoxide ion removes the doubly alpha proton which is formed, giving rise to a new enolate anion (Ferrer et al. 1999). The nucleophilic addition in substitution reactions with the carbon belonging to the carbonyl functional group of the starter units is successive (Ferrer et al. 1999; Jez et al. 2002). Furthermore, C7-C2 Aldol *reaction/cyclization* is produced when nucleophilic attack is produced from the α-carbon of an aldehydic or ketonic enolate upon the carbonyl carbon (Fig. 7.4) of another aldehyde or ketone to form a β-hydroxy product, the enolate ion reacts with a carbonyl compound to form a β-hydroxyaldehyde (aldol) or a β-hydroxyketone (Healy et al. 2018; Austin et al. 2004a, b; Funa et al. 2007). Then, a dehydration step occurs to give a conjugated enone. For the triketide and tetraketide (poly-B-ketone) intermediates, aldol cyclization will take place once the α-carbon atom (C2) and the second carbonyl carbon atom (C7) can bond forming a five- or six-membered ring (Healy et al. 2018; Austin et al. 2004b). The various resulting enolates exert a nucleophilic attack on the more reactive carbonyl carbon atom which usually is the carbonyl carbon atom of a ketone function. Equally important, the mechanistic behind the intramolecular C5-O-C1 lactonization reaction/cyclization (Fig. 7.4) considered as the most general method of cyclization through C–O bond formation (Bisht et al. 2021; Shimizu et al. 2017; Weissman 2009; Hertweck 2009) is related to the stereochemistry of lactone ring formation of the triketide and tetraketide intermediates to produce alkylpyrones (Yu et al. 2012) within the enzymatic active site. It is initiated via the C5 ketoreduction step (Austin and Noel 2003), where the nucleophilic hydroxyl group of the polyketide intermediate (poly-B-ketone)

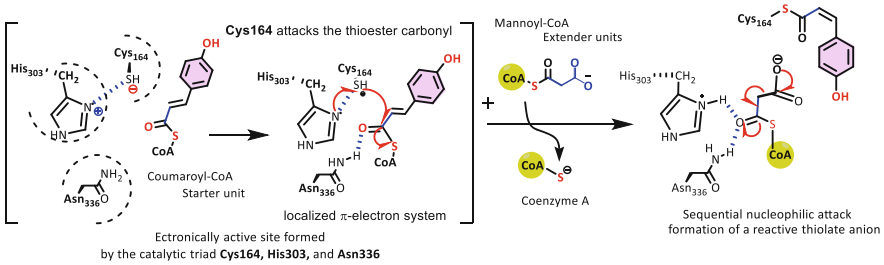

Fig. 7.5 Schematization of the reaction mechanism for the installation of starter substrates and the acetate unit of the malonyl-CoA thioesters within the enzymatic active site. The catalytic triad Cys-His-Asn is solicited for the electronic exchange allowing the substrate to settle and the loading of reaction. Coenzyme A is symbolized as a yellow circle, Hydrogen bonds are emphasized as blue dashed lines, Proton transfers are represented by red arrows

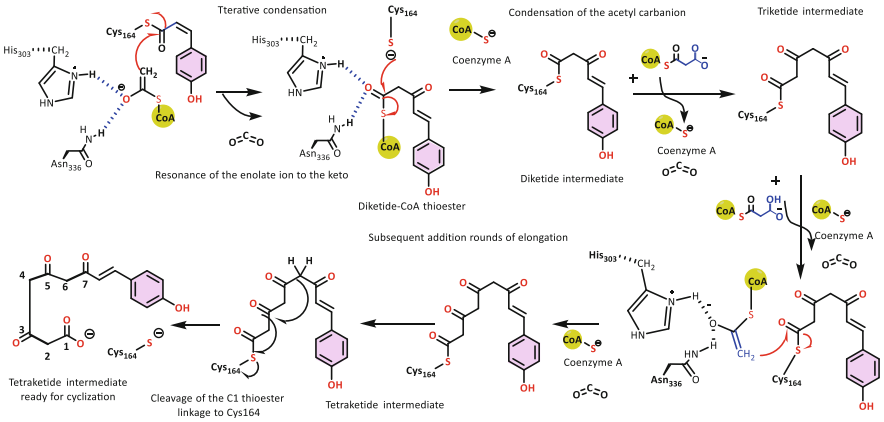

Fig. 7.6 Mechanistic details governing the enzymatic decarboxylation, enolization, and condensation of ketide elongation. The addition of acetate units derived from malonyl-CoA and the flow of electrons is depicted during the elongation of polyketide intermediates. Coenzyme A is symbolized as a yellow circle, Hydrogen bonds are emphasized as blue dashed lines, Proton transfers are represented by red arrows

will be revealed via the reduction of the C5 ketone, which can then intramolecularly attack the C1 carbonyl.**(b) Sequential Steps of PKS III Catalytic/Condensation Reactions**

To discuss PKS III intrinsic reaction's mechanisms in detail, the alfalfa chalcone synthase (CHS2) is proposed here as an illustrative model being considered as a unimodular PKS and carries out a series of decarboxylation, condensation, cyclization, and aromatization reactions at a single active site (Tsai and Ames 2009; Austin and Noel 2003; Ferrer et al. 1999; Jez et al. 2002; Morita et al. 2007; Shi et al. 2008) where three interconnected cavities (Pandith et al. 2020) intersect including a CoA binding tunnel, a coumaroyl binding pocket, and a cyclization pocket as represented by Figs. 7.5, 7.6, and 7.7.

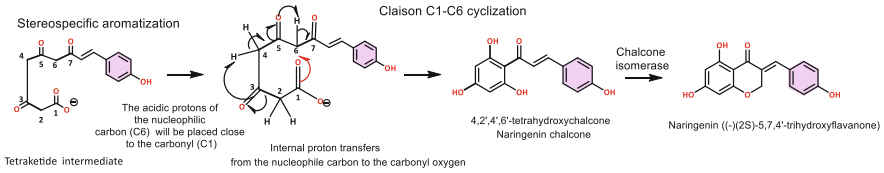

Fig. 7.7 Mechanism of polyketide intramolecular rearrangement and stereospecific cyclization/aromatization of the enzyme-bound tetraketide, cleavage of the (-Cys) enzymatic bound leads to formation of chalcone

- Loading step: instantly occurs within the enzyme sterically and electronically active site (Stewart et al. 2013) formed by the catalytic triad Cys164, His303, and Asn336 (as numbered in *M. sativa* CHS). This step began at the coumaroyl binding pocket by chemical strategy of initial priming (Fig. 7.5) of a monocarboxyl-CoA (p-coumaroyl-CoA) starter and dicarboxyl-CoA extender substrate (malonyl-CoA) through its localized Π-electron system (Austin and Noel 2003; Yu et al. 2012; Morita et al. 2007; Lussier et al. 2012; Stewart et al. 2013). Exhaustively, Cys 164 attacks the thioester carbonyl of the starter resulting in transfer of the starter moiety to the cysteine side chain, while van der Waals contacts dominate the remaining interactions between CHS and p-coumaroyl-CoA at the entrance of the CoA binding tunnel where residues Lys 55, Arg 58, and Lys 62 (Ferrer et al. 1999) make it possible to form H-bonds with two CoA phosphates with a hydrogen bond additional between the NH of the Ala 308 residue (Abe et al. 2005), and the first carbonyl of the pantetheine moiety of CoA positioning terminally bound thioester-bound substrates near to Cys164 (Ferrer et al. 1999; Yu et al. 2012; Wakimoto et al. 2012; Pandith et al. 2020; Weissman 2009; Abe et al. 2004). The latter residue will then serve as a nucleophilic attachment managing the formation of a very reactive thiolate anion from two acidic protons located between the two carbonyls (-O=C-HC$^{(-)}$H-C=O-) of the malonyl-CoA (Ferrer et al. 1999; Pandith et al. 2020; Stewart et al. 2013; Imaizumi et al. 2020). This anion is very likely to perform a sequential nucleophilic attack to the carbonyl group. However, His303 most likely acts as a base when generating the nucleophilic thiolate anion from Cys164 (Ferrer et al. 1999), since it has a amid nitrogen NH within hydrogen bonding distance of the sulfur atom of Cys164 (Ferrer et al. 1999). The instable intermediate thus formed will instantly dislocate the electrons to from the double bond of the carbonyl group up to the oxygen to be negatively charged. The negative charge will reform the double bonds in the thiolate intermediate and the Coenzyme A will leave the starter unit, while Asn336 will be involved in the decarboxylation reaction step (Austin and Noel 2003; Flores-Sanchez and Verpoorte 2009; Dao et al. 2011; Pandith et al. 2020; Abe et al. 2004) by orienting the thioester carbonyl of malonyl-CoA near His303 with Phe215, providing a nonpolar environment for the terminal carboxylate (Ferrer et al. 1999).

- Chain elongation/extension step: the poly-keto chain elongation reaction is absolutely conserved at the catalytic triad (Fig. 7.5), iterative cyclic catalysis of decarboxylative condensations of malonyl-CoA will take place at two carbons on a variety of acyl groups (Fig. 7.6). Actually, the resonance of the enolate ion (Ferrer et al. 1999; Flores-Sanchez and Verpoorte 2009) to the keto form allows condensation of the acetyl carbanion, thus, the tautomerization of the reactive polyketide (Austin et al. 2004a; Stewart et al. 2013; Lajis and Ahmad 2006) and the triggering of carbon-carbon and/or carbon-oxygen bonding is governed by the configuration of the hydrogen bonds of the active-site residues as well as the position of the intramolecular water molecules and the polarization of the polyketide binding cavity (Ferrer et al. 1999; Stewart et al. 2013). Thus, the resulting acetyl-CoA carbanion serves as the nucleophile for chain elongation and the malonyl-derived portion of each molecule occupies a large pocket adjacent to Cys 164 (Ferrer et al. 1999; Flores-Sanchez and Verpoorte 2009; Dao et al. 2011) (Fig. 7.6). The latter recapture the elongated starter-acetyl-diketide-CoA and the release of CoA sets subsequent addition rounds of elongation, resulting in the formation of a final polyketide reaction intermediate (Ferrer et al. 1999; Schröder et al. 1998; Shimizu et al. 2017; Kontturi and Type 2017). In the case of CHS, the size of the active pocket limits the number of acetate additions (to three additions) (Ferrer et al. 1999; Austin et al. 2004a) where a gradual elongation of the polyketide chain assisted by Phe215/Phe265 ensues (Xie et al. 2016; Abe et al. 2004, 2005; Kontturi and Type 2017), and the two-carbon units considerably increase the enzyme intrinsic reactivity and the Polyketide-CoA intermediates. A tetraketide intermediate is then produced following three rounds of starter/extender condensation (Ferrer et al. 1999). Although, in some cases this number may reach several cycles of eight or more condensation (Austin and Noel 2003; Abe 2008; Ping 2016; Abe and Morita 2010). Subsequently, the transiently attached to CoA intermediate undergoes thioester exchange onto the active-site thiolate anion (Cys) stabilized by His and Asn of the canonical type III PKS catalytic triad (Ferrer et al. 1999; Stewart et al. 2013). During successive cycles of polyketide elongation, Phe 265 detaches the coumaroyl bond from the cyclization pocket by acting as a mobile steric gate (Ferrer et al. 1999).

- Chain termination and intramolecular cyclization: The stereospecific cyclization occurs (Morita et al. 2007; Wakimoto et al. 2012; Pandith et al. 2020; Schröder et al. 1998) as a final step being of Claisen-type mechanism (Fig. 7.7) where the enzyme stereoelectronic features and the presence or absence of a thioester bond govern the electronic transfer (Ferrer et al. 1999; Stewart et al. 2013). It conveniently results in cleavage of the C1 thioester linkage to Cys164, once the methylene group adjacent to the coumaroyl fragment exerts a nucleophilic attack on the carbonyl carbon of the thioester linked to Cys164 (Austin and Noel 2003; Austin et al. 2004a). Instantaneously, the closure of the cycle occurs following the internal transfer of protons from the nucleophile carbon to the carbonyl oxygen. The tetraketide intermediate is thus configured as a chalcone where one of the acidic protons of the nucleophilic carbon (C6) will be placed close to the target

carbonyl (C1) (Ferrer et al. 1999; Stewart et al. 2013) (Fig. 7.7). Carbanions are formed by the driving force provided by the intermediate, where the protonation of the carbonyl oxygen would also stabilize the negative charge on the tetrahedral intermediate (Tsai and Ames 2009; Ferrer et al. 1999). Thus, the newly formed ring system will subsequently be expelled from Cys164 (Ferrer et al. 1999). Further stereospecific aromatization reaches the trione ring through another series of internal proton transfers converting the 4,2′,4′,6′-tetrahydroxychalcone to naringenin ((−)(2S)-5,7,4′-trihydroxyflavanone) (Ferrer et al. 1999) (Fig. 7.7). At this stage, the CoA binding tunnel serves as a coumaroyl-binding pocket where residues Ser 133, Glu 192, Thr 194, Thr 197, and Ser 338 surround the coumaroyl with van der Waals contacts (Ferrer et al. 1999; Pandith et al. 2020). However, the carbonyl oxygen (C=O) of residue Gly 216 attaches to the phenolic oxygen of naringenin and the hydroxyl (OH) of residue Thr 197 interacts with the carbonyl of naringenin derived from coumaroyl-CoA (Kim et al. 2013; Ferrer et al. 1999). Cyclization pocket residues, including Thr 132, Met 137, Phe 215, Ile 254, Gly 256, Phe265, and Pro 375, act as less potential H-bond donors (Ferrer et al. 1999; Morita et al. 2019). However, since the polyketide has so many hydrogen acceptor bonds, certain interactions are required for appropriate folding to occur during the cyclization step. Thus, the surface topology of the cyclization pocket dictates how the malonyl-derived portion of the polyketide is folded and the stereochemistry of the cyclization reaction leading to chalcone formation (Ferrer et al. 1999; Pandith et al. 2016).

7.2.2.2.2 Plant-PKS III Classification: Structure/Function/Mechanistic/Derivative's Products

On the genetic, biosynthetic, and structural aspects of the end products, the polyketide synthases process many similarities (Bisht et al. 2021; Morita et al. 2007, 2010a; Wakimoto et al. 2012; Abe et al. 2005). However, there are some differences that allow their division into subcategories (Bisht et al. 2021; Morita et al. 2010a). Thus, among the most famous classifications are those based on the mode of cyclization of polyketide intermediates (Table 7.1). A grouping of PKS is however established in 4 superfamilies (*CHS-type*, STS-type, CTAS-type, and Miscellaneous type PKS) involving one or more rounds of cyclization (Bisht et al. 2021; Pandith et al. 2020).

CHS-Type *Plant Polyketide Synthase's Superfamily*

The classification of this enzyme family under the name CHS-like enzymes (Austin and Noel 2003) is entirely related to their biosynthetic mechanisms and PKs ring closure based on Claisen-type (C6-C1) cyclization (Bisht et al. 2021). Being of relatively different structures, all CHS-like proteins share in common the property of conservation of residues shaping the geometry of the active site, namely, Pro 138, Gly 163, Gly 167, Leu 214, Asp 217, Gly 262, Pro 304, Gly 305, Gly 306, Gly 335, Gly 374, Pro 375, and Gly 376 (Ferrer et al. 1999; Pandith et al.

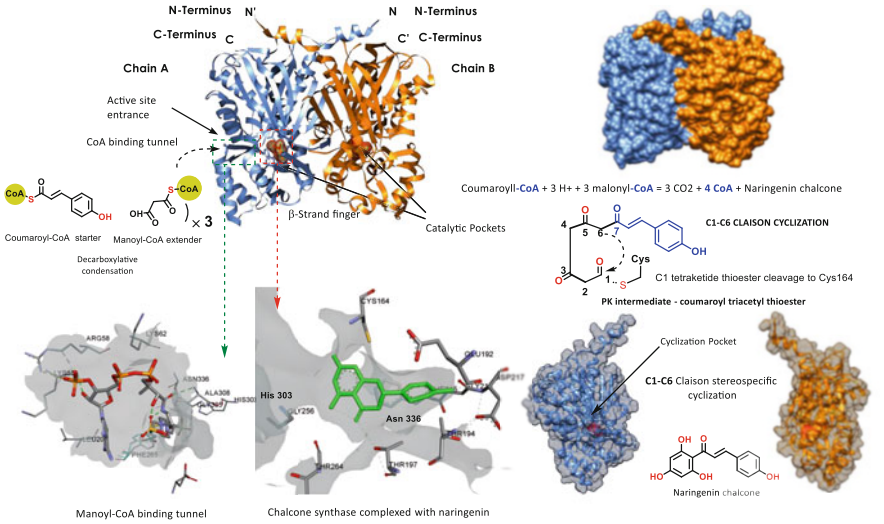

Fig. 7.8 Representation of the reaction mechanism of the CHS (PDB: 1CGK) of *Medicago sativa*, the enzymatic structure, the rearrangement/cyclization of the substrates and the polyketides produced

2016, 2020). Although they share a mode of formation of polyketides or polyketide-CoA thioester intermediates very similar to that of CHS (Austin and Noel 2003; Pandith et al. 2020; Schröder and Schröder 1999), while, their sequence variation in both the coumaroyl-binding pocket and the cyclization pocket imposes steric differences resulting in specificity substrate and resulting product. The main enzymes falling into this category are as follows:

1. Chalcone synthase (CHS)

The CHS is a homodimer of two 42 kDa polypeptides (Ferrer et al. 1999; Dao et al. 2011; Pandith et al. 2020; Imaizumi et al. 2020), ubiquitously present in almost all plant species (Bisht et al. 2021; Rammohan et al. 2020) such as *Medicago sativa* (alfalfa) (Ferrer et al. 1999), *Malus domestica* (Stewart et al. 2017), and *Freesia hybrida* (Sun et al. 2015). It forms a symmetric dimer where each monomer consists of two superimposed structural domains (Pandith et al. 2020) (Fig. 7.8): the upper domain or thiolase and the lower domain (CHS monomer) (Tan et al. 2020; Dao et al. 2011; Pandith et al. 2020). Structurally distinct, in the lower domain active site is larger than 3-ketoacyl-CoA thiolase (Austin and Noel 2003; Ferrer et al. 1999; Pandith et al. 2020; Gayen et al. 2020) and revealed to provide space for the polyketide reaction intermediates required for chalcone formation. Functionally independent, thiolase generates two molecules of acetyl-CoA from acetoacetyl-CoA and free CoA (Ferrer et al. 1999). In particular, the Claisen-type condensation within *CHS-type* enzymes is pivotal to offer a wide family of phytochemicals such as the chalcones, aurones, and flavonoids (Bisht et al. 2021; Rammohan et al. 2020). Based on the information schematized in Fig. 7.8, the active site of CHS is

represented by a CoA binding tunnel, a starter substrate binding pocket, and a cyclization pocket and characterized by three amino acid residues (Cys164, His303, and Asn336) which are almost conserved in all plant-PKS III being the key to selecting starters and to define the center of the reaction of initiation, elongation, and condensation (Austin and Noel 2003; Pandith et al. 2020; Abe et al. 2004). As depicted in the section above, the reaction mechanism within CHS active site consists of sequential catalyses of three iterative decarboxylative condensations of the malonyl-CoA as decarboxylated extender with the p-coumaroyl-CoA starter to yield a linear tetraketide, typically the p-coumaroyl triacetyl thioester. This cysteine-bound intermediate undergoes Claisen-type (C6-C1) intramolecular cyclization (common mode of cyclization found in CHS and similar CHS-like PKSs and a variety of plant type III PKSs) to form an aromatic ring system and produce a chalcone. The chalcone thus produced will then be stereospecifically transformed into naringenin which is a main precursor of flavonoids, phytoalexins, and anthocyanins in plants (Bisht et al. 2021; Dao et al. 2011). Authors (Bisht et al. 2021; Morita et al. 2010a; Shi et al. 2008) had reported that when a CHS enzyme reaction is carried out in vitro two lactone derivatives, bis-noryangonin (BNY) and 4-coumaroyltriacetic acid lactone (CTAL) may be formed as early-released derailment by-products. Interestingly, unnatural methylated C9 triketide lactone was found to be produced by the CHS of *S. baicalensis* and *R. palmatum* by sequential decarboxylative condensations of three molecules of (2RS)-methylmalonyl-CoA (Austin and Noel 2003; Shi et al. 2008; Flores-Sanchez and Verpoorte 2009; Abe 2008).

However, mutations affecting the genes responsible for the formation of this enzyme can have a direct consequence on the functionality of the latter. In a way that X-ray crystallographic structures of non-mutated CHS (Fig. 7.8) as well as mutant CHS such as CHS G256A, G256V, G256L, and G256F show a reduction in the size of the active-site cavity without significant alterations in the conformations of the polypeptide backbones (Bisht et al. 2021; Morita et al. 2010a; Abe et al. 2005). Consequently, these alterations may influence both the number of condensation reactions during the polyketide chain extension phase and the stereochemical conformation of the triketide and tetraketide intermediates during the cyclization reaction.

CHS PK-derived products and Post CHS enzymes derivatization: In relation to the biotic and abiotic environment of the plant, chalcones which are a precursor's intermediates derived from CHS open the way to several classes of compounds in plant secondary metabolism. The major classes thus derived from chalcones are the flavonoids/isoflavonoids (Dao et al. 2011; Jan et al. 2021; Bhattacharya 2019; Tauchen et al. 2020), flavones (apigenin, luteolin), flavonols (quercetin), flavandiols, flavanonol (taxifolin), flavanones (hesperitin, naringenin, hesperidin, naringin, eriodictyol, and dihydrotricin), aurones, anthocyanins/proanthocyanidins (Austin and Noel 2003; Dao et al. 2011; Niaz and Khan 2020; Gao et al. 2010; Tauchen et al. 2020; Rammohan et al. 2020), and condensed tannins (Austin and Noel 2003; Niaz and Khan 2020; Ralston et al. 2005; Tauchen et al. 2020) that are renowned for their undeniable biological effects (Kumar and Pandey 2013) to have compelling

medicinal properties, and broadly representative of phenolic compounds in higher plants and bryophytes. A variety of chalconoids compounds may be produced in different plants such as naringenin chalcone (Pandith et al. 2016; Abe et al. 2005; Rencoret et al. 2022), trihydroxychlacone, 2′,4′-dihydroxychalcone, flavokawains (including flavokavain A, B, and C) in *Piper methysticum* (Abu et al. 2013; Jasim et al. 2021), butein (3,4,2′,4′-tetrahydroxychalcone) in *Rhus verniciflua, Dahlia pinnata,* and *Butea monosperma* (Jasim et al. 2021), xanthoangelol/4-hydroxyderricin in *Angelica keiskei* (Yoshioka et al. 2020), cardamonin (2′,4′--dihydroxy-6′-methoxychalcone) in Alpinia species (Gonçalves et al. 2014), isosalipurposide (chalcone 2'-*O*-glucoside) in Acacia genus (Ghribi et al. 2021), Isoliquiritigenin (4,2′,4′-trihydroxychalcone) found in various edible plants, licorice (*Glycyrrhiza glabra* L.), soy beans (*Glycine max* L.), and shallots (*Allium ascalonicum* L.) (Christensen 2018), okanin (2′,3,3′,4,4′-Pentahydroxychalcone) and okanin-4′-glucoside, from flowers of *Coreopsis tinctoria* (Huang et al. 2020). Subsequently, depending on plant secondary metabolism, isomerization reactions occur on chalcones to transform them to various derivative compounds where the biosynthetic pathway diverges into several branches, each resulting in a different class of flavonoids. The chalcone flavanone isomerase (CHI) present in a variety of plants (Dao et al. 2011; Ralston et al. 2005; Dixon 1999; Forkmann and Heller 1999) exerts isomerization (cycle closure of 6-atom ring) of naringenin chalcone and trihydroxychlacone to flavanones such as (2S)-4′,5,7-trihydroxyflavan-4-one (naringenin) in grapefruits and vegetables and to (2S)-4′,7-dihydroxyflavan-4-one or liquiritigenin (such as in *Glycyrrhiza uralensis* and *Glycyrrhiza glabra*), respectively(Dao et al. 2011; Pandith et al. 2020; Ralston et al. 2005; Tauchen et al. 2020; Rencoret et al. 2022). Dihydroflavonols are then formed from the naringenin by hydroxylases such as the flavanone 3-hydroxylase (F3H), flavonoid 3′ hydroxylase (F3′H), and flavonoid 3′5′ hydroxylase (F3′5′H) and the dihydroflavonols thus produced subsequently transform to flavanol by flavonol synthase (FLS) (Liu et al. 2021). Besides, dihydroflavonol 4-reductase (DFR) and flavone synthase 1 and 2 (FS1/FS2) produce, respectively, flavanols and flavones (Liu et al. 2021). Other enzymes subsequently take control of the molecular transformation of these metabolites into other structurally varied derivatives. For example, during anthocyanin biosynthesis, the flavanone 3-hydroxylase (F3H) catalyzes the stereospecific 3-β-hydroxylation reaction of (2S)-flavanones to dihydroflavonols, that is also reduced to flavan-3,4-diols (leucoanthocyanins) by the dihydroflavonol reductase (DFR) (Dao et al. 2011). The dihydroflavonols are subsequently converted into anthocyanidins by anthocyanidin synthase (ANS) (Tohge et al. 2013). Then, more molecular complexion reaction continues at the level of secondary metabolism forming more stable derivatives such as UDP glucose-flavonoid (Dao et al. 2011; Kontturi and Type 2017; Kumar and Pandey 2013; Liu et al. 2021) produced by the 3-*O*-glucosyltransferase (UFGT), which stabilizes anthocyanidins by 3-*O*-glucosylation reaction (Dao et al. 2011; Liu et al. 2021).

Those derivation steps of chalcones to flavonoids and anthocyanidins offer additional biological and physiological particularities to the plants, such as the pigments/odors of flowers for the attraction of pollinators and repulsion of pests as

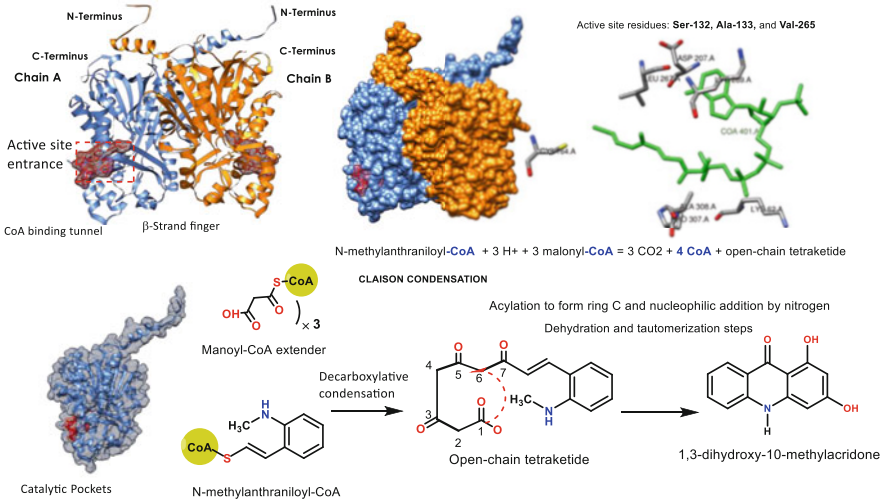

Fig. 7.9 Representation of the reaction mechanism of the ACS (PDB: 3WD7) of *Citrus x microcarpa*, the enzymatic structure (Flat ribbon and 3D surface), the catalytic domain, the rearrangement/cyclization of the substrates, and the polyketides produced

well as proprieties against photo-oxidative damage caused by UV radiation (Ferrer et al. 1999; Shimizu et al. 2017; Tauchen et al. 2020). In another aspect, chalcones aureusidin synthase (*AS*) that manage the oxidative cyclization of 2′--hydroxychalcones (naringenin chalcone and trihydroxychlacone) to aurones (Bisht et al. 2021; Rammohan et al. 2020; Liu et al. 2021) produce heterocyclic compounds where the chalcone-like structure closes into a 5-atom ring instead of the more typical 6-atom ring (C ring) offering various aurones responsible for flowering plant pigmentation including aureusidin (4,6,3′,4′-tetrahydroxyaurone) found in *Antirrhinum majus* (Pandith et al. 2020; Seemann et al. 2006; Li et al. 2020b; Schro 1999), and *Cosmos bipinnatus*, 4,5,6-Trihydroxyaurone, bracteatin (4,6,3′,4′,5′-pentahydroxyaurone), Hispidol (6,4′-dihydroxyaurone), and Leptosidin in *Coreopsis grandiflora* (Mazziotti et al. 2022), sulfuretin (6,3′,4′--trihydroxyaurone) in *Rhus verniciflua* (Lee et al. 2012).

2. Acridone synthase (ACS)

The ACS was originally isolated from *R. graveolens* (Bisht et al. 2021; Austin and Noel 2003; Ferrer et al. 1999; Resmi et al. 2013; Shimizu et al. 2017; Taura et al. 2016; Matsui et al. 2017) that reported to produce 14 different acridones along with four others isolated from Ruta species and *Boenninghausenia albiflora* (Bisht et al. 2021). As anthranilate-derived alkaloids the acridones are biosynthesized by polyketide pathways in plants by the ACS. This enzyme uses both N-methylanthraniloyl-CoA and three malonyl-CoA units as starter and extender substrates, respectively (Bisht et al. 2021; Xie et al. 2016; Shi et al. 2008; Shimizu et al. 2017) (Fig. 7.9), within the catalytic pocket associated with the Cys164, His303, and Asn336

residues. In the same way as CHS, ACS is thought to catalyze the formation of an open-chain tetraketide to produce acridones (Bisht et al. 2021; Michael 2017; Resmi et al. 2013) serving as a common intermediate for virtually all of the known alkaloids (Michael 2017). Structurally, the active-site residues Ser132, Ala133, and Val265 (Bisht et al. 2021; Morita et al. 2010a) have been reported as essential machinery in the substrate specificity for N-methylanthraniloyl-CoA and its further cyclization of the tetraketide intermediate (Fig. 7.9). The latter undergoes successive acylation to form ring C and nucleophilic addition by nitrogen yielding a tricyclic intermediate further transformed to 1,3-dihydroxy-10-methylacridone by dehydration and tautomerization steps (Austin and Noel 2003; Jez et al. 2002; Flores-Sanchez and Verpoorte 2009; Resmi et al. 2013; Schro 1999) which leads directly to the formation of more complex acridones such as rutacridone produced in *R. graveolens* (Bisht et al. 2021; Shi et al. 2008). However, ACS had a larger catalytic pocket cavity consequently allowing various starter affinity and higher ability to accommodate larger N-methylanthraniloyl-CoA as starter molecules (Bisht et al. 2021; Morita et al. 2010a). Plant species belonging to the genera Ruta, Citropsis, Glycosmis, Severinia, Swinglea, Vepris, Conchocarpus, Zanthoxylum (= Fagara), and especially Citrus are a well-established source of a wide range of acridone alkaloids (Bisht et al. 2021; Michael 2017) varying from simple to more complicated molecules such as the C-prenylacridones, furo[3,2-b]- and furo[2,3-c]acridones, pyrano [3,2-b]- and pyrano[2,3-c]acridones, and dimeric alkaloids containing acridone moieties. Moreover, certain species of Rutaceae, for example *Citrus microcarpa*, have an ACS with a remarkable promiscuity of substrates producing 4-hydroxy-N-methylquinolone, 1,3-dihydroxy-N-methylacridone and acid lactone (Bisht et al. 2021; Austin and Noel 2003; Jez et al. 2002; Flores-Sanchez and Verpoorte 2009; Resmi et al. 2013), N-methylanthranilriacetic with additional possibility of producing aromatic tetraketides such as the chalcones, benzophenones, and phloroglucinols (Bisht et al. 2021; Zhou et al. 2016; Abe et al. 2005).

ACS Polyketides derivatives: The aroused biosynthetic pathway will offer a variety of molecules varying from simple acridone alkaloids (Bisht et al. 2021; Michael 2017; Matsui et al. 2017) to name some, arborinine from *Almeidea coerulia, A. rubra, C. articulate, C. inopinatus, C. marginatus, Glycosmis parva, Ruta angustifolia*, citrusamine from *Atalantia buxifolia*, citrusinine-II from *Atalantia wightii, Severinia buxifolia,* and *Swinglea glutinosa*, evoxanthine from *R. graveolens, Teclea amaniensi,* and *Vepris sclerophylla*, Xanthevodine from Melicope vitiflora, 1-Hydroxy-10- methylacridone from Glycosmis mauritiana and Thamnosma rhodesica, Evoxanthidine (norevoxanthine), xanthoxoline, 1,2,3-trihydroxyacridone, furoacridone alkaloids such as furacridone and rutacridone as well as pyranoacridone alkaloids, dimeric acridone alkaloids, miscellaneous alkaloids, azacridone-A, and acrimarines (Bisht et al. 2021; Michael 2017; Matsui et al. 2017).

3. Valerophenone synthase (VPS)

The VPS is an homodimeric enzyme highly homologous to CHS, with subunits of 45 kDa (Bisht et al. 2021; Yu et al. 2012; Morita et al. 2010a; Flores-Sanchez and

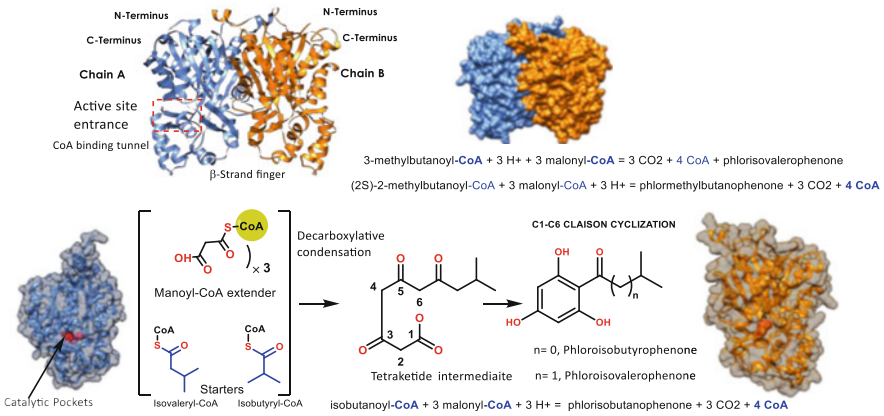

Fig. 7.10 Representation of the reaction mechanism of the VPS (PDB: 6dxb) of *Humulus lupulus*, the enzymatic structure (flat ribbon and 3D surface), the catalytic domain, the rearrangement/cyclization of the substrates, and the polyketides produced

Verpoorte 2009; Zhou et al. 2016; Taura et al. 2016). It uses isovaleryl-CoA or isobutyryl-CoA and three molecules of malonyl-CoA (Bisht et al. 2021; Morita et al. 2010a) (Fig. 7.10) to form the acylphloroglucinol cores, phlorisovalerophenone or phlorisobutyrophenone (Bisht et al. 2021; Austin and Noel 2003; Morita et al. 2010a; Zhou et al. 2016; Shimizu et al. 2017) known as intermediates in the biosynthesis of hop bitter acids (acids a and b) (Bisht et al. 2021; Austin and Noel 2003; Schro 1999). This enzyme is well known to produce humulone and lupulone in hops (*Humulus lupulus* L.) (Bisht et al. 2021; Zhou et al. 2016) which are responsible for the bitter taste of beer and exhibit notable pharmacological effects. Thus, as a bifunctional enzyme, VPS contributes to the biosynthesis of resin and prenylflavonoids in the lupulin gland of *H. lupulus* (Bisht et al. 2021; Flores-Sanchez and Verpoorte 2009). While, its bifunctionality affects the relative amounts of resin and prenylflavonoid in the gland. In *Gerbera hybrida* the VPS also produces bioactive 4-hydroxy-6-methyl-2-pyrone (Zhou et al. 2016; Schröder et al. 1998) (Fig. 7.10).

VPS Polyketides derivatives: In *H. lupulus* lupulin gland, VPS produces phlorisovalerophenone and phlorisobutyrophenone to be considered as starting materials of prenylflavonoids (Gonçalves et al. 2014; Ramawat and Mérillon 2013) including xanthohumol, isoxanthohumol (Rammohan et al. 2020; Ramawat and Mérillon 2013), plus phloroglucinol derivatives such as the α-acids (mainly humulone, cohumulone, adhumulone) (Bisht et al. 2021; Austin et al. 2004a) and β-acids (mainly lupulone, colupulone, and adlupulone) obtained from the deoxyxylulose pathway (Clark et al. 2013), where prenylation of the benzenoid occurs. Those compounds are, however, responsible for a bitterness of the resin of mature hops (Zhou et al. 2016). Phloroglucinol (benzene-1,3,5-triol) and derivatives (monomeric, dimeric, trimeric, higher phloroglucinols, and phlorotannins), acryl phloroglucinols, phloroglucinol-terpene adducts in Eucalyptus species (Niaz and

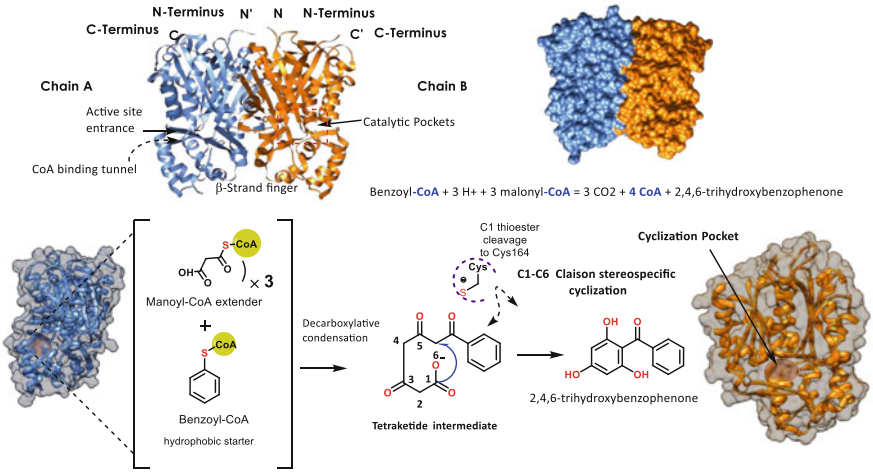

Fig. 7.11 Representation of the reaction mechanism of the BPS (PDB: 7CBF) of *Garcinia mangostana*, the enzymatic structure (Flat ribbon and 3D surface), the catalytic domain, the rearrangement/cyclization of the substrates and the polyketides produced

Khan 2020), phloroglucinol glycosides, halogenated phloroglucinols, prenylated phloroglucinols, and cyclicroup polyketides widely exist in several species belonging to myrtaceae rutaceae, compositae, rosaceae, euphorbiaceae, aspidiaceae, lauraceae, crassulaceae, cannabinaceae, and fagaceae, botanical families (Niaz and Khan 2020), Moreover, these compounds also occur in marine plants such as *Myriophyllum spicatum* where Phloroglucinol and its two hydroxyquinol (1,2,4-benzenetriol) and pyrogallol (1,2,3-benzenetriol) are produced (Kráľová and Šeršeň 2012).

4. Benzophenone synthase (BPS)

Despite sharing 60% sequence identity with CHS (Bisht et al. 2021; Abe et al. 2004, 2005), BPS exhibit affinity toward relatively smaller hydrophobic starters such as benzoyl-CoA and its derivatives by catalyzing a decarboxylative condensation with three malonyl-CoA molecules to produce the tetraketide intermediate 2,4,6-trihydroxybenzophenone (Bisht et al. 2021; Austin and Noel 2003; Franklin et al. 2009; Abe et al. 2004; Songsiriritthigul et al. 2020; Stewart et al. 2017) (Fig. 7.11). Possible mutations in the active site of the BPS enzyme (the case of BPS from *Hypericum androsaemum*) impair its ability to utilize larger substrate molecules (Bisht et al. 2021; Franklin et al. 2009; Stewart et al. 2017). Certain plant species have BPS with varying degrees of affinity toward benzoyl-CoA, for example BPS of *Centaurium erythraea* preferably uses 3-hydroxybenzoyl-CoA as a starting substrate to produce 2, 3′, 4, 6-tetrahydroxybenzophenone, the precursor of xanthones (Bisht et al. 2021; Flores-Sanchez and Verpoorte 2009; Franklin et al. 2009; Schro 1999).

BPS-Polyketides derivatives: the 2, 4, 6-trihydroxybenzophenone (Bisht et al. 2021; Songsiriritthigul et al. 2020) is the biosynthesized intermediate by BPS in the same way as the benzophenones, it further gives rise to various polyprenylated polycyclic benzophenones (Bisht et al. 2021; Morita et al. 2010a; Abe et al. 2005) through intramolecular cyclization to produce sulfonated xanthones/hyperxanthone and by polyprenylation reactions to produce prenylated xanthones such as the α-mangostin (Bisht et al. 2021), 1,5,8-trihydroxy-3-methoxy-2-(3-methylbut-2-enyl) xanthone and 1,6-dihydroxy-3-methoxy-2-(3-methyl-2-buthenyl)-xanthone in *Hypericum androsaemum* (Crockett et al. 2011), and other xanthones, namely, sampsoniones from *Hypericum sampsonii* (Chen et al. 2014) as well as the 7-epi-clusianone, peroxysampsone A, sampsonione A and I, hypersampsone D and E (Zhu et al. 2014).

5. Homoeriodictyol/eriodictyol synthase (HvCHS)

This HvCHS produces chalcone by the same mechanism as that of CHS with a difference in their substrate selectivity (Austin and Noel 2003; Shimizu et al. 2017; Dunstan et al. 2020). HvCHS has more affinity toward feruloyl-CoA and caffeoyl-CoA to produce high levels of homoeriodictyol chalcone and eriodictyol chalcone, respectively (Bisht et al. 2021; Morita et al. 2010a), while other substrates such as 4-coumaroyl-CoA and cinnamoyl-CoA are less favorable in the case of HvCHS (Bisht et al. 2021; Morita et al. 2010a).

HvCHS Polyketides derivatives: Eriodictyol ((2S)-3′,4′,5,7-Tetrahydroxyflavan-4-one), homoeriodictyol (3′-methoxy-4′,5,7-trihydroxyflavanone), and sterubin (7-methoxy-3′,4′,5-trihydroxyflavanone) are bitter-masking flavanones in *Eriodictyon californicum* (Ley et al. 2005). Besides, Eriodictyol-derived phytoalexin alongside Eriodictyol-glycosides derivatives such as saponarin and lutonarin are reported to majorly be present in Barley leaves (Kamiyama and Shibamoto 2012).

STS-Type Plant Polyketide Synthase's Superfamily

This grouping of STS-type enzymes (Bisht et al. 2021; Shimizu et al. 2017) is established according to the common scaffolding for the condensation of the substrates and for the cyclization reaction (Table 7.1). Catalysis of ring-closure reactions is in particular of C2-C7 Aldol type (aromatic or heterocyclic) (Bisht et al. 2021; Abe 2020; Morita et al. 2010a; Austin et al. 2004b), involving different atoms to give rise to different products. Ferrer et al. (1999) report that structurally two residues can vary between CHS-like and STS-like enzymes (Asp255 and Leu268). Thus, sequence variations of these residues can determine the conformation of the tetraketide-CoA thioester intermediate prior to ring formation. The folding of intermediate bound to this type of enzymes before the closing of the new aromatic ring is responsible for the formation of different products according to the structural and functional specificity of each enzyme active site.

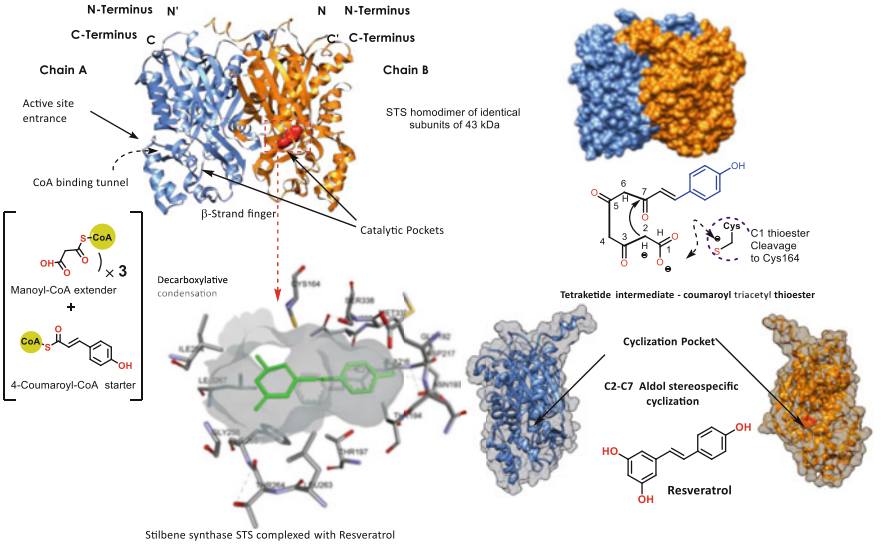

Fig. 7.12 Representation of the reaction mechanism of the STS (PDB: 1Z1F) of *Arachis hypogaea*, the enzymatic structure (flat ribbon and 3D surface), the catalytic domain linked to resveratrol, the rearrangement/cyclization of the substrates and the polyketides produced

(1) Stilbene synthase (STS)

The STS is the second most studied type III PKS after the CHS (Bisht et al. 2021; Austin et al. 2004a, b; Gao et al. 2010; Schröder et al. 1998; Shimizu et al. 2017; Abe et al. 2004; Schro 1999), present in a wide range of plants and formerly known as resveratrol synthase. As homodimer of identical subunits of 43 kDa, STS structural analyzes report that there is no consistent difference in the residues (75–90% of sequence identity) lining the active site with that of CHS (Bisht et al. 2021), while the only difference lies in the residues evoked by the site of cyclization (Bisht et al. 2021; Healy et al. 2018; Austin et al. 2004b). This enzyme uses the same precursor molecules (sequential condensation of 4-coumaroyl-CoA with three C2 units of malonyl-CoA (Fig. 7.12) and reaction mechanism to create a common tetraketide intermediate as CHS typically a p-coumaroyl triacetyl thioester (Bisht et al. 2021; Morita et al. 2010a; Lim et al. 2016; Abe et al. 2004). Besides, the Aldol-specific ring-closing of the tetraketide intermediate initiated by the STS cyclization pocket involves nucleophilic attack of the methylene group (C2) closest to the thioester bond at Cys 164 on the carbonyl carbon (C7) of the coumaroyl moiety (Fig. 7.12) through engaging an internal proton transfer mechanism where the network of hydrogen bonds called "aldol-switch" plays an essential role in determining the specificity of the stilbene product (Bisht et al. 2021; Yu et al. 2012; Healy et al. 2018; Flores-Sanchez and Verpoorte 2009; Shimizu et al. 2017; Austin et al. 2004b). A particularity of STS is that the cyclization of the tetraketide-CoA thioester remains covalently attached to the STS (Austin et al. 2004b). Alternatively, hydrolysis from

Cys164 and an additional decarboxylation step are required for completion of the resveratrol-forming reaction sequence (Bisht et al. 2021; Healy et al. 2018; Austin et al. 2004a, b; Shimizu et al. 2017) (Fig. 7.12). Therefore, alterations in the surface topology of the cyclization pocket of STS can affect the stereochemistry of the cyclization reaction and modulate product selectivity (Bisht et al. 2021). Authors (Shi et al. 2008) report that STS is less common in higher plants and occurs in distant species such as groundnut (*Arachis hypogaea*) (Gao et al. 2010), grapevine (*Vitis vinifera*), and pine (*Pinus sylvestris* and *P. strobe*) (Shi et al. 2008).

STSs-Polyketides derivatives: The chemical core of stilbene-type phytoalexins is biosynthesized by STS that gives rise to the naturally occurring stilbene hydroxylated derivatives including the resveratrol (3,4′,5-trihydroxy-stilbene; RESV) (Bisht et al. 2021; Gao et al. 2010; Tauchen et al. 2020; Hertweck 2009), pterostilbene (Luo et al. 2021; Liu et al. 2021), rhapontigenin (Luo et al. 2021; Liu et al. 2021), piceatannol (astringenin) (Bisht et al. 2021; Niaz and Khan 2020), astringin, gnetol, piceid (polydatin) (Zuiter and Zarqa 2014), pinosylvin (Bisht et al. 2021; Flores-Sanchez and Verpoorte 2009; Resmi et al. 2013; Gao et al. 2010; Shimizu et al. 2017) being considered as main derivatives and shares a common diphenylethylene backbone (Zuiter and Zarqa 2014). The base molecule can be further modified according to specific derivatization reactions, to name a few: methylation of hydroxyl groups that yields pterostilbene (E-3,5-dimethoxy-4′-hydroxystilbene) (Liu et al. 2021; Zuiter and Zarqa 2014), C-alkylation with isoprenyl groups to yield 2-isopentenyl-resveratrol (Yang et al. 2018), dimerization gives rise to e-viniferin (Jan et al. 2021), and/or polymerization or glycosylation on hydroxyl groups to produce glycolyzed derivatives (Watts et al. 2006). In addition to the above-mentioned stilbenes other derivatives including oxyresveratrol, RESV trimethylether, pinosylvin monomethylether, resveratroloside, desoxyrhapontigenin, isorhapontigenin, isorhapontin, pinostilbenoside, rhaponticin (rhapontin), and rhapontigenin can be found in several edible plants, such as berries (cranberry, blueberry, and strawberry), grape, and nuts like hazelnut and pistachio (Zuiter and Zarqa 2014).

(2) Stilbenecarboxylate synthase (STCS)

Stilbene carboxylates (hydrangeic acid and lunularic acid) (Flores-Sanchez and Verpoorte 2009; Schro 1999; Eckermann et al. 2003) are found in several plants such as *Hydrangea macrophylla* and liverwort *Lunularia cruciata* reported to be synthesized by STCS via the phenylpropanoid-polymalonate pathway (Eckermann et al. 2003). This enzyme has an acyltransferase activity, transferring groups other than amino-acyl groups and catalyzes the condensation reaction of 4-coumaroyl-CoA or dihydro-4-coumaroyl-CoA starter residues with three units of malonyl-CoA extender (Bisht et al. 2021; Flores-Sanchez and Verpoorte 2009; Eckermann et al. 2003) (Fig. 7.13) and operates the Aldol ring-folding without removal of the terminal carboxyl group (Eckermann et al. 2003). However, the biosynthesis of the natural product includes a reducing step of the carbonyl function of the PK intermediate (coumaroyl triacetyl thioester) to a hydroxyl function (Bisht et al. 2021; Morita et al. 2010a). Thus, the new configuration allows an Aldol ring-folding

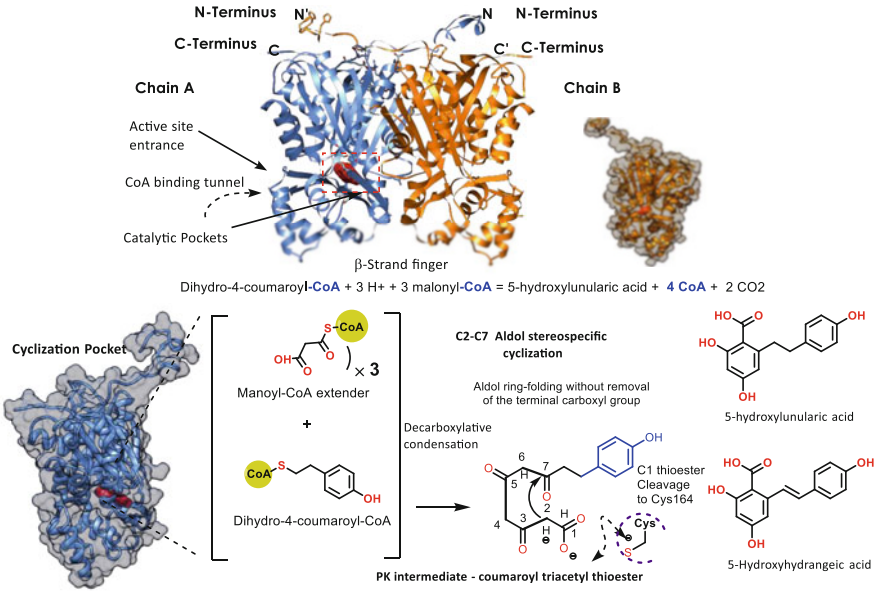

Fig. 7.13 Representation of the reaction mechanism of the STCS (entry: 2P0U) of *Marchantia polymorpha*, the enzymatic structure (flat ribbon and 3D surface), the catalytic domain, the rearrangement/cyclization of the substrates, and the polyketides produced

reaction (Austin et al. 2004b) within the cyclization pocket. Besides, in the absence of the reduction step the enzyme may produce other hydroxylated acids (Austin et al. 2004b). Particularly, authors (Bisht et al. 2021; Morita et al. 2010a) reported that if this enzyme uses coumaroyl-CoA as a starter, only bisnoryagenin and coumaroyl triacetic acid lactone (CTAL) are produced.

STCS-Polyketides derivatives: Several stilbene carboxylates and/or isomeric stilbene derivatives arise from this biosynthetic pathway exerted by STCS (Bisht et al. 2021; Morita et al. 2010a; Eckermann et al. 2003). In the presence of the reducing step, the main produced stilbene carboxylates include lunularic acid being related to the phenyldihydroisocoumarin, hydrangenol (Flores-Sanchez and Verpoorte 2009; Eckermann et al. 2003), and its isomer hydrangeic acid which have been isolated from extracts of the common garden hydrangea (*H. macrophylla*) and the liverwort *L. cruciate (*Eckermann et al. 2003*)*. 5-Hydroxyhydrangeic acid and 5-hydroxylunularic acid are the STCS products expected in absence of the reducing step (Austin et al. 2004b).

(3) Biphenyl Synthase (BPS) and Bibenzyl Synthase (BBS)

The BPS (Bisht et al. 2021; Shi et al. 2008; Flores-Sanchez and Verpoorte 2009; Schro 1999; Stewart et al. 2017) shares almost 60% amino acid sequence identity enzymes of the CHS-superfamily with a high preference for the benzoic acid-derived starter molecules (Bisht et al. 2021; Morita et al. 2010a). It catalyzes three rounds of

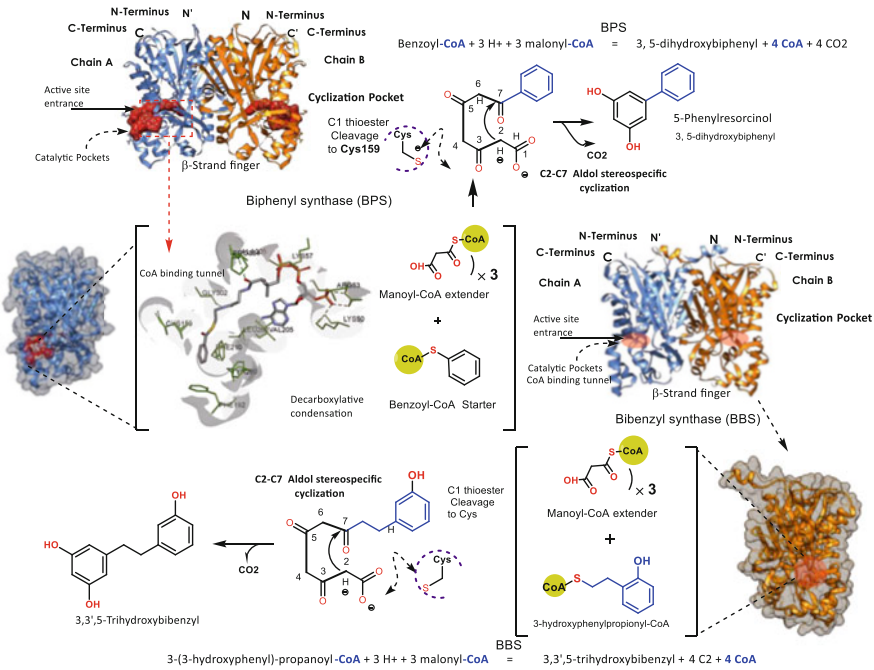

Fig. 7.14 Representation of the reaction mechanism of the BPS (PDB: 5WC4) of *Malus domestic* and BBS (PDB: 7SGY) of *Cannabis sativa*, the enzymatic structure (flat ribbon), the catalytic domain, CoA binding tunnel of BPS, the rearrangement/cyclization of the substrates, and the polyketides produced

condensation reaction of benzoyl-CoA starter with malonyl-CoA extender (Bisht et al. 2021; Morita et al. 2010a; Shi et al. 2008; Flores-Sanchez and Verpoorte 2009; Abe et al. 2004) (Fig. 7.14). The intermediate thus produced undergoes intramolecular aldol condensation to produce 3, 5-dihydroxybiphenyl (Bisht et al. 2021; Shi et al. 2008; Flores-Sanchez and Verpoorte 2009; Songsiriritthigul et al. 2020), being considered as precursor molecule for two major classes of plant phytoalexins, e.g., dibenzofuran and biphenyl aucuparin isolated from *S. aucuparia (*Bisht et al. 2021*)*. BBS, on the other hand, was cloned from *Phalaenopsis* sp. (Flores-Sanchez and Verpoorte 2009; Abe 2008; Pandith et al. 2016; Abe et al. 2004) and catalyzes the production of the tetraketide intermediate 3, 3′5-trihydroxybibenzyl (Bisht et al. 2021; Flores-Sanchez and Verpoorte 2009) by condensation of 3-hydroxyphenylpropionyl-CoA and three malonyl-CoA molecules followed by STS-like aldol ring closure and further producing 9,10-dihydrophenanthrene (Bisht et al. 2021; Austin and Noel 2003; Ferrer et al. 1999; Flores-Sanchez and Verpoorte 2009). This enzyme prefers cinnamoyl-CoA derivatives lacking a double bond over 4-coumaroyl-CoA. BPS and BIS had found to share the same condensation mechanism; however, in the ring-closure reactions, aldol-type cyclization, instead of Claisen-type cyclization, proceeds in BIS. Enzymatic features studies

had shown insights into the functional diversification of BIS from *Malus domestica* (Songsiriritthigul et al. 2020). Possible mutations in the active site affect the preferences of the starter substrates and render it incompetent for the use of larger coumaroyl-CoA molecules (Stewart et al. 2017).

BPS and BBS Polyketides derivatives: The phytoalexins biphenyl and dibenzofurans (Bisht et al. 2021) where the chemical core is biosynthesized by the biphenyl synthase are communally found in the rosaceae species. The intermediate bibenzyl compound 3, 3′5-trihydroxybibenzyl synthesized by the BBS is further transformed into dihydrophenanthrenes (batatasins) through methylation reaction induced by a methyltransferase (S-adenosyl methionine-dependent O-methyltransferase) (Bisht et al. 2021). Batatasins are subsequently metabolized to tricyclic phenanthrenoid 9, 10-dihydrophenanthrene derivatives such as hircinol in *Dendrobium thyrsiflorum* (Liu et al. 2011) and Orchinol in *Orchis militaris (*Bisht et al. 2021*)*.

(4) Hexaketide Synthase (HKS) and Octaketide Synthase (OKS)

The HKS and OKS steric modulation of a single Gly207 residue (corresponding to the active-site Thr197 of CHS) directly affects the length of the polyketide chain and the specificity of the product (Bisht et al. 2021; Morita et al. 2010a; Abe et al. 2005). Shorter chain polyketides (triketide to heptaketide) including a pentaketide chromone (Bisht et al. 2021; Yu et al. 2012; Stewart et al. 2013), 2, 7-dihydroxy-5-methylchromone (Bisht et al. 2021; Yu et al. 2012; Flores-Sanchez and Verpoorte 2009; Abe et al. 2005; Shimizu et al. 2017), and a hexaketide pyrone (Abe et al. 2005), 6-(2,4-dihydroxy-6-methylphenyl)- 4-hydroxy-2-pyrone (Wakimoto et al. 2012; Abe et al. 2005; Taura et al. 2016; Kontturi and Type 2017), are formed by substitution of residues controlling the length of the polypeptide chain. HKS catalyzes a series of iterative condensations of five molecules of malonyl-CoA with acetyl-CoA as the starting substrate (Bisht et al. 2021; Morita et al. 2010a) (Fig. 7.15), while OKS uses acetyl-CoA as a starting substrate and condenses with seven units of malonyl-CoA (Bisht et al. 2021; Flores-Sanchez and Verpoorte 2009; Stewart et al. 2013; Kontturi and Type 2017; Huang et al. 2019) (Fig. 7.15). Both enzymes give a linear polyketide intermediate being the hexaketide 6-(2′,4′--dihydroxy-6′-methylphenyl)-4-hydroxy-2-pyrone (Abe et al. 2005; Shimizu et al. 2017) for HKS and a linear octaketide intermediate in the case of OKS that is further converted to emodin anthrone (Martínez and Bermejo 2005) via atrochrysone by three aldol-type condensations and dehydration (Fig. 7.15). Aldol cyclization gives rise to naphthoquinone and naphthalene derivatives in the case of HKS and to anthranoid of anthraquinone for the OKS (Atanasov et al. 2021; Hook et al. 2014; Diaz-Muñoz et al. 2018; Martínez and Bermejo 2005). In the latter, aromatic octaketides SEK4 and SEK4b may be produced following incorrect bending (Bisht et al. 2021; Tsai and Ames 2009; Morita et al. 2007; Shi et al. 2008; Shimizu et al. 2017; Kontturi and Type 2017). Moreover, HKS reveal a promiscuity of starting substrate as shown by in-vitro tests, where the acetogenic naphthoquinone hexaketide was not produced (Bisht et al. 2021). This suggests that this enzyme is originally involved in the biosynthesis of plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone) (Hook et al. 2014; Jan et al. 2021; Załuski et al. 2015) such as in

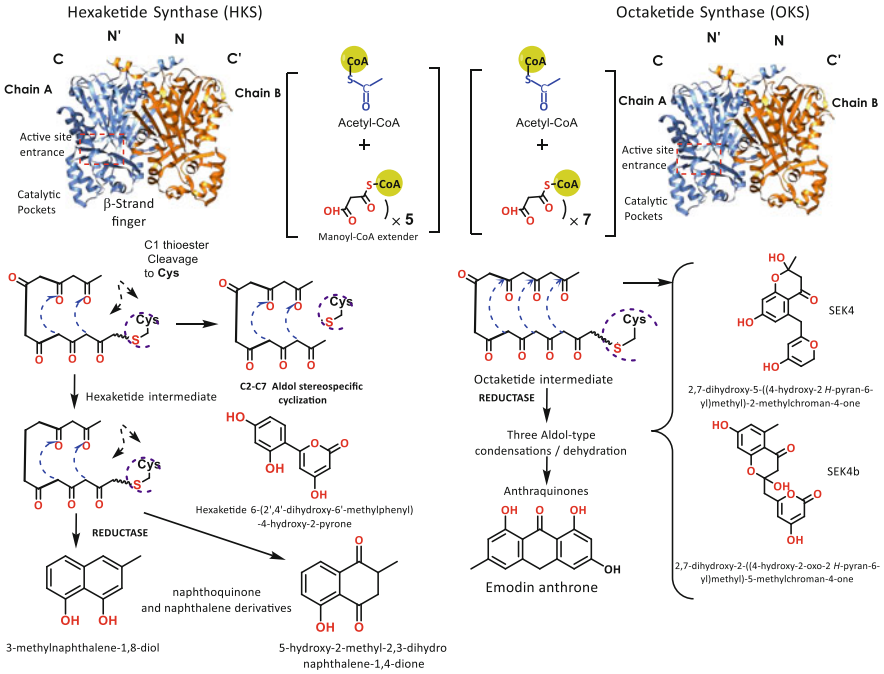

Fig. 7.15 Representation of the reaction mechanism of the HKS and OKS (PDB: 7DTQ) of *Aloe arborescens*, the enzymatic structure (Flat ribbon), the catalytic domain, the rearrangement/cyclization of the substrates, and the polyketides produced

the plant genus Plumbago (Hook et al. 2014; Taura et al. 2016), and the bicyclic tetralones compounds in the presence of adapter enzymes such as cyclase-like cofactor and/or reductase in plants (Morita et al. 2010a; Abe and Morita 2010). Likewise, the OKS of *Aloe arborescens* can accept 4-coumaroyl-CoA to produce the hexaketide stilbene by catalyzing five- and six-ring condensations with malonyl-CoA (Yu et al. 2012; Morita et al. 2010a; Wakimoto et al. 2012). In addition, the enzyme presents a possibility of condensation of n-hexanoyl-CoA as a starting substrate with five and six molecules of malonyl-CoA, in order to produce irregularly folded resorcinol hexaketide and phloroglucinol heptaketide (Morita et al. 2010a, 2019). Interestingly, it has also been shown that *A. arborescens* OKS efficiently accepted (2RS)-methylmalonyl-CoA as substrate to produce 6-ethyl-4-hydroxy-3,5-dimethyl-2-pyrone (Shi et al. 2008; Abe 2008; Pandith et al. 2020).

HKS and OKS Polyketides derivatives: Naphthoquinone hexaketide found in the plant genus Plumbago are precursors of plumbagin (Hook et al. 2014; Geris et al. 2012; Martínez and Bermejo 2005), 3-*O*-methyl droserone (Hook et al. 2014; Geris et al. 2012), 6-hydroxy plumbagin (Geris et al. 2012; Mazziotti et al. 2022), isoshinanolone, isoplumbagolone, chitranone, maritinone, and chitranane (Hook et al. 2014; Geris et al. 2012). Correspondingly, anthrones, anthraquinones,

dianthrones, and emodin anthrone (Diaz-Muñoz et al. 2018; Lajis and Ahmad 2006) widely present in Aloe plants (*A. arborescens, A. nyeriensis,* and *A. cheranganiensis*) are the main derivatives of OKS. In several plant species, anthraquinones derived from anthracene are considered a precursor of anthrone glycosides (ex. barbaloin) (Abe 2008; Abe et al. 2005; Diaz-Muñoz et al. 2018), and anthrone emodin which is in turn a precursor of naphthodianthrones including hypericin present in *Hypericum perforatum* (Jan et al. 2021; Diaz-Muñoz et al. 2018; Martínez and Bermejo 2005). Equally, oxidation of the central cyclohexadienone ring in emodin anthrone yields emodin (Diaz-Muñoz et al. 2018), which can be methylated to physcion (Diaz-Muñoz et al. 2018; Martínez and Bermejo 2005). Moreover, sennoside A and B are two anthraquinone glycosides from *Cassia angustifolia* (Diaz-Muñoz et al. 2018; Martínez and Bermejo 2005). Meanwhile, in-vitro biosynthesis reaction of OKS results in 6-(7-hydroxy-5-methyl-4-oxo-4*H*-chromen-2-yl)-3,5-dioxohexanoic acid and 4-(7-hydroxy-5-methyl-4-oxo-4*H*-chromen-2-yl)-3-oxobutanoic acid as well as SEK4b (Bisht et al. 2021; Morita et al. 2010a; Wakimoto et al. 2012).

(5) STS-type Enzymes (Aldol, Aromatic, Heterocyclic)

It should be noted that during the Aldol condensation conducted by STS-type enzymes (Table 7.1) many other distinguished mechanisms involve nucleophilic addition of an enolate to a ketone to yield heterocyclic β-hydroxyl aldol products (Morita et al. 2010a). The main enzymes assuring this type of reactions are:

(6) Pentaketide Chromone Synthase (PCS)

The PCS shares approximately from 50 to 60% of sequence identity with the enzymes of CHS-superfamily (Bisht et al. 2021) while retaining the Cys-His-Asn catalytic triad as well as most of the CHS active-site residues giving rise to pentaketide chromones (5,7-dihydroxy-2-methylchromone = Noreugenin) in *Pisonia aculeata* (Bisht et al. 2021; Yu et al. 2012; Shimizu et al. 2017) by five steps of decarboxylative condensation of malonyl-CoA molecules as starting substrates followed by cyclization reactions to form an aromatic ring (Bisht et al. 2021; Morita et al. 2010a) (Fig. 7.16). Reported in Aloe (*A. arborescens*) as rich source of chromones (Bisht et al. 2021; Morita et al. 2007; Abe et al. 2004, 2005) and anthraquinones (Bisht et al. 2021; Diaz-Muñoz et al. 2018; Lajis and Ahmad 2006), the PCS had particularity various aromatic starter units affinity such as 4-coumaroyl, cinnamoyl, and benzoyl-CoA esters and middle-chain aliphatic starters such as *n*-hexanoyl, *n*-octanoyl, and *n*-decanoyl CoA esters to yield triketide and tetraketide α-pyrones (Bisht et al. 2021; Morita et al. 2010a). A structural particularity of this enzyme is that the residues Thr197, Gly256, and Ser338 (according to *Medicago sativa* CHS numbering) are substituted by Met207, Leu266, and Val351 (according to *A. arborescens* PCS numbering) that cause steric contraction of the active-site cavity (Morita et al. 2007; Shi et al. 2008). However, Met207 as chemically inert active-site residue (Morita et al. 2007; Shi et al. 2008) solely controls the number of condensations of the extender units depending on the steric bulk of the side chain. Besides, site-directed mutagenesis such as in octaketide-

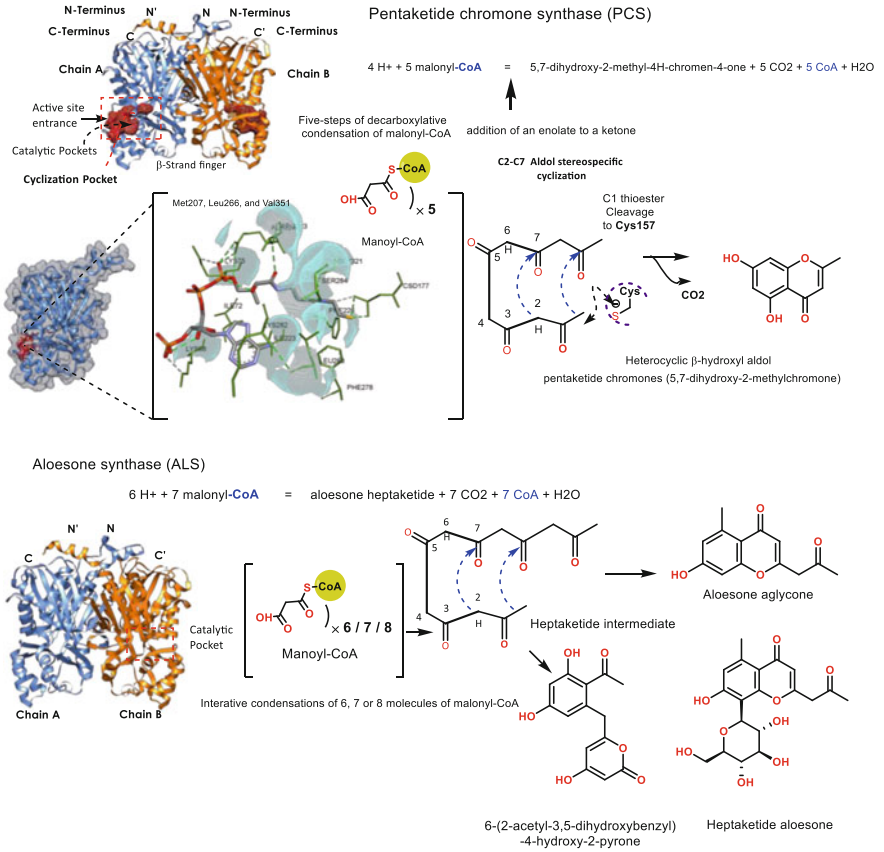

Fig. 7.16 Representation of the reaction mechanism of the PCS (PDB: 2D3M) of *Aloe arborescens*, the enzymatic structure (Flat ribbon), the catalytic domain, malonyl-CoA binding tunnel, the rearrangement/cyclization of the substrates, and the polyketides produced

producing M207G mutant (Morita et al. 2007; Lussier et al. 2012; Abe et al. 2005) revealed that the substitution of a single amino acid residue, Met207 by Gly207 in the active enzymatic sequence (G207A, G207T, G207M, G207L, G207F, and G207W mutant) (Abe et al. 2005) may generate aromatic octaketides such as SEK4 and SEK4b using eight molecules of malonyl-CoA (Morita et al. 2007; Wakimoto et al. 2012; Lussier et al. 2012; Abe et al. 2005; Resmi et al. 2013).

PCS Polyketides derivatives: The biosynthetic pathway driven by the PCS will mainly produce 5,7-dihydroxy-2-methylchromone (Bisht et al. 2021; Yu et al. 2012; Wakimoto et al. 2012; Flores-Sanchez and Verpoorte 2009; Shimizu et al. 2017) which will be converted during secondary metabolism to chromone products of various structures. Those compounds are ubiquitous and distributed in virtually all land plants. They are basically divided into three subgroups, namely: the simple chromones such as the 2-phenoxychromone in *Artemisia rupestris* (Aisa et al. 2006)

and *Epimedium koreanum* (Jin et al. 2014), biflorin from *Pancratium biflorum* (Youssef et al. 2022), and the flower buds of *Syzygium aromaticum* (Lee et al. 2016), then to pyranochromones such as the allopteroxylin, 3,3-dimethylallylspatheliachromene, spatheliabischromene, and 5-*O*-methylcneorumchromone from *Dictyoloma vandellianum* (Alves et al. 2017) and markedly the furanochromones group represented mainly by visnagin and khellin found principally in *Ammi visnaga* (Khalil et al. 2020).

(7) Aloesone synthase (ALS)

The ALS from rhubarb and Aloe species catalyzes sequential condensations of 6, 7, or 8 molecules of malonyl-CoA to produce various aromatic polyketides mainly heptaketide chromone aloesone derivatives (Bisht et al. 2021; Wakimoto et al. 2012; Flores-Sanchez and Verpoorte 2009; Abe 2008; Shimizu et al. 2017; Abe et al. 2004). From 7 molecules of malonyl-CoA, this enzyme will produce aloesone heptaketide (Bisht et al. 2021; Morita et al. 2007; Shi et al. 2008; Abe 2008; Shimizu et al. 2017; Abe et al. 2004; Mizuuchi et al. 2009), the aglycone of aloesin, as a major product. Particularly, ALS accepts aliphatic-CoA esters substrate of long and medium-chain length and has the ability to produce hexaketide pyrone and heptaketide aromatic pyrone (6-(2-acetyl-3,5-dihydroxybenzyl)-4-hydroxy-2-pyrone) (Yu and Jez 2008; Yu et al. 2012; Abe 2008; Jindaprasert et al. 2008). Another heptaketide pyrone 6-(2-(2,4-dihydroxy-6-methylphenyl)-2-oxoethyl)-4-hydroxy-2-pyrone was also reported to be synthesized by ALS using one unit of acetyl-CoA and six units of malonyl-CoA (Mizuuchi et al. 2009). The unstable heptaketide pyrone (or acid form) undergoes subsequent spontaneous isomerization to the ß-ketoacid chromone to be decarboxylated producing therefore an heptaketide aloesone (Abe et al. 2004; Mizuuchi et al. 2009). Similarly, ALS may produce the octoketides SEK4/SEK4b using the same condensation pattern (Bisht et al. 2021; Morita et al. 2007; Flores-Sanchez and Verpoorte 2009; Shimizu et al. 2017).

CTAS-Type Enzymes (Lactonization, Heterocyclic)

The specificity of this group of enzymes is their alkylpyrone-producing activity (Bisht et al. 2021). In contrast, the majority of CTAS enzymes catalyze the C5-C1 oxygen lactonization (Table 7.1) of triketide and tetraketide intermediates to produce pyrones and alkylpyrones (Bisht et al. 2021; Austin and Noel 2003; Shimizu et al. 2017; Taura et al. 2016; Jindaprasert et al. 2008). The residue Trp281 was revealed as a crucial aromatic amino acid in PKS responsible for the production of pyrones, acid lactones, and pyranones, at the level of the cavity of the active site (Mizuuchi et al. 2009; Jindaprasert et al. 2008; Akiyama et al. 1999; Klundt et al. 2009). Therefore, amino acid variation at this position may affect the specificity of cyclization and generate a heterocyclic system. Conversely, different from the other families of PKS, this family generally presents a large cavity of the active site giving flexibility to the tetraketide chain, leading to spontaneous lactonization (Ping 2016; Gayen et al. 2020), where the electrophilic ketone C1 triggers rearrangement by formation of a cyclopyrone and subsequent opening by intramolecular attack of the

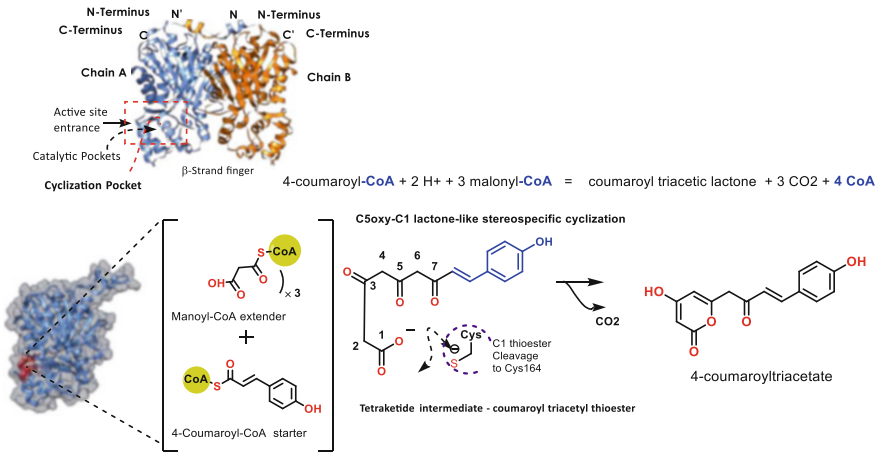

Fig. 7.17 Representation of the reaction mechanism of the CTAS (PDB: 1i86) swiss model O82145 (O82145_HYDMC) of *Hydrangea macrophylla*, the enzymatic structure (Flat ribbon), the catalytic domain, the rearrangement/cyclization of the substrates and the polyketide intermediate and end product

C5 hydroxy group, leading to the formation of the lactone ring (Austin and Noel 2003; Austin et al. 2004a; Jan et al. 2021; Klundt et al. 2009).

(1) 4-Coumaroyltriacetic acid synthase (CTAS)

This enzyme originally separated in *Hydrangea macrophylla* var. thunbergii (Yu and Jez 2008; Eckermann et al. 2003; Akiyama et al. 1999), preferably accepts 4-coumaroyl-CoA as a starter and performs three condensation reactions of malonyl-CoA. It mediates the closure of the coumaroyl triacetyl thioester intermediate by lactonization-like cyclization to yield the 4-coumaroyltriacetate or the coumaroyl triacetic lactone (CTAL) (Bisht et al. 2021; Morita et al. 2010a) (Fig. 7.17) once considered a common by-product of CHS (Austin and Noel 2003; Yu et al. 2012) and STS (Eckermann et al. 2003). Otherwise in nature, lactonization may proceed in a non-enzymatic spontaneous manner after carbonyl ketoreduction of the C5 carbon (Schaub et al. 2019; Tang et al. 2017; Tsai and Ames 2009), producing triketide and tetraketide pyrones after bonding with the carbonyl of the C1 carbon these polyketides are often the derailment products of the PKS reaction main type III. Coumaroyl triacetic acid lactone and dihydroxymethylphenyl methylpyrone compounds are considered as premature hydrolysis products catalyzed by CTAS (Austin and Noel 2003; Jez et al. 2002; Dao et al. 2011; Eckermann et al. 2003; Hertweck 2009; Klundt et al. 2009). In addition, scientific reports have suggested that this enzyme is involved in the biosynthesis of hydramacroside B (Austin and Noel 2003; Eckermann et al. 2003; Akiyama et al. 1999).

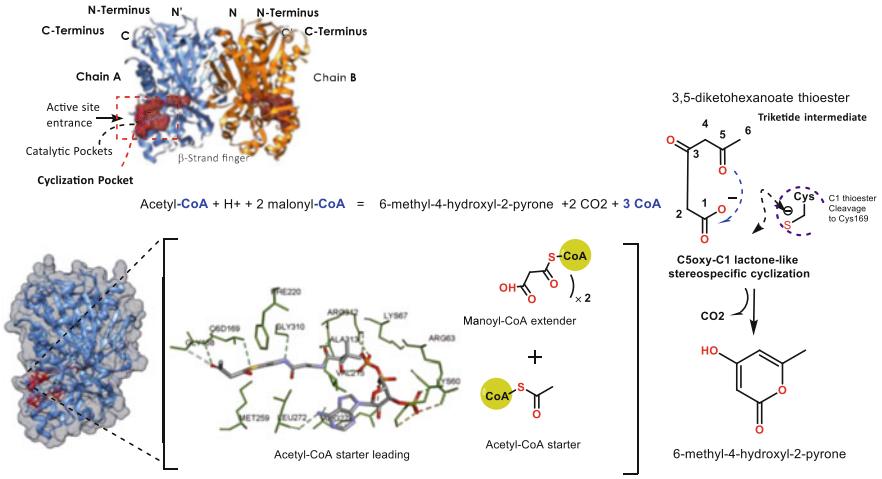

Fig. 7.18 Representation of the reaction mechanism of the 2-PS (PDB: 1EE0) of *Gerbera hybrida*, the enzymatic structure (flat ribbon), the catalytic domain, Acetyl-CoA binding tunnel, the rearrangement/cyclization of the substrates and the polyketide intermediate and end product

(2) C-Methylchalcone synthase (PstrCHS2)

A remarkable particularity of plant-PKS III is the absence of fractions ensuring methylation extension, accordingly these enzymes have the possibility of using methylated starter substrates such as the methylmalonyl-CoA (Xie et al. 2016; Flores-Sanchez and Verpoorte 2009; Shimizu et al. 2017) (Table 7.1). Therefore, the methyl group will consequently be preserved during the C–C bond elongation reaction. In such case, the example of CHS2 isolated from *Pinus strobes* (Shi et al. 2008) is representative of interactive condensation reactions where methylmalonyl-CoA is used as a starting substrate with cinnamoyl diketide NAC to produce methylated styrylpyrone triketide (Jez et al. 2002; Pandith et al. 2020; Schröder et al. 1998). PKS III from several plant species such as CHS from *Scutellaria baicalensis* (Wakimoto et al. 2012; Abe et al. 2005; Jan et al. 2021; Sun et al. 2015), STS from *Arachis hypogaea* (Xie et al. 2016; Tauchen et al. 2020; Akiyama et al. 1999), and BAS from *Rheum palmatum* (Xie et al. 2016; Morita et al. 2010b; Abe et al. 2004) can ensure decarboxylative condensation reactions of (2RS)-methylmalonyl-CoA and 4-coumaroyl-CoA to produce an unnatural C6-C5 aromatic polyketide and 1-(4-hydroxyphenyl)pent-1-en-3-one.

(3) 2-Pyrone synthase (2-PS)

The 2-PS is an enzyme that allows the synthesis of triacetic acid lactone (TAL) by condensing one unit of acetyl-CoA as a starting substrate with two malonyl-CoA extender molecules (two decarboxylative condensation reactions) (Bisht et al. 2021; Morita et al. 2010a; Zhou et al. 2016; Eckermann et al. 2003; Jindaprasert et al. 2008). The triketide intermediate (3,5-diketohexanoate thioester) produced will be cyclized to a 6-methyl-4-hydroxyl-2-pyrone through lactonization (Fig. 7.18) by a nucleophilic attack of C5 keto-enol oxyanion to C1

carbonyl carbon on the enzyme-bound intermediate (Bisht et al. 2021; Schröder et al. 1998; Jindaprasert et al. 2008). The 2-PS has a steric bulk at the active-site residues positions (197 and 338) (Austin and Noel 2003) and accepts also a variety of substrates such as the case of the final product psilotin (Schro 1999; Klundt et al. 2009) where the starting substrate will be benzoyl-CoA condensed with two molecules of malonyl-CoA to produce 6-phenyl-4-hydroxy-2-pyrone (phenylpyrone) and 4-hydroxylated phenylpyrone (psilotinin) present in the pteridophyte *Psilotum nudum* (Klundt et al. 2009). Similarly, other aliphatic-CoA esters such as isovaleryl-CoA, propionyl-CoA, and butyryl-CoA are also used by the 2-PS (Austin and Noel 2003; Eckermann et al. 2003) as a starter substrate to produce triketide pyrones. Long-chain CoA fatty acid esters such as palmitoleoyl (C16)–CoA (Kim et al. 2013; Shi et al. 2008) can also serve as a starting substrate resulting in the formation of alkyl polyphenols such as urushiol in *Rhus verniciflua* (Cheong et al. 2010) and ginkgolic acid (anacardic acid) in *Ginkgo biloba* (Li et al. 2014).

2-Pyrone Polyketides derivatives: α-pyrone moiety is a structural feature found in a huge variety of biologically active metabolites (Schäberle 2016). In plants, monocyclic and α-pyrone including triacetic acid lactone, tetraacetic acid lactone, umbelliferone (in the Umbelliferae family) (Schäberle 2016), and monobenzo α-pyrones such as esculetin in Cichorium intybus (Nwafor et al. 2017) and scopoletin occurring in the genus Scopolia and Djalonensone isolated from the roots of *Anthocleista djalonensis* (Anyanwu et al. 2018; Schäberle 2016) are representative examples of plant pyrones. Other secondary metabolites of α-pyrone plants are ellagitannins and ellagic acid (Schäberle 2016). A class of lactone compounds such as the kavalactones (e.g., yangonin) found in *Piper methysticum* (Hegazy et al. 2019), goniothalamin, dihydrokavain, desmethoxyyangonin, kavalactones kavalactones (+)-methysticin, (+)-kavain, (+)-dihydromethysticin, and (+) dihydrokavain from *Cryptocarya novoguineensis* and Piper methysticum are Pyrone compounds derivatives occurring in several plants (Hegazy et al. 2019; Gurley et al. 2008; Schäberle 2016). Pyrone glycosides (e.g., psilotins) and their derivatives are produced by glycolization of pyrones (Klundt et al. 2009). Accordingly, the 6-methyl-4-hydroxyl-2-pyrone is a biosynthetic precursor of pyrone glycoside gerberin and parasorboside, and Psoralen isolated from *Ficus carica* (Chunyan et al. 2009).

Type III PKS Polyketide's Non-Cyclization

Several PKS III assure connections between the starting CoA substrates and those of extension without regiospecific cyclization (Table 7.1). Intermolecular linking reactions are then produced at the enzymatic level then poly-β-ketones are not cyclized or spontaneously cyclized (stereospecific cyclization) giving rise to various bioactive compounds. Among the PKS IIIs of this class, there may be the Diketide synthase (DKS)_Curcumin Synthase (CURS), β-diketone synthase (DKS), Benzalacetone synthase (BAS), Alkyldiketide-CoA synthase (ADS), Alkylquinolone synthase (AQS), pyrrolidine ketide synthase (PYKS).

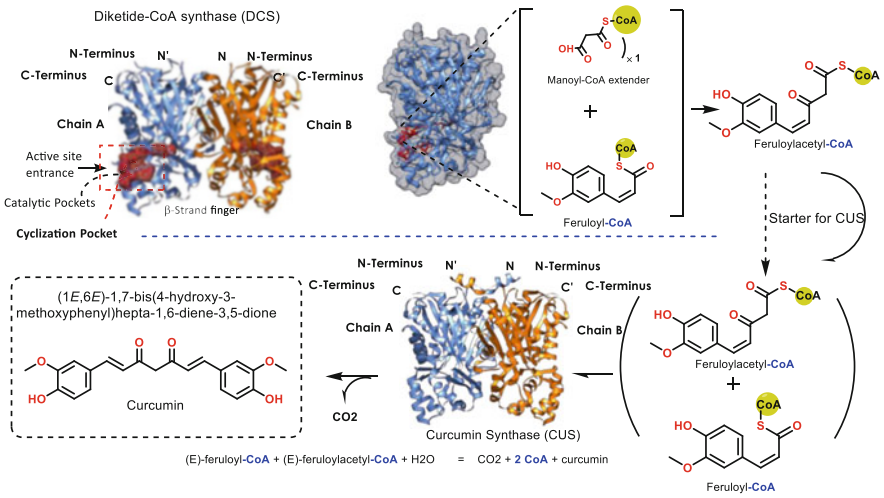

Fig. 7.19 Representation of the reaction mechanism of the DCS (UniProtKB-A0A0A1E5U2 (A0A0A1E5U2_9LILI)) of *Curcuma amada*, and CURS (PDB: 3OV2) of *Curcuma longa*, the enzymatic structure (Flat ribbon), the catalytic domain, the rearrangement/cyclization of the substrates, and the polyketide intermediate and end product

(1) Diketide-CoA Synthase (DCS)_Curcumin Synthase (CURS)

The DCS (Bisht et al. 2021; Morita et al. 2010b; Zhang et al. 2016) and CURS (Abe 2020; Zhang et al. 2016; Pothiraj et al. 2021; Matsui et al. 2017) share around 63% of sequence identity and usually work jointly in producing plant diketides then curcuminoids. DKS in *Curcuma longa* (turmeric) (Li et al. 2020b; Christensen and Christensen 2013; Pothiraj et al. 2021; Matsui et al. 2017) catalyzes the condensation of one unit of feruloyl-CoA starter substrate and one unit of the extender malonyl-CoA to produce feruloyldiketide-CoA (Bisht et al. 2021; Morita et al. 2010a) (Fig. 7.19). Afterward, CURS catalyzes the conversion of feruloyldiketide-CoA (Abe 2020; Yu et al. 2012; Zhang et al. 2016; Matsui et al. 2017) to β-keto acid and sequentially by hydrolysis and condensation with another feruloyl-CoA molecule to produce C6–C7–C6 diarylheptanoid scaffold of curcumin (Fig. 7.19). Moreover, the CURS from *Oryza sativa* (Zhang et al. 2016; Morita et al. 2019) plant has been reported to catalyze the formation of bisdemethoxycurcumin by condensing two molecules of p-coumaroyl-CoA and one molecule of malonyl-CoA (Abe 2020; Flores-Sanchez and Verpoorte 2009; Morita et al. 2019; Matsui et al. 2017). Therefore, the curcuminoids, demethoxycurcumin, and bisdemethoxycurcumin, abundant in the rhizome of *C. longa*, are obtained in conjunction with the synthesis of DCS and curcumin synthase 1 (CURS1) (Shi et al. 2008; Flores-Sanchez and Verpoorte 2009; Christensen 2018). The synthetic scheme as represented in Fig. 7.19 is initiated by a three-step reaction from phenylpropanoids where malonyl-CoA condenses by the action of DCS with feruloyl-CoA, to produce a feruloyldiketide-CoA, then the diketide is converted by the action of CURS1 into a

β-keto acid by hydrolysis and condensation with another feruloyl-CoA molecule to produce curcumin. DCS and CURS1 prefer feruloyl-CoA and can use other starters such as coumaroyl-CoA and malonyl-CoA (Bisht et al. 2021). The hydrophobic interaction between CURS1 and β-keto acid is ensured at the level of the hydrophobic pocket of the CoA binding tunnel following the different orientations of Phe265 and also the substitution of the active-site Ser338 by Gln338 (Katsuyama et al. 2011). The variety of curcuminoids in *C. longa* is related to the presence of different CURS (1, 2 and 3) which use different substrates. In addition, ginger (*Zingiber officinale*) has a specific curcumin synthase (ZoCURS) (Zhang et al. 2016) that accepts 3-(4-hydroxyphenyl)propionyl-CoA as a starting substrate to produce tetrahydrobisdemethoxycurcumin and similar products.

(2) β-Diketone Synthase

The DKS in poaceae species (particularly barley *Hordeum vulgare* and wheat *Triticum sp*) (Bisht et al. 2021; Hen-Avivi et al. 2016) is involved in the synthesis of β-diketones of the epicuticular wax on the plant surface (Table 7.1). In addition to the action of DKS, the process of synthesis of these molecules is closely established in complementarity with other enzymes, namely hydroxylase and lipase/carboxyl transferase to produce β-diketones, hydroxy-β-diketones, and esterified alcan-2-ols (Bisht et al. 2021). In vivo, the DKS uses short chain fatty acyl-CoA (C12-C16) as starting substrates to produce a tetraketide intermediate, and expression a high affinity toward C18 and 3-oxo-C16-CoA in vitro with a great preference for 3-oxo-C18-CoA (Bisht et al. 2021; Hen-Avivi et al. 2016). Elongation reactions of the tetraketide intermediate follows by the fatty acyl elongase to produce a β-diketone carbon skeleton in the end products (Bisht et al. 2021).

β-Diketone and Wax Polyketides derivatives: 1,3-Diketones (β-diketones) are ubiquitous scaffolds present in Poaceae species (Hen-Avivi et al. 2016) such as *Triticum spp*, barley (*H. vulgare*), rye (*Secale cereal*), and oat (*Avena sativa*) which produce very-long-chain β-diketones including hentriacontane-14,16-dione (-β-diketone) and its hydroxy derivatives from epicuticular wax. Long-chain aliphatic beta-dicarbonyl compounds have also been identified form *Vanilla fragrans* and *Vanilla tahitensis* (Orchidaceae) (Ramaroson-Raonizafinimanana et al. 2000) including 16-pentacosene-2,4-dione, 18-heptacosene-2,4-dione, 20-nonacosene-2,4-dione, 22-hentriacontene-2,4-dione, and 24-tritriacontene-2, 4-dione, as well as dibenzoylmethane or *n*-tritriacontane-16,18-dione, naturally obtained from eucalyptus leaves, licorice roots, vanilla beans, and sunflower pollen.

(3) Benzalacetone synthase (BAS)

The BAS mediates the biosynthesis of the C6-C4 moiety of a variety of phenylbutanoids by catalyzing the decarboxylative condensation of malonyl-CoA with 4-coumaroyl-CoA to produce a diketide benzalacetone (Bisht et al. 2021; Morita et al. 2010b; Shimokawa et al. 2012) (Fig. 7.20). The latter is further reduced to 4-hydroxybenzalacetone 4-(4-hydroxyphenyl)-but-3-in-2-one (pHPB) (Flores-Sanchez and Verpoorte 2009; Resmi et al. 2013). This enzyme shares (~70%) sequence identity with CHS (Morita et al. 2010b; Shimokawa et al. 2012), with

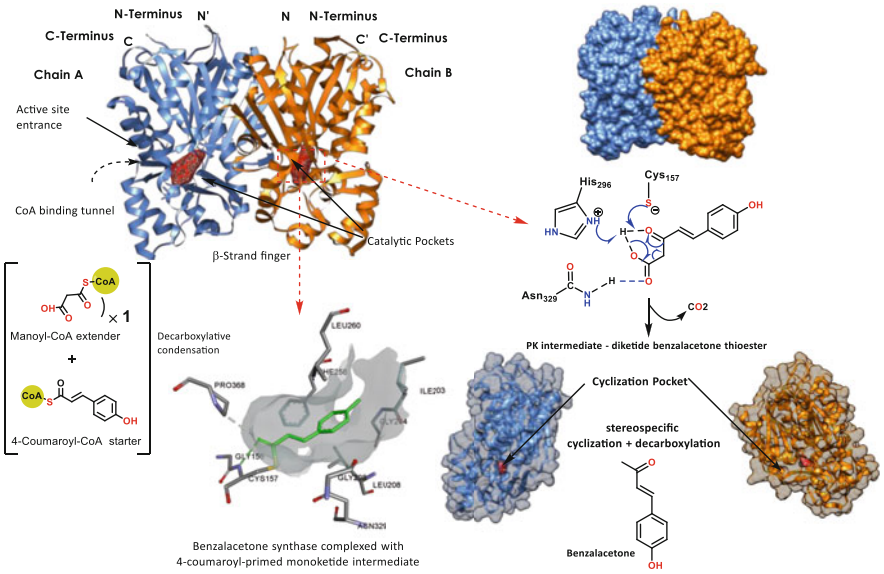

Fig. 7.20 Representation of the reaction mechanism of the BAS (PDB, 3A5R) of *R. palmatum* complexed with 4-coumaroyl-primed monoketide intermediate, the enzymatic structure (flat ribbon, 3D surface), the catalytic domain, 4-coumaroyl-CoA binding tunnel, the rearrangement/cyclization of the substrates and the polyketide intermediate and end product

comparable cavity volumes it proceeds via novel catalytic machinery for thioester bond cleavage of the enzyme-bound diketide intermediate and the final decarboxylation reaction to produce benzalacetone (Bisht et al. 2021; Morita et al. 2010a). Besides, Phe215 residue (*M. sativa* CHS numbering) is mutated and substituted by Leu208 in BAS from *Rheum palmatum* and *Rubus idaeus* (Morita et al. 2010b), causing early termination of the diketide stage within the junction between the active-site cavity and the CoA binding tunnel (Bisht et al. 2021; Morita et al. 2010a). The enzyme-bound diketide is further transformed into benzalacetone by benzalacetone reductase (Austin and Noel 2003). The residue's substitution is strictly crucial in the biosynthesis of benzalacetone and biosynthesis of the C6-C4 moiety of a variety of phenylbutanoids (Shimokawa et al. 2012) (Fig. 7.20). Whereas, the enzyme uses differently another coumaroyl starter-CoA binding pocket following the steric obstruction of the entry in the case of BAS by Leu125, Leu208, and Ser331 (Morita et al. 2010b). It has also been stated that BAS of *R. idaeus* is bifunctional and the enzyme was able to synthesize both the chalcones of benzalacetone and naringenin (Bisht et al. 2021; Morita et al. 2010a). In raspberry BAS has been reported to exhibit broad substrate specificity and can use feruloyl-CoA, 5-hydroxyferuloyl-CoA, 4-coumaroyl-CoA, to produce 3-methoxy-4-hydroxybenzalacetone and 3-methoxy-4,5 dihydroxybenzalacetone (Flores-Sanchez and Verpoorte 2009; Zhang et al. 2016) or malonyl (or methylmalonyl-CoA) in the case of *R. palmatum* along with larger starting units such as N-methylanthraniloyl-

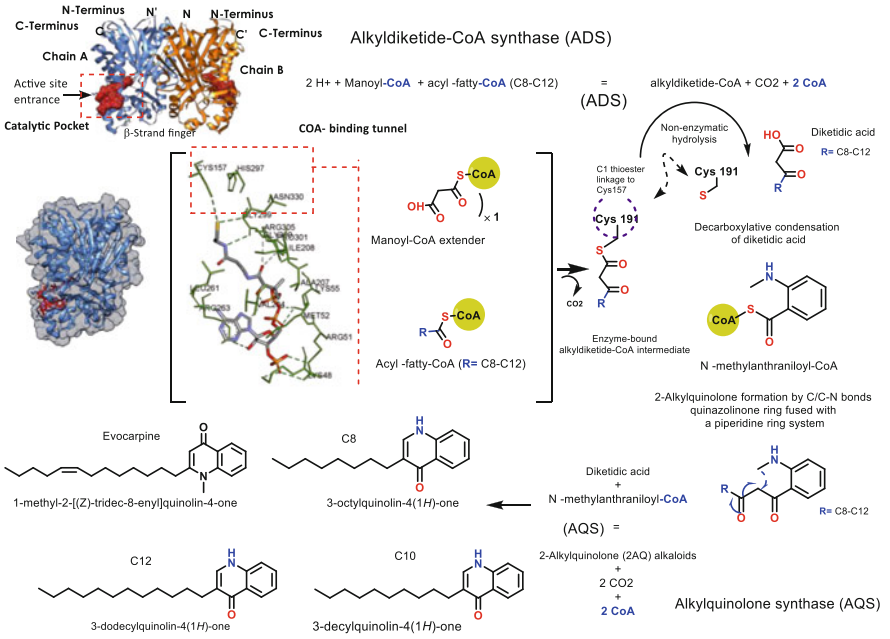

Fig. 7.21 Representation of the reaction mechanism of the ADS (PDB: 5WX7) and AQS (PDB, 5WX5) of *Evodia rutaecarpa*, the enzymatic structure (flat ribbon, 3D surface), the catalytic domain, malonyl-CoA binding tunnel, the rearrangement/cyclization of the substrates and the polyketide intermediate and end products

CoA (or anthraniloyl-CoA) to produce 4-hydroxy-2(1H)-quinolones (Morita et al. 2010b; Shimokawa et al. 2012). The latter is considered as a precursor for the synthesis of quinolone alkaloids widely present in Rutaceae (Resmi et al. 2013; Matsui et al. 2017).

(4) Alkyldiketide-CoA Synthase (ADS)_Alkylquinolone Synthase (AQS)

ADS and AQS are functionally distinct PKS III and share 61% of sequence identity (Bisht et al. 2021; Matsui et al. 2017). They often act in synergy such as the case of the species *Evodia rutaecarpa* (Morita et al. 2019; Matsui et al. 2017), where ADS first catalyzes a decarboxylative condensation of the malonyl-CoA extender with an acyl -fatty CoA (C8-C12) to produce an alkyldiketide-CoA intermediate (Fig. 7.21), whereas AQS subsequently catalyzes the decarboxylative condensation of diketidic acid (formed by non-enzymatic hydrolysis of the alkyldiketide-CoA intermediate) with N-methylanthraniloyl-CoA forming –C/C–N bonds and a quinazolinone ring fused with a piperidine ring system as in the 2-alkylquinolone (2AQ) alkaloids products (Matsui et al. 2017). Comparably, 2AQ are biosynthesized similarly as curcumin in turmeric described above (Zhang et al. 2016; Matsui et al. 2017). The active-site architecture of ADS and AQS features Trp-332 and Cys-191

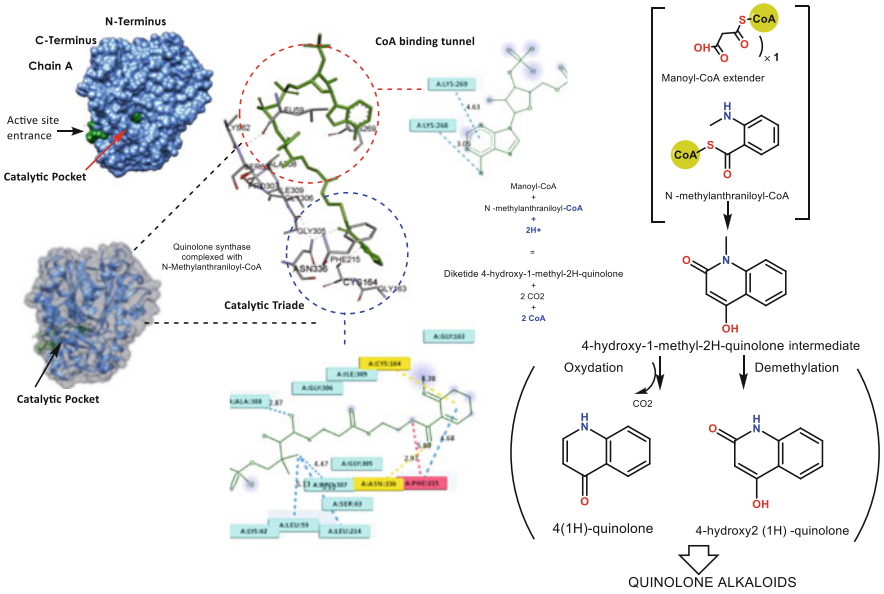

Fig. 7.22 Representation of the reaction mechanism of the QNS (PDB, 6L7J) of *Aegle marmelos* complexed with Coenzyme A, the enzymatic structure (Chain A Flat ribbon, and 3D surface), the catalytic domain, N-methylanthraniloyl-CoA binding tunnel, the rearrangement/cyclization of the substrates and the polyketide intermediate and end products

residues, respectively, in the CoA binding tunnel with Tyr-215 to control specific substrate entry (Matsui et al. 2017).

ADS and AQS Polyketides derivatives: 2-Alkylquinolone (2AQ) alkaloids are the main derivatives of the Alkyldiketide intermediates (Matsui et al. 2017). Likewise, the evocarpine (−methyl-2-(8Z)-8-tridecenyl-4(1H)-quinolinone) was previously reported as a 2-alkylquinolone alkaloid including a 2-tridecylidene group produced by the synergistic action of ADS and AQS in *Evodia rutaecarpa* (Abe 2020; Rebhun et al. 2015), in the same way as quinazolinocarboline alkaloids such as rutecarpine reported in *Tetradium ruticarpum* (Li et al. 2020a) and evodiamine in *E. rutaecarpa* (Rebhun et al. 2015).

(5) Quinolone Synthase (QNS)

The QNS identified from *Aegle marmelos* (Abe 2020; Wakimoto et al. 2012; Resmi et al. 2013; Matsui et al. 2017) is involved in the biosynthesis of the quinolone alkaloid group (Abe 2020; Shen et al. 2019). At the active site, the reaction is chained by a decarboxylative condensation of malonyl-CoA with N-methylanthraniloyl-CoA to form diketide 4-hydroxy-1-methyl-2H-quinolone intermediate (Fig. 7.22), which cyclize spontaneously by formation of amide to give 4-hydroxy2 (1H)-quinolone (Bisht et al. 2021; Abe 2020; Resmi et al. 2013). Structurally, two important amino acid residues (Ser132 and Ala133) have been shown to be essential for the functioning of the QNS active site (Resmi et al. 2013).

The catalytic efficiency of QNS is higher for acyl-coenzyme A substrates compared to smaller precursors (Bisht et al. 2021).

QNS-derived are predominantly represented by quinolones such as quinine, 2(1*H*)-quinolone, 4(1*H*)-quinolone, mefloquine, and casimiroine (Resmi et al. 2013) and are a group of anthranilic acid-derived alkaloids (Shi et al. 2008; Resmi et al. 2013; Ping 2016) exclusively restricted to Rutaceae plants. Moreover, dictamnine, skimmianine from *Skimmia japonica* (Resmi et al. 2013), acronycine in *Acronychia baueri* (Seneca 2007), melicopicine in *Melicope fareana* (Shi et al. 2008; Seneca 2007), and rutacridone in *Ruta graveolens* (Bisht et al. 2021; Shi et al. 2008; Seneca 2007) were reported as quinolone alkaloids.

(6) Pyrrolidine Ketide Synthase (PYKS)

In Solanaceae and Erythroxylaceae plants, the PYKS catalyzes a decarboxylative condensation of two molecules of malonyl-CoA to give diketide 4-carboxy-3-oxobutanoyl-CoA (Morita et al. 2019; Huang et al. 2019) (Table 7.1) covalently linked to the Cys166 catalyst (Morita et al. 2010a, 2019). Hydrolysis of the latter will take place with a non-enzymatic "Mannich type" condensation and directly utilizes N-methyl-Δ1-pyrrolinium cation as the starter substrate involving two rounds of malonyl-Coenzyme A mediated imine-ketide condensation (Bedewitz et al. 2018) where the amine and a carbonyl condensation is followed by a carbonation, which plays the role of the nucleophile in the nucleophilic addition to the compound formed, which is indispensable to tropane skeleton construction and generates racemic 4-(1-methyl-2-pyrrolidinyl)-3-oxobutanoic acid (Abe 2020; Huang et al. 2019; Bedewitz et al. 2018). Stereospecific oxidative cyclization reaction further occurs to form the tropinone. Highly homologous to CHS, this enzyme retains the Cys166-His305-Asn338 catalytic triad residues in the CoA binding tunnel in the same way as the other PKS III, with a particularity of having significantly smaller active-site cavity (Morita et al. 2019).

Pyrrolidine Ketide derivatives: the main derivatives of the PYKS synthesis process are the tropinone and tropane alkaloids (Abe 2020; Tsai and Ames 2009; Huang et al. 2019; Bedewitz et al. 2018), precursors of tropine, pseudotropine (Bedewitz et al. 2018), 2-carbomethoxytropinone, ecgonine, and ecgonidine in *Erythroxylum coca* (Seneca 2007; Huang et al. 2019; Bedewitz et al. 2018), hyoscyamine in *Hyoscyamus niger* (Shah et al. 2020), and atropine and scopolamine in *Atropa belladonna* (Passos and Mironidou-Tzouveleki 2016).

7.3 Biological Activities of Plant PK and Derivatives

The polyketides and derivatives are naturally produced by the plants in order to fulfill several adaptive roles (Stewart et al. 2013; Bhattacharya 2019), including protection against external aggressions such as UV radiation and light intensity. Correspondingly, they are involved in the architecture of plants, the color of flowers (Bisht et al. 2021; Geris et al. 2012; Wang et al. 2019a; Kontturi and Type 2017), the

development of pollen (Pothiraj et al. 2021), the nodulation of the roots (Xie et al. 2016), and mainly in the defense against the attacks induced by the other living organisms. Plant polyketides also contribute to the astringent and bitter flavors (Clark et al. 2013; Ley et al. 2005; Mhaske and Argade 2006) of many foods and drinks. However, the role of each chemical family of polyketides is revealed by the in-vivo functions of type III PKSs, this is therefore reflected in the diversity of medicinal properties of their products. From another point of view minor plant-derived flavonoids (Ramawat and Mérillon 2013), comprising chalcones, dihydrochalcones, aurones, and dihydroflavonols (or flavononols), may be considered as bioactive compounds, chemopreventive and anticancer agents (Christensen and Christensen 2013; Rammohan et al. 2020; Kumar and Pandey 2013; Jasim et al. 2021; Christensen 2018; Ramawat and Mérillon 2013; Ouyang et al. 2021; Xiao et al. 2008).

– *Chalcones, aurones, and their derivatives bioactivities*: Chalcones have received special consideration because of their possibilities for synthetic and biosynthetic production and also because of the extent of their biological activities (Christensen and Christensen 2013; Rammohan et al. 2020; Ouyang et al. 2021). Repeatedly reported to have a structural heterogeneity (Ouyang et al. 2021), and they act mainly as intermediates in the biosynthesis of flavonoids (Forkmann and Heller 1999; Liu et al. 2021). On the biological side, they act on various drug targets and can have anticancer effects (Rammohan et al. 2020; Jasim et al. 2021; Ouyang et al. 2021), anti-inflammatory (Rammohan et al. 2020; Jasim et al. 2021; Ramawat and Mérillon 2013), antidiabetic (Mahapatra et al. 2015), cancer chemopreventive (Orlikova et al. 2011; Rammohan et al. 2020; Ouyang et al. 2021), antioxidant (Gacche et al. 2008), antibiotic (Okolo et al. 2021; Xu et al. 2019), antileishmanial (Osman et al. 2022), and antimalarial (Sinha et al. 2019). Their anticancer effects are reported to actuate a selective inhibitory effect on cell cycle inducing apoptosis (Rammohan et al. 2020; Ouyang et al. 2021), thus they may be considered as chemopreventives. In-vitro bioassyas have proven that chalcones exhibit a broad spectrum of bio-activities, concluding their promising development as pharmaceuticals to be an efficient alternative to dreadful chemical treatments. Certain chalcones such as the chalcones, metochalcone and sofalcone, are already used clinically as choleretic and anti-ulcer agents (Ouyang et al. 2021). Khellin and dehydrokhellin derived from the seeds of *Ammi visnaga* had been proven phototoxic and vasorelaxant inhibitors of cAMP phosphodiesterase (Bisht et al. 2021). Besides, khellin and visnagin had been previously reported to have a pleiotropic effect on urolithiasis (Bhagavathula et al. 2015), vasodilating properties due to its calcium antagonistic activity inducing the inhibition of the contractile reactions intermediated by Ca^{2+} entry through L-type Ca^{2+} channels. In photochemotherapy, khellin is known for the treatment of vitiligo (Khalil et al. 2020; Chunyan et al. 2009; Bhagavathula et al. 2015). While visnagin present in *A. visnaga* could induce an anti-inflammatory response by down-regulation of the transcription factors AP-1 and NF-κB (Lee et al. 2010), it could decrease the

mRNA expression and the release of TNF-α, IL-1β, and IFNγ. Moreover, the LPS-induced IL-6 and MCP-1 mRNA levels were reduced considerably through the action of visnagin. Isoliquiritigenin (20,4-trihydroxychalcone) isolated from licorice roots acts on several types of cancers, including gastrointestinal and colon cancer, lung cancer, breast cancer, ovarian cancer, leukemia, and melanoma (Ouyang et al. 2021). This chalcone can suppress cancer cell proliferation and inhibit their migration by inducing apoptosis and autophagy (Chen et al. 2017; Lin et al. 2020). It also permits arresting the cell cycle, by inhibiting angiogenesis and metastases (Wang et al. 2019b). Similarly, butein derived from the bark of *R. verniciflua* exhibits anticancer activity by inducing anticarcinogenic action in non-small cell lung cancer (NSCLC) and apoptosis both in vivo and in vitro (Di et al. 2019; Zhou et al. 2016). Besides, Zhou et al. (2018) demonstrated the action of the butein on the G2/M phase inducing cell cycle arrest and inhibiting Aurora B and histone H3 phosphorylation in hepatocellular carcinoma (HCC). Additionally, butein has been shown to increase phosphorylation of ataxia telangiectasia mutated (ATM) and kinase-1/2 checkpoints (Chk1/2), thereby reducing cell division cycle 25C levels (cdc25C) in HCC (Moon et al. 2010). In organic chemistry, their chemical scaffold of chalcones formed of 1,3-diaryl-2-propene-1-one (as trans-(4) or cis-(5) isomers with two aromatic rings (A and B) joined by an unsaturated three-carbon α,β-carbonyl system) offers them the possibility of being modified in favor of a particular biological activity (Ouyang et al. 2021). Thus, the addition of various functional groups such as aryls, halogens, hydroxyls, carboxyls, phenyl, etc. may help to specify the molecular target by improving their positioning within the target active site (Ouyang et al. 2021; Gomes et al. 2017). Moreover, the chalcone moiety hybridizations with different anticancer or antibiotic pharmacophores could produce a new range of potentially active molecules acting as a solution to drug resistance and improving therapeutic specificity (Ouyang et al. 2021). Isobavachalcone is abundant in species belonging to the Fabaceae family (Rammohan et al. 2020; Jasim et al. 2021; Ramawat and Mérillon 2013; Ouyang et al. 2021). It is one of the most reputed chalcones due to its biological activity, mainly antitumor, stopping the proliferation of many cancerous cells. Its antiproliferative and proapoptotic/apoptotic activities are revealed in HCC by targeting the signaling pathway of extracellular signal-regulated (ERK)/ribosomal S6 kinase 2 (RSK2) kinases (Li et al. 2019b), and in colorectal cancer cells via inhibiting the kinase 3β AKT/GSK-3β/β-catenin pathway of cell proliferation (Li et al. 2019a). Scientific works have been able to reveal its apoptotic action mediated by ROS thioredoxin reductase 1 (TrxR1) in human prostate cancer (Li et al. 2018). The aurones [2-benzylidenebenzofuran-3 (2H)-ones] and benzofuranone (heterocyclic compounds containing phenyl group linked through a carbon–carbon exocyclic double bond) (Sui et al. 2021) are structural isomerides of flavones widely abundant in many species of the scrophulariaceae and compositae families (Kumar and Pandey 2013; Ramawat and Mérillon 2013), they have been reported pharmacologically active scaffolds being considered as antiviral (Haudecoeur et al. 2011), antibacterial (Olleik et al. 2019), antifungal, anti-inflammatory, antitumor, antimalarial, antioxidant,

neuropharmacological, etc. (Sui et al. 2021). Aureusidin was reported to act on thyroid hormones inducing the inhibition of iodothyronine deiodinase (Bisht et al. 2021). Aurones obtained by chemical synthesis were found as potential cancer chemotherapeutic agents (Bisht et al. 2021; Mazziotti et al. 2022; Sui et al. 2021). Molecularly, an antiproliferative effect is revealed through the inhibition of the cyclin-dependent kinases by binding with the nucleotide-binding domain of P-glycoprotein (Bisht et al. 2021; Mazziotti et al. 2022). Aurones characterized in *Uvaria hamiltonii* were reported to possess an anticancer activity via a tubulin-binding action (Bisht et al. 2021; Mazziotti et al. 2022). Hemmerling and Hahn (2016) have determined their inhibitory activity of ATP-dependent enzymes and proteins by mimicking the adenine of ATP, essential for the function of enzymes and receptors and an arrest of tumor growth such as arresting the growth of human chronic myeloid leukemia cells. Hence, scientific reports (Mazziotti et al. 2022; Sui et al. 2021; Haudecoeur et al. 2011; Olleik et al. 2019) have repeatedly indicated that aurone derivatives have interesting biological activities, namely antitumor, antioxidant/metal chelating, anti-tyrosinase, antibiotic, anti-inflammatory, and antidiabetic effects.

– Acridones and their alkaloid derivatives *bioactivities*: Acridones due to their electron-deficient tricyclic aromatic scaffold, which incorporates a π-conjugated electron (Chan et al. 2020), have a versatile range of bioactivities and unique optical biological properties. It has natural medicinal efficacy and finds various pharmacological applications. Glyfoline isolated from *Glycosmis citrifolia* has been shown to be a potent antineoplastic drug; it inhibits nucleoside transport and interferes with the G2/M phase of tumor cells (Wu et al. 2010). Acronycin, isolated from *Acronychia baueri*, interferes with DNA replication in tumor cells (Michael 2017; Chan et al. 2020), posing possible clinical integration as an antiproliferative therapeutic agent targeting tumor DNA. These properties of acridones open up prospects for synthesis/hemi-synthesis (by incorporating an alkyl chain in the N position, as with pyrimidocarvazones or pyrimidoacridone) in order to obtain several artificial derivatives of acridone with multiple biological actions such as antibacterial and anticancer abilities (Chan et al. 2020).
– *Valerophenones eriodictyol and homoeriodictyol bioactivities*: For the valerophenones, several compounds belonging to them are responsible for the bitter taste in several fruits and plants (Zhou et al. 2016; Clark et al. 2013). They have been reported to have a broad spectrum of biological activities where the adhumulone, cohumulone, humulone, isoadhumulone, and isohumulone isolated from *Humulus lupulus* (Cannabaceae) have been shown to protect against osteoporosis by possessing an anti-osteoporotic effect (Maurya et al. 2008). Thus, in clinical trials, they have been admitted in combination with a vehicle or a pharmaceutical excipient for the treatment of osteoporosis. They are also effective for the treatment of gynecological disorders (Maurya et al. 2008), prostate hypertrophy, and/or cancer such as the case of Xanthohumol (Venè et al. 2012). Certain valerophenones purified from the roots of *Nardostachys jatamansi* (Valerianaceae) as well as deoxymiroestrol isolated from the root of *Pueraria mirifica* (Leguminosae) inhibit bone sorption and hypercalcemia (Tauchen et al.

2020; Maurya et al. 2008). They had been clinically developed for the treatment of postmenopausal disorders of lipid metabolism, menopausal syndrome, and hypogonadism (Bisht et al. 2021; Zuiter and Zarqa 2014; Maurya et al. 2008). Phloroglucinol is potentially an inhibitor of endothelial progenitor cell (EPC) growth and may attenuate tumor growth and angiogenesis in Lewis lung carcinoma (LLC) (Orlikova et al. 2011). Phloroglucinol derivatives and acylphloroglucinol derivatives (i.e., hyperforin) had an antidepressant, antibacterial, and anti-inflammatory activities (Crockett et al. 2011, 2016). In another optic, xanthones (xanthones, vieillardixanthone, isocudraniaxanthone A, 1,3,7-trihydroxyxanthone, cheffouxanthone, smeathxanthone), benzophenones, and polyprenylated benzophenones have remarkable biological activities (Pasaribu et al. 2021). The addition of hydroxyl groups such as the case of hexahydroxybenzophenone and garcinol confers a significant antioxidant power (Mohamed and Ibrahim 2020). In addition, cytotoxic potency has been shown to be induced by xanthones (9-hydroxycalabaxanthone, macluraxanthone, garcinoxanthocins A and B), garcinol (14-deoxygarcinol, isogarcinol), and polyprenylated benzophenones (gutiferone E, guttiferone H, garcinol, and picrorhizone H) as well as garcibractatin A, forbesione, isoforbesione, and gambogic acid (Pasaribu et al. 2021). An antibiotic effect against *P. falciparum* was also noted by benzophenones where Mckeanianones A–C, bannaxanthones E and I, α-mangostin and β-mangostin, pancixanthones A and B and assiguxanthone A, subelliptenone were reported active against several bacterial strains (Pasaribu et al. 2021; Auranwiwat et al. 2016). In addition, eriodictyol and homoeriodictyol have a wide range of pharmacological activities (Deng et al. 2020a) and are involved in several biological treatment processes. Eriodictyol enables activation of B-cell lymphoma-2 (Bcl-2) and Bcl-2-related factor X (BAX) signaling pathway via caspase-3 signaling pathway thereby improving cardiomyocyte damage (Deng et al. 2020a; Xie et al. 2018). It also improves the dysfunction of mitochondria where it was reported as protective molecule of H9c2 cardiomyocytes against the injury induced by hypoxia/reoxygenation (Xie et al. 2018). In addition, its anti-inflammatory action is notable, due to the fact that it decreases the accumulation of NO following the activation of the joint of the C-sensitive protein, p38 and JNK2, which leads to a decrease in the factor of endothelial binding, and vascular stenosis (Deng et al. 2020a).

– *Stilbenoids and derivatives bioactivities*: Stilbenoids have attracted increasing interest especially after the discoveries of combretastatin and resveratrol which have shown clinical chemopreventive and anticancer proprieties (Ferrer et al. 1999; Christensen 2018; Xiao et al. 2008). Simple stilbenes including the methylenedioxy derivatives and glycosides (having oxygen functions on the aromatic rings) (Zuiter and Zarqa 2014; Xiao et al. 2008), prenylated and geranylated stilbenes (Pandey et al. 2018; Xiao et al. 2008), aryl benzofuran derivatives (Xiao et al. 2008; Xu et al. 2019), carbon substituted stilbenes with *C*-glycosides, and hybrid stilbenes had been reported to exhibit remarkable biological properties correlated with cancer prevention and amelioration of diabetes complications and nervous system disorders (Piekuś-Słomka et al. 2019).

Biphenyls and dibenzofurans have particular antimicrobial activities against *V. inaequalis* and *E. amylovora* only unveiled in few reports (Chizzali and Beerhues 2012). Aucuparin and eriobofuran have a remarkable antifungal effect (Sarkate et al. 2018) and an antibacterial effect against human pathogens such as *Pseudomonas syringae* and *S. aureus* (Chizzali and Beerhues 2012; Sarkate et al. 2018; Khalil 2013). Aucuparin, noreriobofuran, and some other biphenyls have interesting anti-inflammatory activity where they act at the molecular level on the production of inflammation agents induced by the N-formyl-methionyl-leucyl-phenylalanine (fMLP) of the superoxide anion, an agent inflammatory mediator produced by neutrophils (Khalil 2013). Achyrofuran, a prenylated dibenzofuran has antidiabetic properties (Carney et al. 2002), while biphenyl glycosides and dibenzofuran isolated from *Pyracantha fortuneana* fruit had tyrosinase inhibitory and neuroprotective activity (Chizzali and Beerhues 2012). Authors (Nandy and Dey 2020) have proven that the bibenzyls and bisbybenzyls of bryophytic origin (Bryophyta, Marchantiophyta, and Anthocerotophyta) and the Orchidaceae family could be considered as therapeutic molecules by revealing their pharmacology and their structure-activity. Their presence in bryophytes and Orchidaceae is at the origin of the medicinal interest related to their species. Besides, bambusifolol, 3-hydroxy-5-methoxy bibenzyl in *Eria bambusifolia*, bulbotetusine in *Bulbophyllum retusiusculum*, chrysotoxin in *Dendrobium pulchellum*, dendrosignatol in *Dendrobium signatum,* and dendrowillol A in *Dendrobium williamsonii* are cytotoxic compounds on human tumor lines (Nandy and Dey 2020). Brittonin A and B, chrysotobibenzyl and dihydroptychantol A, lunularin and Marchantin A have cytotoxicity, proapoptotic, antibiotic activity (Nandy and Dey 2020). Several chemical structures of bibenzyls and bisbibenzyls allow their classification into subtypes, namely cyclic structures found in *Marchantia emarginata* (Huang et al. 2010), macrocyclics such as those present in *Asterella angusta*, *Blasia pusilla*, and *Dumortiera augustan*, also chlorinated structures found in *Riccardia marginata*; and polychlorinated in *Riccardia polyclada* (Nandy and Dey 2020). They can also be prenylated like those present in *Radula perrottetii*, cinnamoylated bibenzyl derivatives revealed in *Polytrichum pallidisetum*, and geranylated bibenzyls in *Radula kojana*, and hydroxybenzyl in *Radula complanata* (Nandy and Dey 2020).

– *Naphthoquinone hexaketide, anthrones, and anthraquinones bioactivities*: Naphthoquinones are highly reactive organic compounds widely present in higher plants (Hook et al. 2014). Several scientific reports highlighted their multiple pharmaco-activities such as cytotoxic, antioxidant, anti-inflammatory, and antibacterial activities, etc. (Hook et al. 2014; Diaz-Muñoz et al. 2018; Martínez and Bermejo 2005; Ramos-Peralta et al. 2015; Wisintainer et al. 2014). Structurally assorted they may be present as simple 1,4-naphthoquinones, furan and pyran naphthoquinones, 1,2-naphthoquinones, naphthohydroquinones, and naphthoquinone polymers. Those diversified structures are responsible for a range of pharmacological effects including cytotoxic, antioxidative, anti-inflammatory, and antibacterial biological activities (Hook et al. 2014; Ramos-Peralta et al. 2015). Recently, Vukic et al. (2018) found that

naphthoquinones present in *Onosma visianii* root induce apoptosis and cell cycle arrest in HCT-116 and MDA-MB-231 cancer cell lines. Anthraquinones being considered as important active compound of rhubarb (Shi et al. 2008; Abe et al. 2004; Diaz-Muñoz et al. 2018; Lajis and Ahmad 2006) may be present as simple compounds mainly represented by rhein, emodin, aloe-emodin, chrysophanol, physcion, isoemodin, chrysaron, isoemodin, laccaic acid D, or as glycolyzed derivatives, such as aloe-emodin-8-glucoside, emodin-8-glucoside, rhein-8-glucoside, physcion diglucoside, emodin-6-glucoside, etc. (Cao et al. 2017). Biologically, the rhein was reported as renoprotective compound acting as inhibitor of renal fibrogenesis and nephropathy (Zhang et al. 2017) and presents strong inhibitory effect on several clinical anaerobes. Emodin has a blood pressure lowering potential (Lim et al. 2014) and regulates/improves microcirculation, alongside an hepatoprotective and renoprotection potential (Deng et al. 2020b). In addition, it was previously reported as antioxidant, anti-inflammatory, antitumor, and antimicrobial (Jan et al. 2021; Diaz-Muñoz et al. 2018; Martínez and Bermejo 2005; Lim et al. 2014; Deng et al. 2020b). Aloe-emodin has cardiovascular, hepatoprotective, and immune regulation activities, alongside antibacterial, antifungal, antiviral, anti-inflammatory, antitumor potentials (Bhattacharya 2019; Pandey et al. 2018; Martínez and Bermejo 2005; Seneca 2007; Chinchilla et al. 2013). Chrysophanol may act as antioxidant reducing the damage of oxygen free radicals to cells and has a neuroprotective effect (Zhao et al. 2016). Physcion induces an anti-inflammatory response after cerebral ischemia and neuroprotective effect through the reduction of nerve damage caused by reperfusion (Dong et al. 2021). Besides, physcion was previously reported as an apoptotic and inhibitor of tumoral cells proliferation when studied on a variety of carcinoma cells (Diaz-Muñoz et al. 2018; Martínez and Bermejo 2005; Chinchilla et al. 2013). On the other hand, anthraquinone glycosides were previously reported as laxative compounds, with other revealed bioactivities such as the antioxidant, anticancer, anti-inflammatory properties (Pandey et al. 2018; Martínez and Bermejo 2005).

– Chromones, Aloesones, and their heterocyclic derivatives: Chromone pentaketide (5,7-dihydroxy-2-methylchrome), chromone heptaketide, and their heterocyclic derivatives (with additional rings, e.g., furano-, pyrano- and oxepino-chromone glycosides) are a class of compounds with varied medicinal and pharmacological properties. For example, aloesin and its analogs, isolated from the genus Aloe, can treat skin hyperpigmentation by inhibiting the tyrosinase (Amen et al. 2021). In addition, certain chromones isolated from Aloe species such as 8-[C-β-D-[2-O-(E)-cinnamoyl]glucopyranosyl]-2-[(R)-2-hydroxypropyl]- 7-methoxy-5-methylchromone), which has shown potent topical anti-inflammatory activity (Amen et al. 2021). Macrolobin, isolated from *Macrolobium latifolium*, induces an inhibitory action on acetylcholinesterase (do Nascimento et al. 2020). Uncinosides A and B, isolated from *Selaginella uncinata*, have an anti-RSV (respiratory syncytial virus) effect (Ma et al. 2003). Heptaketide chromone aloesone derivatives, such as aloesone 7-O-β-d-glucopyranoside in rhubarb and anti-inflammatory aloesone 8-C-β-d-

glucopyranoside in Aloe (*A. arborescens*) (Amen et al. 2021). The heterocyclic chromone glycosides possessing a pyrano-, oxepino-, and pyrido-chromone glycosides were reported to be specifically present in *Saposhnikovia divaricate*, Eranthis species, and *Schumanniophyton magnificum* (Amen et al. 2021). The Biflorin isolated from the roots of *Capraria biflora* had been reported as cytotoxic/antiproliferative on CEM, HL-60, B16, HCT-8, and MCF-7 tumor cells with antimicrobial (against gram-positive) and antimutagenic effects (Lee et al. 2016; Wisintainer et al. 2014).

7.4 PK-Derived Products from Bioengineering to Industrial Applications

Due to their structural simplicity and manageable manipulation, polyketide synthases III (PKS III) in plants have attracted interest in new drug discovery in recent years, they were used in the production of ranges of natural and unnatural modified polyketides solving a variety of health problems (Bisht et al. 2021). With the development of biotechnology and emerged characterization tools, great progress has been made in recent years to optimize type III PKSs for high-level production of plant polyketides to expand their roles as platform chemicals. The functionality of PKSs in vitro (Jez et al. 2002; Wakimoto et al. 2012; Pang et al. 2021; Hertweck 2009) differs from those in vivo (Yu et al. 2012; Weissman 2009; Tsai and Ames 2009; Hertweck 2009), such that affinity to particular substrates changes and enzymatic reactions produce derailment by-products (Austin and Noel 2003; Watts et al. 2006; Austin et al. 2004a; Pandith et al. 2020) with different end result. Since the uses of plant polyketides for industrial or pharmacological applications are mainly limited by their availability and purification issues (Lussier et al. 2012; Gao et al. 2010), the bioengineers often design an artificial biosynthetic pathway nonstereospecifically in solution through several methods using model microorganisms in their favorable conditions by providing specific priming and starting substrates (Wakimoto et al. 2012; Lussier et al. 2012; Abe 2008; Abe et al. 2005; Pandey et al. 2018; Dunstan et al. 2020; Yang et al. 2021).

- Engineered microbes for the synthesis of PPKs: Researchers have developed techniques for reconstructing a plant biosynthetic pathway using microbes as heterologous hosts (Richardson and Khosla 1999; Lussier et al. 2012; Gayen et al. 2020) for polyketide biosynthesis. This environmentally friendly pattern is very competitive compared to biosynthesis in plants or by chemical methods (Lussier et al. 2012), having the advantage of relative speed of production of the target molecules as well as the availability of the substrates and their low cost. Microbial bioengineering techniques require the introduction of several heterologous genes into the host microorganisms and to ensure their stability (Lussier et al. 2012; Gao et al. 2010; Cummings et al. 2014; Kuzuyama and Seto 2012). The transcription and translation of genes will generate functional enzymes ensuring the in-vitro

biosynthesis of polyketides with more or less sufficient yields for commercial production (Lussier et al. 2012). As discussed previously, structural variation in plant-derived polyketides is related to the type and number of starter substrates relative to PKS specificity. This structural diversification will also be dependent on modifications induced by regiospecific condensation (Caldara-Festin et al. 2015; Morita et al. 2007; Kontturi and Type 2017), cyclization, aromatization, hydroxylation, methylation, acylation, prenylation, sulfation, and glycosylation reactions (Lussier et al. 2012).

This bioengineering approach implements new metabolic pathways for the synthesis of polyketides in the host organism in order to improve its productivity. Conducted mainly in two genetically and biochemically well-characterized microorganisms (eukaryotes), namely: *Escherichia coli* and *Saccharomyces cerevisiae*, due to their easy culture and manipulation (Richardson and Khosla 1999; Wakimoto et al. 2012; Lussier et al. 2012; Abe et al. 2005; Gao et al. 2010). *S. cerevisiae* is, however, favored due to possible reconstruction and compartments similar to plant cells (Lussier et al. 2012). Similarly, the eukaryotic cellular environment is also more suitable for the expression of functional membrane proteins, such as cytochromes P450 (Lussier et al. 2012). In fact, the majority of scientific work directed toward the engineering of plant polyketides in microbes is much more focused on CHS (Wakimoto et al. 2012; Abe et al. 2005) and STS (Bisht et al. 2021; Wang et al. 2020), along with some works on curcuminoid synthase (Zhang et al. 2016; Gao et al. 2010) and tetraketide synthase (Abe 2008; Tahir et al. 2021; Fujii 1999). The used microorganisms are originally producers of the elongation molecule (malonyl-CoA), but with regard to the CoA-ester starter molecules necessary for the synthesis of polyketides, supplementation in the growth medium may be established as an important step in the microbial engineering process (Lussier et al. 2012; Cummings et al. 2014; Katsuyama and Horinouchi 2010).

- *Co-expression of specific biosynthetic component*: Several polyketides and derivatives have been obtained in the laboratory by combining microbial bioengineering techniques by the co-expression of certain microorganisms/plants specific enzymes within a host microbe, usually *E. coli* and/or *S. cerevisiae*. For example, naringenin and pinocembrin could be obtained at the level of *E. coli* by promoting the expression of CHS from the plant *Glycyrrhiza echinata* (Lussier et al. 2012; Ralston et al. 2005; Gao et al. 2010), where the CHS was able to use both cinnamoyl-CoA and p-coumaroyl-CoA as starters in the culture medium. Thus, variable yields could be recorded at the end of the bacterial growth cycle (Lussier et al. 2012). Xiao et al. (2008) have also been able to introduce a chalcone isomerase from *Pueraria lobata* to catalyze the isomerization of chalcone naringenin into naringenin. Similarly, authors (Jiang et al. 2005) have been able to implement the phenylalanine ammonia lyase (PAL) of the red yeast *Rhodosporidium toruloides* and 4-coumarate-CoA ligase (4CL) of *Arabidopsis thaliana* to produce naringenin and pinocembrin with relatively high yields by varying the growth conditions and concentrations of the starter substrates mainly tyrosine and phenylalanine. Likewise, Yan et al. (2005) were able to use four

genes derived from plants, namely cinnamate4-hydroxylase (C4H) from *Arabidopsis thaliana*, 4CL from *Petroselinum crispum*, CHS and chalcone isomerase (CHI) from *Petunia* × *hybrida*. The authors carried out the supplementation in variable substrates using trans-cinnamic acid, p-coumaric acid, caffeic acid, and ferulic acid, to obtain yields of pinocembrin (16.3 $mg.L^{-1}$), naringenin (28.3 $mg.L^{-1}$), eriodictyol (6.5 $mg.L^{-1}$), respectively, but not homoeriodictyol (Lussier et al. 2012; Yan et al. 2005). Nevertheless, it was found that when trans-cinnamic acid was the precursor low yields of naringenin were recorded indicating that C4H was a rate-limiting step. Moreover, Leonard et al. (2005) have been able to introduce other genes such as flavone synthases (FSI and FSII) into strains harboring C4H, 4CL, CHS, and CHI to obtain flavones and flavonoids. In the same vein, the authors were able to characterize the production of flavones (apigenin and luteolin) in *E. coli* from the precursor phenylpropanoid acid. For this purpose, the Leonard et al. (2006) used 4CL and FSI from *Petroselinum crispum*, CHS and CHI from *Petunia x hybrida* and 7-O-methyltransferase (OMT) from *Mentha x piperita*. The authors (Miyahisa et al. 2005; Leonard et al. 2005) were able to demonstrate that the availability of the malonyl-CoA elongation molecule in host microorganisms could be the cause of the low yield of polyketides produced by bacterial engineering techniques. Therefore, different strategies have been implemented to increase the intracellular abundancy of malonyl-CoA in *E. coli* such as the overexpression of four ACC subunits (Lussier et al. 2012; Chan et al. 2009) of the bacterium dependent on biotin to lead to an increase in the production of flavones, in particular the pinocembrin. On the other hand, if biotin ligase (BL) (Lussier et al. 2012; Gao et al. 2010) is combined in co-expression with ACC, the polyketides yield increases considerably. In another approach, supplementation of acetate and glucose in the culture medium of host organisms could improve the production of malonyl-CoA following co-expression of acetyl-CoA synthetase (ACS) and ACC in *E. coli*, with this approach the authors were able to increase yields of pinocembrin, naringenin, and eriodictyol (Lussier et al. 2012).

Overall, the integrated strategies for the purpose of polyketide production required precursor supplementation (Lussier et al. 2012; Abe et al. 2005; Gao et al. 2010; Pyne et al. 2019). Scientists (Santos et al. 2011) were able to design combinatorial pathways at the host organism level consisting of the chained expression of TAL, 4CL, CHS, CHI in the host strain *E. coli*. First, the production of tyrosine was a key factor in determining the production of naringenin directly from glucose (Lussier et al. 2012). Then, it was reported that the important precursors managing the polyketide production pathway were either p-coumaric acid or tyrosine, for this, TAL from the yeast *Rhodotorula glutinis* (RgTAL), 4CL from *Petroselinum crispum*, CHS from *Petunia hybrida* and CHI from *Medicago sativa* have made it possible to provide these two precursors considerably for the initiation of elongation reactions in *E. coli* without recourse for a precursor supplementation (Santos et al. 2011).

Additionally, in the case of Stilbene synthases, authors Becker et al. (2003) were the pioneers of the mechanism of resveratrol production in a modified

microorganism. The strain *S. cerevisiae* was used as a host to produce resveratrol from p-coumaric acid provided by co-expressing 4CL from *Populus trichocarpa* × *Populus deltoides* and STS from *Vitis vinifera*. Studies were then carried out to maximize the yields of end products, thus reducing the steps necessary to obtain it (Lussier et al. 2012). A fusion of the 4CL of *A. thaliana* and STS from *V. vinifera* increased considerably the production of resveratrol up to 15 times compared to the co-expression of 4CL and STS (Zhang et al. 2006). Similarly, in *E. coli*, the co-expression of 4CL from *Nicotiana tabacum* and STS from *V. vinifera* or *Arachis hypogaea* has made it possible to obtain considerable yields of resveratrol (Beekwilder et al. 2006). Other enzymes were subsequently solicited such as the integration of PAL and a CPR of *Populus trichocarpa x P. deltoides* and a C4H of *Glycine max* by supplementing the medium with phenylalanine in *S. cerevisiae* to produce resveratrol (Trantas et al. 2009). So, the majority of works of microbial synthesis of resveratrol have either oriented the co-expression of various enzymes or modified the availability of starting substrates in the synthetic culture medium (Lussier et al. 2012). Subsequently, for the case of curcuminoid synthase involved in the formation of curcuminoids and diarylheptanoids, the production of bisdemethoxycurcumin in *E. coli* is obtained by the expression of rice CUS (*Oryza sativa*) using two molecules of p-coumaroyl-CoA and one molecule of malonyl-CoA (Katsuyama et al. 2007). This enzyme can also catalyze the synthesis of dicinnamoylmethane and curcumin from cinnamoyl-CoA and feruloyl-CoA as starting substrates. In addition, bisdemethoxycurcumin, dicinnamoylmethane, and cinnamoyl-p-coumaroylmethane have been reported to be produced in *E. coli* by the co-expression of PAL from *R. rubra* and a 4CL from *Lithospermum erythrorhizon* and CUS from *O. sativa* using tyrosine and/or phenylalanine (Lussier et al. 2012).

Following the multiple research works going in this direction, the variation of the polyketides produced in vitro by microbial engineering is directed by the variation of the enzymes in co-expression and the starting substrates supplemented directly in the medium or as precursors resulting from the synthesis of other enzymes.

- *Action of PKS substrate's variation:* Several studies have shown that CHS, STS, and VPS can effectively utilize various starting CoA-linked thioesters including acetyl-CoA, methylmalonyl-CoA, cinnamoyl-CoA, caffeoyl-CoA, butyryl-CoA, isovaleryl-CoA, hexanoyl-CoA, benzoyl-CoA, and phenylacetyl-CoA (Bisht et al. 2021; Abe 2020; Morita et al. 2010a; Schröder et al. 1998). For example, benzalacetone, bisnoryangonine, and *p*-coumaroyltriacetic acid lactone are reaction by-products of CHS, STS, and STCS using *p*-coumaroyl-CoA as a starter (Bisht et al. 2021; Morita et al. 2010b; Shimokawa et al. 2012). In-vitro engineering (Bisht et al. 2021; Abe 2008; Imaizumi et al. 2020) demonstrated that the CHS possesses large active-site cavity, wide entrance to the active site, and deep binding tunnel (Jez et al. 2002; Morita et al. 2010b; Pandith et al. 2020) that result in a multifunctional activity and broad substrate specificity with remarkable affinity to a series of starters. A variety of non-physiological substrate analogs

(Yu and Jez 2008; Shi et al. 2008; Wakimoto et al. 2012; Taura et al. 2016; Jindaprasert et al. 2008) have been experimentally fixed with CHS to produce a series of unnatural polyketides. Analogs of coumaroyl-CoA where the coumaroyl aromatic ring is substituted by a furan or thiophene ring (Shi et al. 2008) had been converted to new unnatural polyketides. This property that type III PKSs has of being used as structural analogs to the starting substrate is put to use by biochemists in order to diversify the resulting products. In addition, multiple scientific works have emphasized this property to vary the synthetic products by varying the starting substrates. It was noted that the CHS also accepts aliphatic-CoA starters such as isovaleryl, isobutyryl, n-hexanoyl, n-octanoyl, n-decanoyl, and n-dodecanoyl (Lussier et al. 2012; Pandith et al. 2016; Shimizu et al. 2017; Funa et al. 2007; Mizuuchi et al. 2009) to produce triketide and tetraketide lactones in the presence or absence of tetraketide phloroglucinols. Also, this enzyme accepts benzoyl-CoA to produce phlorobenzophenone (2,4,6-trihydroxybenzophenone) (Shi et al. 2008), cinnamoyl-CoA to produce deoxychalcone (Dao et al. 2011; Schro 1999) (pinocembrin chalcone), and phenylacetyl-CoA to provide phlorobenzylketone (2,4,6-trihydroxyphenylbenzyl ketone) (Shi et al. 2008; Pandith et al. 2020). Halogenated structural analogs of cinnamoyl-CoA and p-coumaroyl-CoA (Morita et al. 2010a; Stewart et al. 2013) as well as analogs in which the coumaroyl moiety was replaced by furan or thiophene (Shi et al. 2008; Flores-Sanchez and Verpoorte 2009) were accepted as starter substrates in order to produce new polyketides by an STS. This enzyme also demonstrates a various accommodation to different starters commonly associated with different models of PK synthesis, in order to produce chalcones, benzophenones, and phloroglucinols and many other products (Flores-Sanchez and Verpoorte 2009; Lussier et al. 2012; Abe et al. 2005). For example, a variety of cyclization reactions offer a variety of chalcone, this is possible by varying in-vitro the starting molecule (p-coumaroyl-CoA) in alfalfa CHS by other substrates such as feruloyl-CoA, hexanoyl-CoA, phenylacetyl-CoA, benzoyl-CoA, butyryl-CoA, isobutyryl-CoA, and isovaleryl-CoA (Jez et al. 2002; Dao et al. 2011) to generate corresponding chalcone products, chalcone analogs, tetraketide lactone and triketide lactone, phlorobenzyl ketone methylpyrone (Dao et al. 2011). Furthermore, in-vitro experiments demonstrated that VPS and CHS also synthesize analogs of 4-hydroxy-6-methyl-2-pyrone (triacetic acid lactone), 4-hydroxy-6-isobutyl-2-pyrone, and 4-hydroxy-6-isopropyl-2-pyrone (Zhou et al. 2016), using isobutyl or isopropyl at position 6 with isovaleryl-CoA or isobutyl-CoA as starting unit and malonyl-CoA as starting unit ‘extension.

7.5 Conclusion

Plant polyketides are considered as valuable metabolites for several research and industrial areas. All of the studies targeting their understanding provide fundamental knowledge on the mechanisms and regulations involved in their synthesis and derivatization. They emphasize their chemical structures and molecular synthesis mechanisms highlighting the specificity, mechanical aspects, and functional structure of the responsible enzymes of their synthesis. Polyketides as natural compounds correspond to a wide range of chemical structures and are characterized by an unequal qualitative and quantitative distribution according to species but also organs, tissues, and physiological stages. The characterization of the polyketide synthase enzymes equipped to create polyketide chemodiversity, in particular the functionally related PKSs III from plants, would be advantageous for better understanding the biosynthesis of the different chemical families derived from the plant species. In particular, scientific studies have been able to prove that the functional diversity of plant-PKS III evolves continuously due to the steric modulations of the chemically inert residues of the active-site cavity, while preserving the main characteristics of the catalytic site. This evolution is, however, taken advantage of by bioengineering applications where the enzymes belonging to this category will be oriented toward the biosynthesis of molecules with multiple interests. As such, PKS IIIs offer various possibilities for artificially biosynthesizing pharmaceutically important plant polyketides to be introduced into the pharmaceutical and dermocosmetic industry. On the other hand, despite the promising chemical nature of these molecules, their industrial applications remain lacking. This encourages studies to concentrate more on the development of these molecules toward a framed introduction in the industrial field while controlling their large-scale production. Besides, the current and future importance of natural plant polyketides will depend on their experimental, structural study and on their biological activities developed in laboratories. Their implementation is a way to achieve turnover in several industrial fields led by pharmaceutical companies setting up bioprospecting and extensive screening programs for the main bioactive polyketides. It will however be possible to develop a virtual screening approach, to reposition known molecules and to practice the natural fragment approach for the valorization of several families of characterized and purified polyketides. Recent metabolomics techniques and in particular bioengineering techniques also offer promising prospects. Plants therefore still present a promising future as a source of active molecules for human health despite the intrinsic technical difficulties and the complexity of recent regulations related to access to genetic resources and benefit-sharing.

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Chapter 8
Plant Tocopherols and Phytosterols and Their Bioactive Properties

Pradip Poudel, Spyridon A. Petropoulos, and Francesco Di Gioia

Abstract Tocopherols and phytosterols are plant-derived fat-soluble bioactive compounds with several health beneficial properties. Tocopherols are mainly known for their antioxidant activity, while phytosterols are well known for their capacity to lower blood cholesterol levels in the human body. Plants produce these compounds for their own protection against oxidative damages and to maintain cell integrity. Including plant-based food in the daily meal plan, especially vegetable oil, fresh vegetables, nuts, and fruits, helps to fulfill our dietary needs for tocopherols and phytosterols. After briefly describing the biochemistry, biosynthesis and the important role these two categories of compounds play in the plant physiology, this chapter provides an overview of (i) the primary plant sources of tocopherols and phytosterols, summarizing some of the factors that determine their concentration in plants; and (ii) the main health-promoting effects that have been reported recently for both categories of bioactive compounds. While more research is needed to unravel the health effects of tocopherols and phytosterols, additional research effort is needed to identify alternative low-cost sources of these valuable compounds, using, for example, by-products and waste of the agri-food industry. Future research should also focus on the development of functional food products employing sustainable biofortification techniques that may allow to enhance the content and bioavailability of tocopherols and phytosterols in commonly consumed plant and plant-derived food products.

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8.1 Introduction

Tocopherols and phytosterols are fat-soluble secondary metabolites of plant origin increasingly studied for their health beneficial properties. These compounds are generally extracted from the unsaponifiable lipid fraction of plant-based food (Ryan et al. 2007). As tocopherols and phytosterols are not synthesized in the animal body, humans fully rely on plant-based food for their daily dietary intake of these compounds. Vegetable and edible oils are particularly rich in tocopherols and phytosterols; however, they can also be found in significant amounts in nuts, fresh vegetables, and fruits. Both compounds have strong antioxidant activity because of the phenolic head and electron-donating methyl and ethyl group attached to it (Lesma et al. 2018; Wallert et al. 2019). They can scavenge free radicals through breaking the oxidation chain reaction. Further, they can protect from lipid peroxidation, oxidative stress, cancer, and neurogenerative diseases. In addition, phytosterols are mainly known for the cholesterol-lowering ability in the blood plasma, thus preventing obesity and cardiovascular diseases (Poli et al. 2021). Including around 2–3 g of plant sterols in the daily diet could decrease low-density lipoproteins (LDL) levels by 10–15% and reduce the chance of coronary heart disease over the lifetime by 20% (MacKay and Jones 2011; Chawla et al. 2016; Yang et al. 2019). Similarly, a daily intake of 15 mg of α-tocopherol is recommended by the Institute of Medicine (US) Panel on Dietary Antioxidants and Related Compounds (2000).

Tocopherols are the forms of a molecule that comprise vitamin E. Vitamin E includes eight different forms of a molecule (α-, β-, γ-, and δ-), among which four are tocopherols and other four are tocotrienols (Niki and Abe 2019). The basic tocopherols structural units contain a chromanol ring and a hydrophobic carbon chain (16 C) attached to C2 position (Niki and Abe 2019; Ali et al. 2022). The main chemical difference among α, β, γ, and δ tocopherol is the number and position of the methyl group in the chromanol rings (Fig. 8.1). Alpha forms of tocopherols contain three methyl groups at C5, C7, and C8 position, while β-tocopherols contains only two methyl group at C5 and C8 position. Similarly, γ-tocopherols also contain two methyl groups at C7 and C8 positions, while δ-tocopherols only

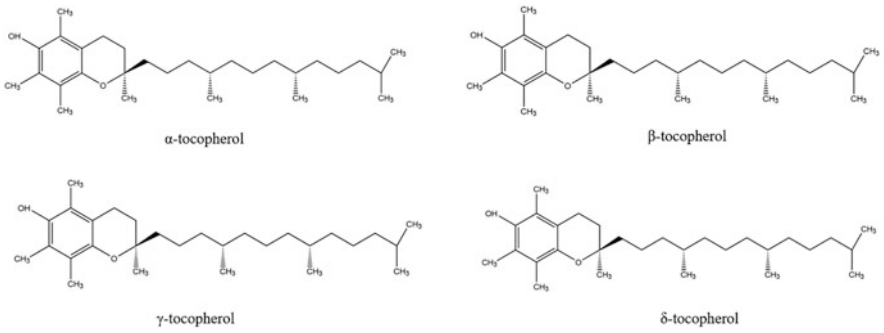

Fig. 8.1 Chemical structure of tocopherol isomers

contain one methyl group at C8 position of the chromanol ring. Because of the presence of the phenyl and electron-donating methyl group, tocopherols (vitamin E) are mainly known for their neutralizing role in lipid peroxidation and oxidative stress (Frankel 1989; Wallert et al. 2019; Bora et al. 2022). These structural differences between the different forms of tocopherols are responsible for their varying antioxidant and biological activities (Bora et al. 2022).

Among the four different isomers of tocopherol, α-tocopherol is the most abundant one based on their presence in different plants and plant-based food products. Comparatively to the other forms, α-tocopherol has higher biological activity as it is retained at high levels in plasma and body tissues (Szewczyk et al. 2021), due to the active selection by the α-tocopherol transfer protein (α-TTP) and slow degradation by the cytochrome P450. In contrast the other isomers of tocopherols are regarded as xenobiotics and are actively degenerated by the cytochrome P450 and secreted through bile and urine (Azzi 2018). This higher biological activity makes α-tocopherol the most important tocopherol and this is why it is generally recommended or referred to as vitamin E, and tocopherol level is expressed or measured in the level of α-tocopherol and α-tocopherol equivalent (EFSA Panel on Dietetic Products, Nutrition, and Allergies (NDA) 2015).

Phytosterols are another important class of bioactive compounds widely studied because of their cholesterol-lowering activity in the human body. Phytosterols comprise plant sterols and stanol naturally found in the plant cell membrane. Plant stanol and sterols have similar chemical structures, with the exception that stanols do not have a double bond in their chemical structure (Chawla et al. 2016). Hydrogenation of plant sterols results in the respective plant stanol; for example, hydrogenation of β-sitosterol results in β-sitostanol. The main function of stanols in plants is the formation of cell membrane structures. There are around two hundred different phytosterols reported, however, major phytosterols found in different sources are β-sitosterol, campesterol, and stigmasterol (Lagarda et al. 2006; Wang et al. 2018). Phytosterols have a similar structure to the cholesterol, however, they have one extra methyl or ethyl group at C24 position of the sidechain (Chawla et al. 2016). The chemical structures of the most common phytosterols (β-sitosterol, campesterol, and stigmasterol) found in plants are shown in Fig. 8.2.

Phytosterols are synthesized mainly in plants and marine animals but cannot be synthesized in the human body. Phytosterols can be found in different forms in plants, for example, free phytosterol, esterified with a fatty acid, steryl glycosides, and acylated glycosides (Yang et al. 2019). The structure and different forms of the phytosterols affect the biological activity, including their cholesterol-lowering capacity and antioxidant activity (Wang et al. 2018). Around 50% of the dietary intake of phytosterols includes β-sitosterol, however, generally, campesterol concentration is higher in blood possibly due to higher absorption in the intestine (Schött et al. 2017; Wang et al. 2018). Plant oils, vegetables, and nuts are rich sources of phytosterols; therefore, great importance is given to include these plant products in our daily diet.

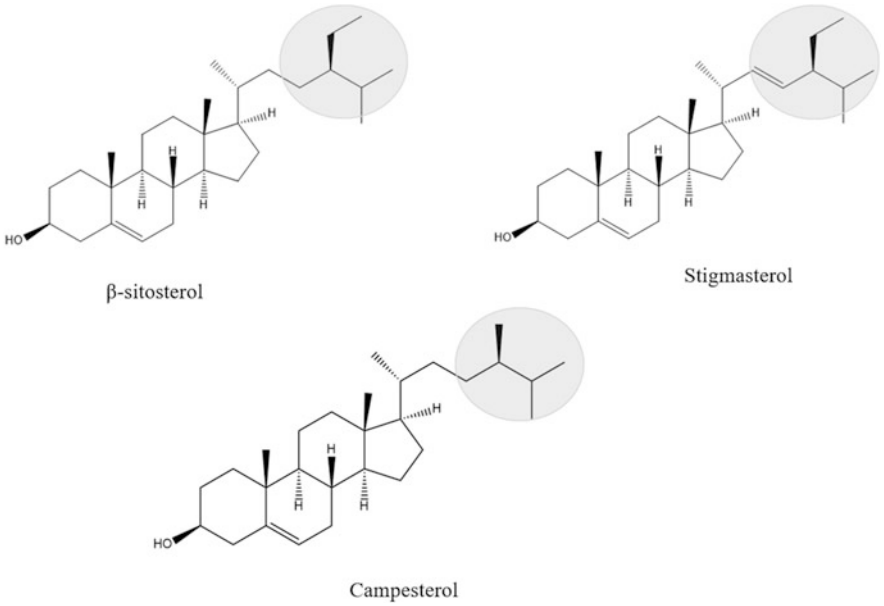

Fig. 8.2 Chemical structures of β-sitosterol, stigmasterol and campesterol

8.2 Biosynthesis and Physiological Role in Plants

The biosynthesis of tocopherols in plants involves different pathways: cytosolic shikimate, plastid methylerythritol phosphate and tocopherol-core pathways as shown in Fig. 8.3. Tocopherols derive from two precursors: 2,5-dihydroxyphenylacetate (HGA) and phytyldiphosphate (PDP) which are derived from two different pathways. The HGA forms the aromatic head of the tocopherols and is derived from the cytosolic shikimate pathway, while PDP forms the hydrophobic carbon chain of the tocopherols, and is derived from the plastid methylerythritol phosphate pathway (Lushchak and Semchuk 2012; Vinutha et al. 2017). HGA and PDP condensation is the following step forming 2-Methyl-6-phytyl-1,4-benzoquinone (MPBQ), which is catalyzed by the homogentisate phytyl transferase (VTE2). This marks the starting of the tocopherol-core pathway. Tocopherol cyclase (VTE1) converts the MPBQ to the δ-tocopherol, and from δ-tocopherol to the β-tocopherol via γ-tocopherol methyl transferase (VTE4). On the other hand, the formation of the 2,3-dimethyl-5-phytylbenzoquinonol (DMPBQ) is facilitated by the MPBQ/MSBQ methyltransferase (VTE3), and from DMPBQ to γ-tocopherol by the VTE1. Gamma-tocopherol is converted into α-tocopherol by the VTE4 (Lushchak and Semchuk 2012; Vinutha et al. 2017; Kusajima et al. 2021; Ali et al. 2022).

Tocopherols play an important role in plants both during stress and non-stress conditions. They help to maintain the integrity and fluidity of the photosynthetic cell

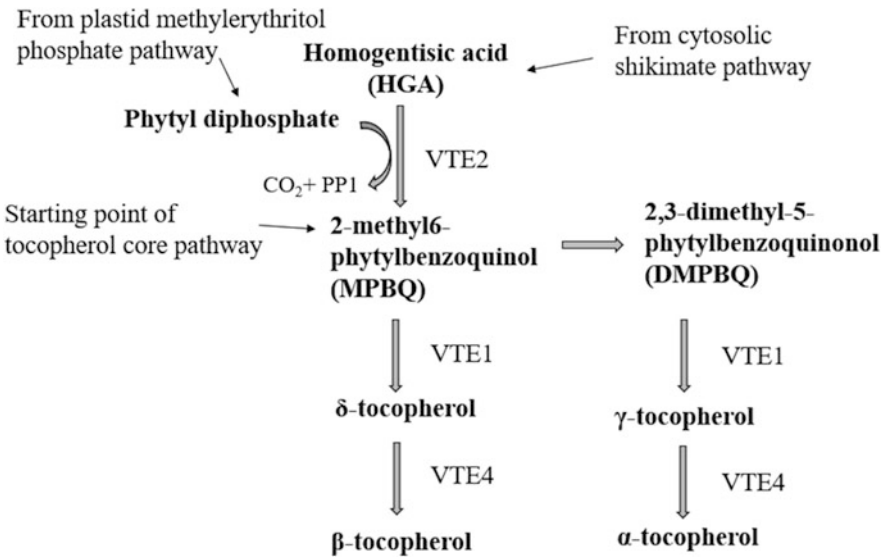

Fig. 8.3 Biosynthetic pathway of tocopherols in plants. VTE1, tocopherol cyclase; VTE2, homogentisate phytyltransferase; VTE3, MPBQ/MSBQ methyltransferase; VTE4, γ-tocopherol methyl transferase

membrane throughout the plant life cycle through its free radical quenching capacity (Sadiq et al. 2019). Biosynthesis of tocopherols may vary throughout the plant life cycle based on growth and development stages. Plants produce tocopherols in presence of various biotic and abiotic stress conditions, as a mechanism of self-protection from oxidative damages. For example, Kusajima et al. (2021) reported an increase in tocopherols concentration in *Arabidopsis thaliana* plants after the application of heat shock, through the activation of the corresponding biosynthetic pathways. Similarly, tocopherols level increased in *A. thaliana* when plants were subjected to drought stress by overexpressing VTE1 ((Liu et al. 2008). Plant tocopherols levels could also increase during other environmental stress conditions determined by high light levels, salinity stress, heavy metal ion, ozone, and UV-B radiation as they play a critical role in protecting plants from oxidative damages (Lushchak and Semchuk 2012). Stahl et al. (2019) reported an increased expression of genes involved in the tocopherol's biosynthesis and increased concentration of γ-tocopherol and δ-tocopherol when they inoculated *A. thaliana* leaves with *Pseudomonas syringae*. Another important example of the antioxidant function of tocopherols in plants is given by the presence of α-tocopherol in the leaf chloroplasts. Alpha-tocopherols present in leaf chloroplasts trap the reactive oxygen species (ROS) produced during photosynthesis and further prevent the lipid peroxidation in thylakoid membranes by scavenging lipid peroxyl radicals (Munné-Bosch 2005). Other than its antioxidant role in plants, tocopherols also play a role in plant cell signaling activities. Munné-Bosch (2019) have discussed the stress sensing and signaling activities of tocopherols. Tocopherols signal the accumulation of

3′-phosphoadenosine 5′-phosphate in chloroplasts which helps preventing the degradation of primary messenger RNA and promotes the production of mature messenger RNA in nucleus. According to Munné-Bosch (2019), the mechanism behind the modulation of 3′-phosphoadenosine 5′-phosphate by tocopherols is still unknown. Further, tocopherols present in vegetable oils, exert their anti-oxidation function protecting the oil from oxidation processes, thus contributing to increase the stability and shelf-life of the oil (Kamal-Eldin 2006; Mishra et al. 2021).

A recent study revealed that exogenous application of tocopherols in wheat via foliar application not only enhanced wheat tolerance to drought stress but also increased plant growth, yield, seed phenolics and flavonoids content, activity of antioxidant enzymes, and content of α-, β- and γ-tocopherols (Ali et al. 2019). Similarly, seed priming of carrots with α-tocopherol increased growth parameters, yield, chlorophyll, proline, ascorbic acid and total phenols content, and also the antioxidant activity in carrots (Hameed et al. 2021). This also suggests a potential application of tocopherols for the potential agronomic biofortification of food crops.

The biosynthesis of plant sterols is a multi-stage complex process, which mostly occurs in the endoplasmic reticulum. A simplified biosynthesis pathway of common phytosterols in plant is presented in Fig. 8.4. The biosynthesis of the plant sterols starts with acetyl-CoA, which later converts into the squalene through mevalonate (MVA) pathway (Zhang et al. 2020). It involves an important enzyme, HMGR (3-hydroxy-3-methylglutaryl-CoA reductase), which catalyzes the conversion of 3-hydroxy-3-methylglutaryl-CoA to mevalonic acid (Valitova et al. 2016). The following process is the formation of cycloartenol through the cyclization of squalene facilitated by the squalene epoxidase and cycloartenol synthase, respectively. C24 methylation of the cycloartenol is the other important process of sterol formation which is catalyzed by the SMT (C24-sterol methyltransferase). SMT has two different forms, SMT1 and SMT2, which are involved in primary and secondary methylation activities. SMT1, SMT2, and DWF1 (Dimunito/Dwarf1) catalyze the synthesis of campesterol and β-sitosterol from the cycloartenol as shown in Fig. 8.4. Conversion of the β-sitosterol to the stigmasterol is facilitated by the enzyme protein CYP710A1. Plant sterols concentration and their composition differ in different plant species, and is potentially modulated by the enzymes SMT and CYP710A (Zhang et al. 2020).

An important function of phytosterols in plants is to maintain the cell membrane integrity and fluidity (Grosjean et al. 2015). The importance of phytosterols becomes even more critical for plants during stress conditions. Plants respond to stress through different biochemical and physiological changes, which includes the increased production of phytosterols. Kumar et al. (2018) reported an increase in the sterol and steryl ester concentration in drought-stressed plants. The importance of phytosterol in seeds for storage and germination was discussed by Zhou et al. (2019). Yu et al. (2021) have reported reduced lipids droplets in seeds with lower phytosterol levels which are critical for energy storage and lipid metabolism in seeds. This suggests an important role of sterols in seed growth and development. Similarly, excess phytosterol accumulation in seeds delayed the seed germination due to thicker seed coats and irregular seed coat formation in *Arabidopsis thaliana*

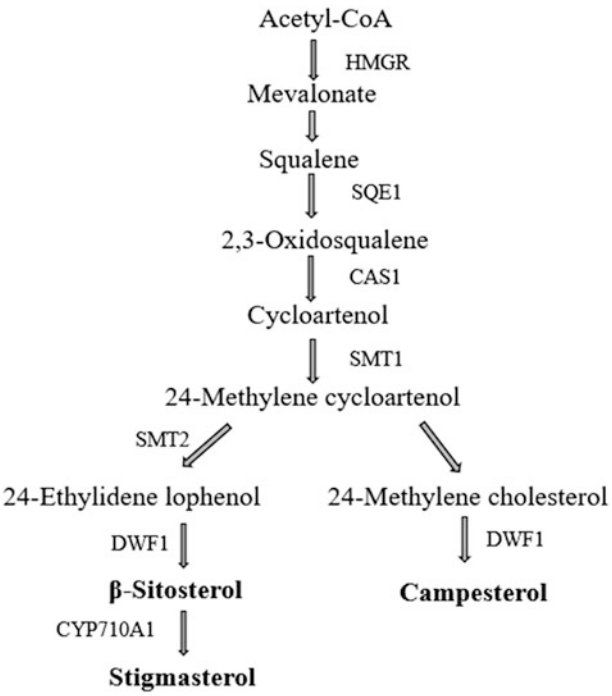

Fig. 8.4 Simplified biosynthesis pathway of plant sterols. HMGR, 3-hydroxy-3-methylglutaryl-CoA reductase; SQE, squalene epoxidase; CAS, cycloartenol synthase; SMT, C24-sterol methyltransferase; DWF, Dimunito/Dwarf1; CYP710A, cytochrome P710 A

(Shimada et al. 2021). Further, the effect of phytosterol composition on cotton fiber length and secondary cell wall deposition was studied by Niu et al. (2019) who found a decrease in fiber cell length and promotion of secondary cell wall formation in cotton with high sitosterol and a low ratio of campesterol to sitosterol. Phytosterol also helps to promote plant innate immunity against biotic stress factors. Wang et al. (2012) reported an increased resistance of the plant to external pathogens by restricting the nutrient flow to the apoplast. Maintenance of cell membrane integrity and fluidity contributes to protect plants from external stress. Other studies have also shown the potential role phytosterols plays on plant reproductive growth, seed formation, and ultimately in determining crop yield (Du et al. 2022).

8.3 Tocopherols and Phytosterol Content in Plants

Plant-derived products and foods are the major sources of tocopherols and phytosterols required for the human body, as both classes of compounds are not synthesized de novo in the human body (Chen et al. 2009; Chawla et al. 2016; Azzi 2018).

Interconversion of different forms of tocopherols through methylation and demethylation also does not take place inside the human body (Azzi 2018; Bora et al. 2022). Tocopherols are produced exclusively by photosynthetic organisms like plants, algae, and cyanobacteria, while major sources of tocopherols and phytosterols are seeds of oleaginous crops such as sunflower and canola, soybean seeds, nuts, leafy vegetables, and some fruits.

The amounts and types of tocopherols intake throughout the world are different and are influenced by different dietary habits. For example, around 70% of the vitamin E uptake from food sources in the USA is in the form of γ-tocopherol due to the consumption of high proportions of soybean and other vegetable oils like canola oil rich in γ-tocopherol (Dietrich et al. 2006). Likewise, the Mediterranean diet which is regarded as one of the healthiest dietary habits includes olive oil (extra-virgin olive oil) as an important ingredient that is rich in α-tocopherol (Shahidi and De Camargo 2016; Chatzopoulou et al. 2020). In addition, the Mediterranean diet includes different diverse vegetable species and fulfills one-tenth or more calories required by vegetables and fruits, which are rich in different micronutrients along with tocopherols. However, the bio-accessibility of the tocopherol present in vegetables is comparatively low due to the low level of lipid content, considering that the presence of fat components in the food matrix increases the bio-accessibility of the tocopherols (Azzi 2018; Bora et al. 2022). For example, raw vegetables consumed along with canola oil or eggs increase the absorption of tocopherols compared to the consumption without any fat component (Bora et al. 2022). Different commonly used vegetable oil, fresh vegetable, and fruits that are rich in tocopherols content are summarized in Table 8.1. The range of tocopherols concentration varies not only between species but also within the same species in function of the crop cultivar or variety, maturity stage, and is influenced by environmental conditions including pedoclimatic and light conditions, as well as by crop management practices and harvesting procedures (Bauernfeind and Desai 1977; Chun et al. 2006). Even in post-harvest stages, factors like vegetable processing, storage, samples preparation, and laboratory analysis procedures were found to affect tocopherols level in vegetable oil, fruits, and vegetables (Bauernfeind and Desai 1977; Chun et al. 2006; Knecht et al. 2015).

Olive oil, canola oil, and sunflower oil are commonly used and important sources of tocopherols as they contain high levels of α-tocopherols. They contain 3.7–27.7, 11.7–41.85 and 43.23–91.6 mg α-tocopherol/100 mg of oil, respectively (Table 8.1). Soybean generally contains high amounts of total tocopherols (73.61–171.5 mg/100 g oil) compared to olive (4.99–31.81 mg/100 g oil), canola (26.58–113.15 mg/100 g oil), and sunflower oil (45.3–111.48 mg/100 g oil); however, it is not considered an excellent source of tocopherols and vitamin E compared to sunflower, canola, and olive oil (Chun et al. 2006) because a large proportion of total tocopherols in soybean is in γ-tocopherol form and only a small fraction is represented by α-tocopherol. When considering what are the best sources of tocopherols and vitamin E, particular attention has been dedicated to α-tocopherol being the isomer with the highest retention in the blood plasma and tissues compared to other tocopherol isomers that are metabolized faster and are excreted out of the human

Table 8.1 Content ranges of tocopherols in different plant and plant-derived sources

Species	α-tocopherol	β-tocopherol	γ-tocopherol	δ-tocopherol	Total tocopherol (mg/100 g)	References
Vegetable and edible oils						
Sunflower oil	43.23–91.6	2.07–8.45	0–9.23	0–2.2	45.3–111.48	Grilo et al. (2014), Ergönül and Köseoğlu (2014), Ayerdi Gotor et al. (2015), Cruz and Casal (2018), Wen et al. (2020), Xu et al. (2020)
Rapeseed (canola oil)	11.7–41.85	6.43	12.2–46.07	2.68–18.8	26.58–113.15	Grilo et al. (2014), Ergönül and Köseoğlu (2014), Xu et al. (2020)
Soybean	6.96–55.73	5.3	49.52–79.91	11.83–30.56	73.61–171.5	Grilo et al. (2014), Ergönül and Köseoğlu (2014), Wen et al. (2020)
Maize oil	2.2–50.53	1.1–5.9	25.97–75.99	4.35–18.4	55–119.27	Grilo et al. (2014), Ergönül and Köseoğlu (2014), Wen et al. (2020), Xu et al. (2020)
Sesame oil	0.8–1.0	–	37.6–39.4	2.9–3.3	21.4–42.5	Xu et al. (2020)
Linseed oil	0.7	–	39.8–41.6	1.0–1.2	42.5	Xu et al. (2020)
Rice bran oil	0.59–50.3	0–5.3	0–19.4	0–8.49	3.23–62.2	Wen et al. (2020)
Cottonseed oil	44.93–63.4	–	28.45–51.8	2–2.96	62.6–111.8	Wen et al. (2020)
Hemp oil	2.61–2.65	–	61.17–61.18	3.04–3.11	66.82–66.94	Cruz and Casal (2018)
Peanut oil	13.32–36.64	–	11.33–31.45	2.28–3.83	21.97–71.9	Wen et al. (2020)
Camelia oil	12.34–20.1	–	1.3–3.9	0.48–1.92	14.12–25.92	Zhang et al. (2019), Wen et al. (2020)
Olive oil	3.7–27.7	–	0.8–2.6	0.49–1.51	4.99–31.81	Zhang et al. (2019)
Walnut oil	1.24–2.88	–	26.95–33.15	–	28.95–37.15	Maguire et al. (2004)
Hazelnut oil	27.9–34.12	–	3.14–9.1	–	31.04–43.22	Maguire et al. (2004)
Almond oil	43.47–44.43	–	1.04–1.46	–	44.51–45.89	Maguire et al. (2004)
Macadamia oil	9.78–14.68	–	–	–	9.78–14.68	Maguire et al. (2004)
Fresh vegetables						
Kale	3.4–5.8	0.06–0.12	0.3–1.3	0.02–0.04	3.78–7.26	Isabelle et al. (2010), Wen Lee et al. (2022)

(continued)

Table 8.1 (continued)

Species	α-tocopherol	β-tocopherol	γ-tocopherol	δ-tocopherol	Total tocopherol (mg/100 g)	References
Spinach	1.3–5.90	0.01–0.014	0.15–4.18	0.005–0.007	1.47–10.10	Chun et al. (2006), Kim et al. (2007), Knecht et al. (2015), Lee et al. (2018), Wen Lee et al. (2022)
Pea shoots	0.86–1.04	0.005–0.007	0.009–0.0143	Trace	0.87–1.06	Wen Lee et al. (2022)
Lettuce	0.22–0.55	0.01	0.11–0.74	–	0.32–1.06	Chun et al. (2006), Cruz and Casal 2013)
Celery	0.26–0.47	0.01–0.018	Trace	Trace	0.27–0.49	Chun et al. (2006), Kim et al. (2007), Knecht et al. (2015)
Chayote leaves	0.6–1.6	0.03–0.13	0.7–2.5	0.1–0.5	0.83–4.73	Wen Lee et al. (2022)
Turmeric leaves	3.6–6.6	0.16–0.26	1.5–3.1	0.08–0.13	5.34–10.09	Wen Lee et al. (2022)
Green Amaranthus	0.14–0.24	0.02–0.35	0.17–0.33	0.007–0.011	0.34–0.93	Wen Lee et al. (2022)
Garlic chives	2.1–2.7	0.09–0.15	0.7–1.1	0.1–0.5	2.99–4.45	Wen Lee et al. (2022)
Cassava leaves	6.9–7.3	0.06–0.1	0.13–0.29	0.002–0.006	7.09–7.67	Wen Lee et al. (2022)
Fenugreek leaves	3.03–3.21	0.014–0.016	0.01	0.01	3.06–3.25	Wen Lee et al. (2022)
Red sweet pepper	2.72–3.78	0.1–0.19	0.03–0.17	0.020–0.04	2.87–4.18	Knecht et al. (2015)
Sweet potato	0.25–0.56	0.01	–	–	0.26–0.57	Chun et al. (2006), Lee et al. (2018)
Eggplant	0.11–0.34	–	0.5–0.7	0.08–0.11	0.69–1.15	Kim et al. (2007)
Bamboo shoot	0.15–0.33	–	0.26–0.53	–	0.41–0.86	Kim et al. (2007)
Soybean sprout	0.11–1.09	–	1.62–3.96	0.15–1.99	1.88–7.04	Kim et al. (2007), Isabelle et al. (2010)
Broccoli	1.22–3.75	0.02–0.03	0.19–0.43	–	1.43–4.21	Chun et al. (2006), Knecht et al. (2015), Lee et al. (2018)
Cabbage	0.07–0.21	–	–	–	0.12–0.69	Chun et al. (2006)

Carrot	0.37–1.30	0–0.02	–	–	0.37–1.03	Chun et al. (2006), Knecht et al. (2015), Lee et al. (2018)
Coriander	5.16	–	0.06	0.007	5.23	Isabelle et al. (2010)
Cauliflower	0.08–0.16	–	0.20–0.44	0.005	0.34–0.61	Chun et al. (2006), Isabelle et al. (2010)
Cucumber	0.03	0.01	0.04	–	0.08	Chun et al. (2006)
Red chili	5.65	–	0.22	0.008	5.88	Isabelle et al. (2010)
Tomato	0.53–1.02	0.04	0.07–0.38	0.03	0.53–1.47	Chun et al. (2006), Knecht et al. (2015)
Fresh fruits						
Apple	0.04–0.38	0.01	0.04	0.01	0.04–0.44	Chun et al. (2006), Kim et al. (2007)
Grape	0.06–0.10	–	0–0.32	–	0.06–0.42	Chun et al. (2006), Kim et al. (2007)
Peach	0.11–0.94	–	0–0.04	0–0.06	0.11–1.04	Chun et al. (2006), Kim et al. (2007)
Plum	0.09–0.39	–	0–0.13	–	0.09–0.52	Chun et al. (2006), Kim et al. (2007)
Avocado	1.33–2.66	0.03–0.08	0.13–0.69	0.03	1.98–3.13	Chun et al. (2006)
Banana	0.3–0.23	–	–	–	0.06–0.24	Chun et al. (2006)
Blackberries	0.69–2.17	0.02–0.06	1.27–1.57	0.52–1.08	2.8–4.68	Chun et al. (2006)
Blueberries	0.37–0.79	–	0.29–0.47	0–0.05	0.74–1.36	Chun et al. (2006)
Cantaloupes	0.04–0.08	–	0.08–0.12	–	0.13–0.19	Chun et al. (2006)
Cranberries	1.03–1.43	–	0.04	–	1.34–1.88	Chun et al. (2006)
Grapefruit	0.1–0.22	–	–	–	0.10–0.24	Chun et al. (2006)
Kiwi	1.28–1.34	–	0.02–0.04	–	1.40–1.50	Chun et al. (2006)
Oranges	0.25	–	–	–	0.25	Chun et al. (2006)
Raspberries, red	0.66–1.04	0.06–0.12	1.06–1.72	0.74–1.56	3.10–3.82	Chun et al. (2006)
Dry fruits						
Walnut	2.87–5.57	0.33	42.73–45.67	4.17–6.31	50.1–57.98	Hejtmánková et al. (2018)
Hazelnuts	76.9–83.7	0.72–0.96	3.01–3.29	0.13–0.35	80.76–88.3	Hejtmánková et al. (2018)
Cashew nut	<0.5	–	6.68–12.42	0.63–0.67	7.31–13.09	Hejtmánková et al. (2018)
Pistachios	3.58–4.86	–	57.69–59.31	0.98–1.14	62.25–65.31	Hejtmánková et al. (2018)
Brazil nuts	14.4–18	–	36.29–49.11	140.1–319.5	190.79–386.6	Hejtmánková et al. (2018)

(continued)

Table 8.1 (continued)

Species	α-tocopherol	β-tocopherol	γ-tocopherol	δ-tocopherol	Total tocopherol (mg/100 g)	References
Macadamia nuts	39.4–47.6	–	17.8–19.55	0.64–0.76	57.84–67.91	Hejtmánková et al. (2018)
Pecans	1.89–11.09	–	10.5–66.9	1.12–4.48	13.51–82.47	Hejtmánková et al. (2018)
Pea nuts	26.4–28.4	–	9.46–12.94	0.56–0.69	36.42–42.03	Hejtmánková et al. (2018)

body system by the liver. However, recently the scientific community devoted more attention toward other tocopherols like γ- and δ-tocopherols, aiming to investigate further their possible antioxidant activity and physiological functions (Wagner et al. 2004; Dietrich et al. 2006; Blair 2018; Zheng et al. 2020). Oils from peanuts, cottonseeds, hazelnuts, almonds, macadamia, and rice brans are other excellent sources of α-tocopherols (Table 8.1). Other edible oils like maize oil, sesame oil, linseed oil, hemp oil, and walnut oil are richer in γ-tocopherol. In general, seed oils are the major sources of tocopherols and vitamin E for the human body. Nevertheless, people obtain significant amounts of tocopherols also through the inclusion of vegetables in their diet.

Among vegetables, leafy vegetables like kale and spinach, or sweet pepper and broccoli are major sources of α-tocopherols. Kale contains 3.4–5.8 mg of α-tocopherol per 100 g fresh weight, while spinach, sweet red pepper, and broccoli contain 1.3–5.9, 2.72–3.78, and 1.22–3.75 mg of α-tocopherol per 100 g fresh weight, respectively. Similarly, spices and condiments like red chili, coriander, turmeric leaves, cassava leaves, and fenugreek leaves are also found to have a higher level of α-tocopherol and with possible applications in diet and pharmaceutical uses as a source of tocopherols. Among fruits, avocado, cranberries, red raspberries, and kiwi (1.33–2.66, 1.03–1.43, 0.66–1,04, and 1.28–1.34 mg/100 g fresh weight, respectively) have relatively high concentration of α-tocopherols. Tree fruits like apple, peach, and plum also contain α and γ-tocopherol but at relatively lower concentrations. Further, a recent study suggested the possibility to use tree fruit leaves as a low-cost source of tocopherols (Wojdyło et al. 2022). These authors studied α-tocopherol content in the leaf of tree fruits like apples, pears, plums, and cherries. The time the leaves were collected, and the species affected the content of tocopherols more than the cultivars. Apricot leaves (203.34–260.86 μg/g dry weight in spring and 23.83–235.62 μg/g dry weight in autumn) had the highest tocopherols content, followed by peach, plum, and apple (Wojdyło et al. 2022). Different dry fruits and nuts are also excellent sources of tocopherols and vitamin E. Hazelnuts, macadamia nuts, and peanuts have a higher proportion of α-tocopherols, whereas walnut, cashew nuts, pistachios, Brazil nuts, and pecans have comparatively higher γ-tocopherols content.

Vegetable oils are also rich in phytosterols. Daily intake of phytosterol varies based on regional and country dietary patterns. For example, within China, total phytosterols intake varies between 257.7–473.7 mg/day in different regions (Wang et al. 2018). Similarly, the Mediterranean diet usually includes phytosterol within the range of 377–550 mg/day due to the inclusion of vegetable oil (olive oil) and a variety of vegetables. The phytosterol content in different edible and vegetable oil, fresh vegetable, and fruits are presented in Table 8.2. Concentration level widely varies as it depends on many factors like species, variety, management practices, environmental conditions, extraction, and analysis method just like for the tocopherols' level.

Rice bran oil contains the highest amount of total phytosterols among the commonly studied edible oils followed by sesame oil (Table 8.2). Rice bran oil total phytosterol concentration ranges between 1230.9 and 2392.58 mg/100 g fresh

Table 8.2 Content range of phytosterols in different plant and plant-derived sources

Species	β-sitoserol (mg/100 g)	Campesterol (mg/100 g)	Stigmasterol (mg/100 g)	Total phytosterol[a] (mg/100 g)	References
Vegetable and edible oils					
Sunflower seed oil	182.4–245.8	13.1–65.73	15.9–41.8	197–440.6	Ayerdi Gotor et al. (2015), Wang et al. (2018), Yang et al. (2019), Xu et al. (2020), Almeida et al. (2020)
Rapeseed (canola oil)	109.1–394.1	46.2–270.79	2.2–25.67	290–673	Yang et al. (2019), Xu et al. (2020), Almeida et al. (2020)
Soybean	165.3–174.89	62.4–96.7	62.81–87.28	100.4–355.67	Wang et al. (2018), Yang et al. (2019), Almeida et al. (2020)
Maize oil	251–540.62	39.6–219.02	22.9–56.72	343.1–743.65	Yang et al. (2019), Xu et al. (2020), Almeida et al. (2020)
Sesame oil	322.73–467.7	41.4–90.4	48.1–86.89	457.01–818.19	Mariod et al. (2011), Wang et al. (2018), Yang et al. (2019), Xu et al. (2020)
Linseed oil	97.3–162	33.1–65.2	16.7–26.5	171–363.5	Wang et al. (2018), Xu et al. (2020)
Rice bran oil	590.8–735.17	20.7–226.43	21.8–132.9	1230.9–2392.58	Wang et al. (2018), Yang et al. (2019)
Peanut oil	136.33–189.12	19.83–41.19	16.33–48.16	243.25–395.9	Maguire et al. (2004), Wang et al. (2018), Yang et al. (2019)
Camelia oil	48.1–50.09	16.5–16.52	22.11–23	91.78–193.5	Wang et al. (2018), Yang et al. (2019)
Olive oil	152.05–185.61	14.31–25.85	7.4–21.13	195.42–380.62	Yang et al. (2019), Almeida et al. (2020)
Walnut oil	66.2–165.23	3–31.53	0.7–32.80	80.6–379.45	Wang et al. (2018), Yang et al. (2019)
Flaxseed oil	133.42–182.16	88.32–142.72	5.03–20.21	406.08–527.38	Yang et al. (2019)
Cottonseed oil	402.8–403.8	43.6–44.2	5.2–5.4	492.4	Mariod et al. (2011)
Grapeseed oil	131.96–161.3	24.04–34.46	31.8–39.74	234.95–312.65	Yang et al. (2019)

(continued)

Table 8.2 (continued)

Species	β-sitoserol (mg/100 g)	Campesterol (mg/100 g)	Stigmasterol (mg/100 g)	Total phytosterol[a] (mg/100 g)	References
Penoy oil	240.27–277.16	7.02–35.62	0.34–4.8	325.06–409.32	Yang et al. (2019)
Hazelnut oil	48.5–99.1	2.5–6.67	0.6–3.81	70.0–117.99	Maguire et al. (2004), Wang et al. (2018)
Almond oil	58.2–207.1	0.7–8.1	0–5.17	109.5–221.87	Maguire et al. (2004), Wang et al. (2018)
Macadamia oil	45.3–152.5	2.8–9.2	0.5–3.83	114.1–177.04	Maguire et al. (2004), Wang et al. (2018)
Fresh vegetables					
Lettuce	29.7	2.5–29.9	0.6–6.2	25.5–50.3	Wang et al. (2018)
Celery	0.6–13.2	1.44–29	0.5–6.0	0.74–38.0	Kaloustian et al. (2008), Han et al. (2008), Wang et al. (2018)
Green pepper	26.4–45.9	3.0–4.9	14.8–26	46.8–79.6	Wang et al. (2018)
Sweet potato	22.4	23.3	15.2	85.7–195.1	Wang et al. (2018)
Eggplant	10.2–19.4	7.1–17.1	1.8–4.4	25.5–50.7	Wang et al. (2018)
Bamboo shoot	53.2–55.6	65.7–71.1	15.7–18.5	147.1–158.3	Wang et al. (2018)
Cabbage	1.5–14.5	0–1.1	0–6.8	6.89–13.4	Kaloustian et al. (2008), Wang et al. (2018)
Carrot	4.8–14	0.99–10.9	0.8–4.8	7.35–26.5	Kaloustian et al. (2008), Han et al. (2008), Wang et al. (2018)
Cauliflower	1.2–6.9	0.2–2.31	0.7–0.56	26.82–27.98	Kaloustian et al. (2008), Wang et al. (2018)
Cucumber	0.5–3.8	0.2–0.9	1.1–2.9	35.0–106.2	Han et al. (2008), Wang et al. (2018)
Onion	3.66–9.4	0.21–0.9	0.028–2.2	1.22–16.4	Kaloustian et al. (2008), Han et al. (2008), Wang et al. (2018)
Radish	3.6–23.4	0.2–1.0	1.4–8.6	6.2–35.4	Wang et al. (2018)
Tomato	2.9–6.6	0.6–7.2	1–1.9	9.6–19.1	Han et al. (2008), Wang et al. (2018)
Fruits					
Apple	0.1	ND	0.3	2.4–3.6	Wang et al. (2018)

(continued)

Table 8.2 (continued)

Species	β-sitoserol (mg/100 g)	Campesterol (mg/100 g)	Stigmasterol (mg/100 g)	Total phytosterol[a] (mg/100 g)	References
Lemon	11.9–18.7	35.8–46.2	6.5–8.1	59.4–79.8	Wang et al. (2018)
Pomelo	1–4.4	19.3–20.3	9.4–16.2	36–40	Wang et al. (2018)
Peach	0.9–11.6	0–0.5	0–1.6	1.0–13.7	Han et al. (2008), Wang et al. (2018)
Plum	0.5–0.7	0–0.2	0–0.2	0.7–0.9	Wang et al. (2018)
Blueberries	0.1–0.3	0.5–1.1	0–0.2	5.9–7.5	Wang et al. (2018)
Kiwi	13.4	1.1	2.0	17.5	Han et al. (2008)
Oranges	8.8–19.6	30.8–43	6.2–9.2	49.0–73.4	Han et al. (2008), Wang et al. (2018)
Strawberries	10.9	0.3	0.2	11.8	Han et al. (2008)

[a]Total phytosterols content is the range of sum of plant sterols reported in the cited manuscript and not the sum of the three phytosterols reported in this table

weight of oil, and β-sitosterol holds the highest share, followed by campesterol and stigmasterol. Sesame, flaxseed, maize, canola, and cottonseed oil contain total phytosterol in the range of 457.01–818.19, 406.08–527.38, 343.1–743.65, 290–673, and 492.4 mg/100 g fresh weight of oil, respectively. All the above-mentioned vegetable oils are rich in β-sitosterol followed by campesterol. Soybean oil which is the most consumed oil in North America contains 100.4–355.67 mg total phytosterols/100 g fresh weight of oil, while olive oil which is one of the main ingredients of the Mediterranean diet contains total plant sterols in the range of 195.42–380.62 mg /100 g fresh weight of oil. Other vegetable oils like sunflower, linseed, camelia, grapeseed oil, and nut oils also contain a significant amount of phytosterols (Table 8.2).

Fresh vegetables and fruits also contribute to the daily intake of phytosterols. They contain a relatively small concentration of plant sterols on a fresh weight basis, however, could play a significant role in human health due to a relatively higher consumption in the human diet compared to vegetable oil. Sweet potato, bamboo shoot, cucumber, green pepper, eggplant, and lettuce are the major phytosterol-containing vegetables (Table 8.2). Sweet potato and bamboo shoots contain 85.7–195.1 and 147.1–158.3 mg phytosterol/100 g of fresh weight, respectively, where campesterol is more abundant followed by the β-sitosterol and stigmasterol. Cucumber, green pepper, eggplant, and lettuce have phytosterol concentrations in the range of 35.0–106.2, 46.8–79.6, 25.5–50.7, and 25.5–50.3 mg/ 100 g of fresh weight, respectively. Other vegetables that are rich in plant sterols are radish, carrot, celery, cauliflower, cabbage, tomato, and onion. Among fruits commonly studied fruits such as lemon, orange, pomelo, kiwi, and strawberries contain a relatively higher amount of total phytosterols. Lemon has 59.4–79.8 mg/100 g fresh weight of total phytosterols, whereas orange, pomelo, kiwi, and strawberries have 49–73.4, 36–40, 17.5, and 11.8 mg/100 g fresh weight of total phytosterols. Other fruits rich in phytosterols are peach, blueberries, apple, and plum.

As mentioned above, several factors could affect the tocopherols and phytosterol content even within species. Genetics is a major component as varieties and cultivars of the same species could contain different levels of tocopherol and phytosterols. Radenkovs et al. (2018) evaluated the by-product of apple processing industries as possible sources of tocopherols and phytosterols and found different α-tocopherol and phytosterol levels in different *Malus* species and varieties. "Bernie Prieks" had higher δ-tocopherols (around 72% of total tocopherols) and total phytosterols. β-Sitosterol was detected in all studied Malus species, while other phytosterols were not identified in some species. A wide range of genetic variability on tocopherol composition is being utilized for breeding for the development of varieties with high nutritional oil quality (Rani et al. 2007). Growing environment and crop management are other factors affecting tocopherol and plant sterol concentration. For example, the level of tocopherols in sunflower seed decreased over the production year with higher average air temperature, especially during grain filling time (Ayerdi Gotor et al. 2015). In addition, a negative correlation ($r = -0.61$) has been observed between the tocopherol concentration and temperature during summer (grain filling period). Further, Zhang et al. (2007) found light during germination may have an effect on the tocopherol level in canola oil. Light increased total tocopherols, especially α-tocopherol during seed germination, compared to seeds germinated in dark conditions. In general, total tocopherols increased during germination. Further, interconversion of isomers was also seen; γ isomer changed to the α and disappearance of the δ isomer and appearance of the β isomer after two days of germination also suggest their interconversion. Total phytosterol also increased during germination in rapeseed but was higher in the presence of light than in dark conditions. Increased concentration of α-tocopherol, total tocopherols, and total phytosterols during germination suggest that oil extraction after germination could be a viable option for concentrating such beneficial phytochemicals in the oil fraction, however, there is a depletion of oil reserve during germination, and seedling growth. Shi et al. (2010) also found similar results in soybean seeds. Oil extraction two to three days after the seed soaking increased tocopherol and phytosterol levels in the soybean oil, however, their level decreases five to seven days after soaking.

Seguin et al. (2010) have reported an effect of seeding rate, row spacing, and seeding date on the tocopherols level in soybean. A seeding rate at 40 seeds/m^2 and wide row spacing (more than 36 cm) resulted in a higher α-tocopherol level in soybean oil. The earlier seeding date resulted in an almost 45% higher α-tocopherol concentration compared to the mid to late-May seeding. Carrera and Seguin (2016) have also mentioned the effect of irrigation and fertilization strategy on the tocopherol levels of edible oil. This suggests that tocopherols concentration in seed oil could vary based on the different management practices and growing environment. The enhanced concentration of tocopherols and phytosterols during the germination process may justify the growing interest of consumers in the consumption of sprouts and microgreens of various species that are considered rich sources of phytonutrients and bioactive compounds (Kyriacou et al. 2016; Di Gioia et al. 2017, 2021). Moreover, several factors like processing, cooking, and method of sample extraction and analysis make a major difference in the tocopherol and phytosterol levels. Naz

et al. (2011) have reported a decrease in the individual and total tocopherol levels (37.9%) during neutralization, bleaching, and deodorization processes, indicating loss of a major portion of tocopherols during oil processing. Improvement in the processing technology is needed to preserve tocopherols in the oil during processing. Heat treatment of the oil also decreases tocopherol levels. For example, higher temperatures and prolonged heating/frying/cooking time degrade tocopherols present in the oil (Kmiecik et al. 2019). However, in the case of vegetables, research has shown a higher level of tocopherols and phytosterols in slightly heated or cooked, or steamed vegetables compared to raw fresh vegetable. For example, higher tocopherols were found in steamed broccoli compared to raw and fresh broccoli (Chun et al. 2006; Kaloustian et al. 2008). This is often related to the increased extractability of the compounds, from steamed/heat treated vegetables, due to cell disruption (Knecht et al. 2015). Lee et al. (2018) compared the effect of different cooking methods (boiling vs. blanching vs. steaming vs. microwave) on the level of tocopherols in different vegetables and found that all cooking methods had higher total tocopherols levels compared to the raw vegetable; however, the effect of cooking methods varies based on the vegetable species. Steaming was better for spinach, blanching for broccoli, microwaving for sweet potato, carrot, mallow, and boiling was better for chard. Kaloustian et al. (2008) compared the phytosterols level before and after cooking (boiling) in different vegetables (cabbage, celery, red carrot, white cauliflower, yellow onion, and red pepper) and found an increase in level in all studied vegetables. Different sample extraction methods also affect the levels of tocopherols and phytosterols measured as different methods differ in their precision and sample extraction procedures (Kaloustian et al. 2008; Almeida et al. 2020). For example, the sample extraction method of acid hydrolysis resulted in a notably higher sterol value compared to alkaline saponification alone (Kaloustian et al. 2008). Different analysis methods were found to differ in their sensitivity to the plant sterol levels present (Péres et al. 2006; Xu 2008; Saini and Keum 2016).

8.4 Tocopherols and Phytosterols Health Effects

Tocopherols are mainly known for their antioxidant properties and their role in reducing cardiovascular and neurodegenerative diseases. The effect of different tocopherols on human health is summarized in Table 8.3. Due to the higher retention in human plasma and tissue compared to other tocopherols, α-tocopherol has higher biological activity, and most of the studies on tocopherols are mainly focused on α-tocopherol and its potential health benefits. Alpha-tocopherol has more prominent antioxidant activity as it contains one or two more electron-donating methyl groups in the chromanol group compared to other tocopherols. It offers protection from lipid peroxidation and various oxidative stresses. An in vivo study has shown the antioxidant capacity of tocopherols to be in the following order: $\alpha > \beta > \gamma > \delta$ (Bora et al. 2022). Higher antioxidant activity of the α-tocopherol inside living organisms is possibly due to the hepatic α-tocopherol transfer protein as it only recognizes

Table 8.3 Health benefits of the tocopherols

Tocopherols	Health benefits	References
α-Tocopherol	Antioxidant; neutralization of lipid peroxidation and oxidative stress	Adami et al. (2018), Wallert et al. (2019), de Carvalho et al. (2019), Villalón-García et al. (2022)
	Anti-inflammation	Wallert et al. (2019), Liu et al. (2021b), Schubert et al. (2022), Kopańska et al. (2022)
	Reduced risk of heart and cardiovascular disease	Wallert et al. (2019), Violi et al. (2022)
	Protection against neurodegenerative disease	Elfakhri et al. (2019), Berardesca and Cameli (2021), Zakharova et al. (2021)
	Antitumor activity and lung cancer	Yano et al. (2000), Tam et al. (2017), Fernandes et al. (2018)
	Protection of kidney function	Tasanarong et al. (2009, 2013), Kongkham et al. (2013), Monami et al. (2021)
	Reduced depression and anxiety	Lee et al. (2022)
	Protection of eye function	Engin (2009), Wang et al. (2011), Xin et al. (2016)
	Gene regulation	Fischer and Rimbach (2019), Gugliandolo et al. (2019)
	Improved immune system	Wu et al. (2000), Mojani et al. (2013)
β-Tocopherol	Antioxidant; protection from oxidative stress and lipid peroxidation	Brigelius-Flohé (2006), Azzi (2018)
	Increased immunity	Wu et al. (2000)
γ-Tocopherol	Antioxidant; protection from oxidative stress	Brigelius-Flohé (2006), Jiang et al. (2022)
	Anti-inflammatory activity	Lee and Lim (2019), Liu et al. (2021a), Jiang et al. (2022)
	Protection from cardiovascular disease	Masterjohn et al. (2012)
	Protection against nitrosative stress	Takahashi et al. (2006), Das Gupta et al. (2015)
	Protection against neurodegenerative disease	Pahrudin Arrozi et al. (2020)
	Anticancer	Betti et al. (2006), Smolarek and Suh (2011), Das Gupta et al. (2015), Chen et al. (2017)
	Protection against cognitive decline and dementia	de Leeuw et al. (2020)
	Gene regulation	Torricelli et al. (2013)
	Increased immunity	Wu et al. (2000)
	Protection against asthma	Wagner et al. (2007, 2008)
	Protection of kidney function	Tasanarong et al. (2013)
δ-Tocopherol	Antioxidant activity	Li et al. (2011)
	Anticancer	Betti et al. (2006), Li et al. (2011), Smolarek and Suh (2011), Chen et al. (2017), Blair (2018)
	Anti-inflammatory activity	Smolarek and Suh (2011)
	Increased immunity	Wu et al. (2000)
	Neuronal differentiation	Deng et al. (2015)

α-tocopherol, while they have maximum retention in plasma and tissue levels. In a zebrafish study, de Carvalho et al. (2019) found a reduction in oxidative stress and anxiety induced through caffeine uptake. Similarly, α-tocopherol supplementation protected human spermatozoon from induced oxidative stress (Adami et al., 2018). A study by Wallert et al. (2019) found an inhibition effect of α-tocopherol on ischemia/reperfusion injury-induced oxidative and inflammatory responses, maintaining normal cardiac function. They suggested the use of vitamin E (especially α-tocopherol) as an acute therapy for a patient with myocardial infarction. In addition, α-tocopherol is very helpful against neurodegenerative diseases like Alzheimer's disease. Neuroinflammation and oxidative stress were further found to exacerbate Alzheimer's disease progression (De Felice and Lourenco 2015; Elfakhri et al. 2019). Elfakhri et al. (2019) found a possible curating strategy for Alzheimer's disease through the administration of etodolac and α-tocopherol in a concurrent manner as they found a significant reduction in Alzheimer's disease-related pathology in the brains of mice through the α-tocopherol application. A recent study by Villalón-García et al. (2022) showed a reduction in lipid peroxidation and ROS generation in *PLA2G6*-Associated Neurodegeneration through the application of α-tocopherol. Likewise, α-tocopherol also protected the cultured cortical neurons from oxidative stress and the brain cortex of rats during cerebral ischemia/reperfusion injury (Zakharova et al. 2021). However, at higher concentrations, the antioxidant capacity of the α-tocopherol could decrease (Liu et al. 2021b). β-tocopherol, a close homologous of the α-tocopherol, is not studied much compared with other tocopherols for its health effects. In recent studies, β-tocopherol showed similar antioxidant activity and protection from lipooxidation and oxidative stress, although the efficiency was comparatively lower than α-tocopherol (Brigelius-Flohé 2006; Azzi 2018).

Despite having a good antioxidant capacity, α-tocopherol can not trap reactive nitrogen species, unlike γ-tocopherol which has an unsubstituted C-5 position making it more active to trap relative nitrogen species (Saldeen and Saldeen 2005). Therefore, γ-tocopherol could trap reactive nitrogen species and form 5-nitro-γ-tocopherol which protects the mitochondrial function more efficiently than other tocopherols (Jiang et al. 2022). In addition, Pahrudin Arrozi et al. (2020) found comparative effectiveness of γ-tocopherol to α-tocopherol on the reduction of the amyloid-beta (Aβ) and amyloid precursor protein (APP) contents which are higher in Alzheimer's disease patients. Further, γ-tocopherol also reduced mitochondrial permeability as suggested by the reduction in CypD protein and pro-caspase-3 protein expression, which was not seen in the α-tocopherol treatment (Pahrudin Arrozi et al. 2020). de Leeuw et al. (2020) found a positive correlation of γ-tocopherol with the presynaptic protein levels in the elderly human midfrontal cortex, suggesting an important role in preserving cognitive power and preventing dementia problems. A lower presynaptic protein level is generally used to be recognized as a clinical diagnosis of dementia. Supplementation of α and γ tocopherols could help preventing oxidative stress, the reason behind the decreasing level of presynaptic protein levels, thus protecting from dementia and deterioration of cognitive power (de Leeuw et al. 2020). A previous research suggested a better

action against myocardial infarction with the combination of α and γ-tocopherol (Hensley et al. 2004), while Deng et al. (2015) have reported a potential role of δ-tocopherol on neuronal differentiation through the l-type calcium channels.

Tocopherol also helps to protect against cancer cells. Many studies have shown the important role of γ- and δ-tocopherol against cancer cells, however, they did not find conclusive evidence of anticancer activity determined by α-tocopherol (Abraham et al. 2019; Retzlaff et al. 2021). A meta-analysis even shows a potential negative role of α-tocopherol on the effectiveness of chemo and radiotherapy further worsening the survival of cancer patients (Retzlaff et al. 2021). However, many articles have mentioned the effectiveness or lower effectiveness of α and β-tocopherol as inhibitors of cancer cells proliferation compared to the γ- and δ-tocopherol (Galli et al. 2004; Yang et al. 2012; Azzi 2018). Galli et al. (2004) compared α and γ-tocopherol and their carboxy-ethyl-hydroxychroman metabolites on prostate cancer cell proliferation and found that γ-tocopherol and its precursors were more effective in the inhibition of PC-3 growth through the downregulation of cyclin expression. Similarly, Li et al. (2011) did a comparative study of α-, γ-, and δ-tocopherol on inhibiting lung tumorigenesis through a xenograft model. δ-tocopherol was more effective in inhibiting tumor growth possibly through trapping more reactive oxygen and nitrogen species compared to γ- tocopherol while α-tocopherol was ineffective. δ-Tocopherol inhibited oxidative DNA damage and nitrotyrosine formation and further increased apoptosis in tumor cells (Li et al. 2011). Similarly, colon tumor formation was reduced by the dietary supplementation of δ- and γ-tocopherol which also suppressed markers of oxidative and nitrosative stress (Chen et al. 2017). γ- and δ-tocopherols were also effective in preventing breast cancer, while a-tocopherol was ineffective (Smolarek and Suh 2011). Despite several studies have shown the ineffectiveness of α-tocopherol in a cancer-preventing role, Mahabir et al. (2008) have found an inverse relation between α-tocopherol intake and risk of lung cancer. Increasing the intake of α-tocopherol decreased lung cancer risk by 34–54%, through the analysis of epidemiology data from 1088 patients with lung cancer cases. Nevertheless, α-tocopherol succinate has shown antitumor activity. In particular, Fernandes et al. (2018) and Tam et al. (2017) observed antitumor activity of the α-tocopherol succinate against human breast cancer cells, both in vitro and in vivo.

Tocopherols and especially α-tocopherols are involved in many gene regulatory functions (Rimbach et al. 2010; Fischer and Rimbach 2019; Gugliandolo et al. 2019). For example, Gugliandolo et al. (2019) found the modulation effect of α-tocopherol on non-amyloidogenic pathways and autophagy in an in vitro study of Alzheimer's disease. Likewise, α-tocopherol modulates the expression of selective Tumor Necrosis Factor-Alpha-Induced (TNF) genes in primary human aortic cell lines (Ranard et al. 2019). γ-tocopherol also has gene regulatory activities, and it upregulates a transglutaminase 2 (TG2) and its activity and decreases cyclin D1 and cyclin E (Torricelli et al. 2013). In this way, γ-tocopherol helps the inhibition of prostate cancer cells in humans.

α- and γ-tocopherols also protect kidney function. A meta-analysis by Monami et al. (2021) showed a reduction in the incidence of contrast-induced nephropathy

(CIN) after the treatment with α-tocopherol. Another study also found a decrease in rat renal contrast-induced nephropathy with the pretreatment with α-tocopherol (Kongkham et al. 2013). A similar result was also found by Tasanarong et al. (2013) who compared an oral administration of α- and γ-tocopherol with the placebo in patients affected by chronic kidney disease. α-tocopherol was the most effective in preventing contrast-induced acute kidney injury compared to γ-tocopherol, while both are effective against placebo. γ-tocopherols also protect against allergic rhinitis and asthma (Wagner et al. 2007, 2008). Supplementation of γ-tocopherol for four days protected from the inflammatory effect induced by allergen (Wagner et al. 2008).

Tocopherols were also found to protect eye functions as studies have shown the role of vitamin E in the prevention of cataracts and glaucoma (Tanito 2021). Orally administered α-tocopherol protected eyes from ultraviolet radiation-induced cataract in rats, however, this effect was dose-dependent (Wang et al. 2011). Similarly, α-tocopherol application as eye drops was found to prevent ocular oxidative damage improving the ocular stability and efficiency (Xin et al. 2016). According to Pastor-Valero (2013), a lower prevalence of cataracts could be associated with a higher intake of vitamin E (α-tocopherol) and vitamin C through a high consumption of fruit and vegetables following the Mediterranean diet.

The immunoregulatory role of tocopherols has also gained attention. Supplementation of tocopherols increases the function of the immune system, thus reducing the chance of infection, especially in the older population (Lewis et al. 2019). Recently Wu et al. (2000) showed a lymphocyte proliferation capacity of α, β, γ, and δ tocopherols in a mice study. In general, the order of efficiency among the four tocopherol isomers was β-tocopherol ≈ δ-tocopherol > γ-tocopherol > α-tocopherol. Similarly, Mojani et al. (2013) reported a lymphocyte proliferation activity of α-tocopherol alone or in combination with mixed-tocotrienols. These studies suggest a potential beneficial role of tocopherols in strengthening the body immune system under different stress conditions.

Phytosterols have several health-promoting activities, including a reduction in blood cholesterol levels, antioxidant, and anti-inflammatory activities, as summarized in Table 8.4. Phytosterols help lowering the total cholesterol and low-density lipoprotein cholesterol (generally regarded as “bad cholesterol”) in blood by reducing their absorption (Lagarda et al. 2006; Trautwein and Demonty 2007; Vezza et al. 2020). The exact mechanism behind the reduction of LDL cholesterol is still under investigation, however, many theories mentioned the precipitation of cholesterol in the presence of added phytosterol and competition for absorption (MacKay and Jones 2011; Chawla et al. 2016). The efficiency of phytosterols in lowering the LDL cholesterol levels in blood plasma is higher when their intake is associated with fat intake, as bile secreted in the presence of fatty food facilitates the fat mixed micelles formation, important for plant sterols absorption (Trautwein and Demonty 2007). The same study revealed that there is cholesterol in the bile that is not completely reabsorbed in the presence of phytosterols and is later excreted in feces. Outside of the micellar phase, cholesterol is not soluble, and forms a co-crystal with unabsorbed phytosterols and they are excreted together. Cholesterol and phytosterol share the

Table 8.4 Health benefits of phytosterols

Health benefits	References
Reduced blood cholesterol level	Li et al. (2018), Reaver et al. (2019), Trautwein and Demonty (2007), Wu et al. (2014)
Anti-obesity activity	Thornton et al. (2011), Li et al. (2018), Masuzaki et al. (2019), Vezza et al. (2020)
Anti-diabetic activity	Ramalingam et al. (2020), Krishnan et al. (2021), Jayaraman et al. (2021)
Antioxidant activity and reduction of oxidative stress	Koc et al. (2021), Lesma et al. (2018), Paniagua-Pérez et al. (2008)
Anti-inflammatory activity	Othman and Moghadasian (2011), Valerio and Awad (2011), Kurano et al. (2018), Teixeira et al. (2021), He et al. (2022)
Anti-atherogenic activity	Nashed et al. (2005), Moghadasian et al. (2016), Ghaedi et al. (2020)
Anticoagulant	Salunkhe et al. (2018), Gogoi et al. (2018)
Anticancer activity	Awad et al. (2007), Jiang et al. (2019), Blanco-Vaca et al. (2019)
Immune system modulation	Paniagua-Pérez et al. (2008), Boukes and Van de Venter (2016), Le et al. (2017), Hu et al. (2017)

same transporter protein and process, there is also a competition for sterols uptake and transportation that contributes to reduce the cholesterol level in the body (Trautwein and Demonty 2007). In a randomized, placebo-controlled study, Reaver et al. (2019) reported a 10.2% decrease in low-density lipoprotein cholesterol (LDLc) through the dietary supplementation of 1.5 g/day phytosterol equivalents. Similarly, an association of lower total cholesterol and LDLc with phytosterol intake was found by Li et al. (2018). They also found a lower-body max index, waist circumference, and prevalence of overweight/obesity in the population with higher phytosterol intake in their diet. Many other studies have also shown the inverse relationship between phytosterols intake and obesity (Vezza et al. 2020). In a diet-induced obesity mouse model, Thornton et al. (2011) found a lower mass accumulation in a high-fat diet with phytosterols. Masuzaki et al. (2019) also reported a reduced preference for a high-fat diet in mice after phytosterol intake (brown rice-specific c-oryzanol) through modulation in striatal dopamine D2 receptor and further changing metabolic function. They described phytosterol as a possible approach to protect against obesity and diabetes. In addition, Jayaraman et al. (2021) found β-sitosterol may play a role in the downregulation of the IKKβ/NFκB and c-Jun-N-terminal kinase (JNK) signaling pathway, which helps to reduce obesity-induced insulin resistance. Daily supplementation of 20 mg/kg of body weight of phytosterols stabilized the level of blood glucose, serum insulin, and marker of oxidative stress in high-fat diet-fed diabetic rats (Krishnan et al. 2021). A similar result was also observed by Ramalingam et al. (2020). Administering daily 15 mg/kg of body weight of β-sitosterol to rats on high-fat feeding for up to 30 days, they found lower plasma glucose and increased levels of insulin.

Further, phytosterols are also found to have antioxidant, anti-inflammatory, anticoagulant, and atherosclerotic properties. A study by Koc et al. (2021) suggests the potential use of β-sitosterol on renal and cardiac necrosis and apoptosis due to the anti-inflammatory and antioxidant properties of phytosterols which further reduce oxidative stress. Paniagua-Pérez et al. (2008) have demonstrated the antioxidant properties of phytosterols and found that β-sitosterol could trap up to 78.12% of free radicals at 250 μg/mL of phytosterol, through DPPH assay. Lesma et al. (2018) also reported the antioxidant activity of phytosterols, γ-oryzanol, and their conjugates. Anti-inflammatory activity is the other important role of phytosterols. Teixeira et al. (2021) reported the potential anti-inflammatory effect of phytosterol as it reduced TNF-α and IL-6 in inflammation induced by lipopolysaccharide in the macrophages. Another study also found the anti-inflammatory activity of the β-sitosterol in the macrophages through the inactivation of STAT1 and NF-κB (Valerio and Awad 2011). He et al. (2022) also reported on the anti-inflammatory role of phytosterols, which reduced lipopolysaccharides-induced inflammation of acute lung injury through the activation of the LXRs/ABCA1 pathway. Reduction in IL-6, TNF-α, and MCP-1 levels in the adipose tissue in the mice with obesity-induced chronic inflammation also shows the potential anti-inflammatory activities of phytosterols.

The anti-atherogenic role of phytosterols and their effect on cardiovascular diseases is one of the most discussed and highly controversial topics. Some studies have shown positive effects, while others have shown a detrimental effect on cardiovascular health. Moghadasian et al. (2016) reported a positive effect on atherosclerotic lesion size and severity compared to control when they supplement low-density lipoprotein receptor knockout (LDL-r-KO) mice with a wild rice and phytosterol combination. They attributed the result seen to the decrease in plasma LDL and the increase in fecal cholesterol extraction. Nashed et al. (2005) reported an anti-atherogenic activity of phytosterols and inhibition of proinflammatory cytokine production as a possible pathway for such effect in apolipoprotein E (apoE) deficient mice. apoE is the protein which plays important role in lipid transportation in plasma (Hatters et al. 2006). Further, anticoagulant activities of phytosterols were reported by Gogoi et al. (2018) and they suggested a possible use of soybean-extracted β-sitosterol to prevent thrombosis-associated cardiovascular disorder. There are some studies in which authors have discussed the possible negative effects of phytosterols on coronary atherosclerosis. In particular, a genome-wide meta-analysis by Scholz et al. (2022) revealed a detrimental effect of phytosterols on coronary artery disease (CAD). They found a positive relationship between increased serum phytosterol levels with CAD after performing a Mendelian randomization analysis. Similarly, a study on mice found an increased rate of ventricular arrhythmia, impaired cardiac function, and sudden death with the increased plasma level of phytosterols (Ge et al. 2021). Therefore, further studies, in vivo and in vitro, are required to better understand the effect of phytosterols on cardiovascular health.

Anticancer properties and immune system stimulation are other possible health benefits of phytosterols. Phytosterols may have a role in reducing cancer through the modulation of proliferation and apoptosis of tumor cells (Blanco-Vaca et al. 2019). Awad et al. (2007) observed a reduction in tumor cell growth and an increase in the transformed cell membrane in human breast cancer cells. They found an increase in

Fas protein level and caspase-8 activity and discussed them as a possible cell signaling pathway in the protection mechanism against a cancer cell. A meta-analysis by Jiang et al. (2019) also revealed an inverse relationship between phytosterols intake and cancer risk. Another commonly discussed health benefit of phytosterols is the stimulation of the immune system. Boukes and Van de Venter (2016) reported phagocytosis and increased innate immune response in the U937 leukemia cells, in vitro study, after the pretreatment with phytosterols extracted from *Hypoxis* spp. Similarly, Hu et al. (2017) also reported an increase in immunity with the phytosterols supplementation in weaned piglets. There was an increase in lymphocyte production in the mice after the administration of phytosterols in the study conducted by Paniagua-Pérez et al. (2008). A study has also shown the immunotherapeutic potential of phytosterols after observing immunosuppression activity of β-sitosterol and stigmasterol in murine cells (Le et al. 2017). Because of their multiple health beneficial effects, nowadays plant sterols have become an important part of the development of functional foods (Poli et al. 2021).

8.5 Conclusions

Tocopherols and phytosterols are plant fat-soluble bioactive compounds which play an important role in the plant physiology, ranging from the protection against oxidative stress to cell membrane stability. Tocopherols are mainly known for their antioxidant activity, while phytosterols are primarily known for their capacity to lower blood cholesterol levels in the human body. Recently, several randomized in vitro and in vivo studies have shown their multiple beneficial effects on human health. As these important bioactive compounds are not synthesized by animals and the human body, the daily recommended intake of these compounds should be fulfilled using plant and plant-derived food sources. Vegetable oil, fresh vegetables, nuts, and fruits are the primary sources of both tocopherols and phytosterols, and their regular inclusion in the diet is recommended, although the same compounds have been also successfully extracted from by-product of agri-food industry or from commonly non-edible plant portions such as the leaves of fruit trees. Considering the proven health beneficial properties of these compounds, more research is needed to identify low-cost widely available sources of tocopherols and phytosterols to enhance their availability at global scale. Further, future research work should focus on the development of plant-based functional food products investigating sustainable biofortification approaches for increasing the level of tocopherols and phytosterols in commonly consumed crops and plant products.

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Chapter 9
Anthra-, Benzo-, and Naphthoquinones

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Abstract Natural products represent important drugs since ancient times, nowadays as endless opportunities to find new compounds with pharmacological activities, a great number of new drugs are still derived from natural sources. Quinones and their derivatives have a fundamental role in several applications, i.e., pharmaceutical, medicinal, and food industries. Quinones represent a large class of compounds showing fascinating chemistry: they interact as electron transfer agents with biological targets by the formation of covalent bindings in redox reactions. Quinones constitute an important class of natural and synthetic compounds. A wide variety of synthetic quinones were prepared for developing structures with pharmacological activities.

Their structure makes them interfere/udergo in chemical transformations. A great interest by the scientific community is given to quinone-based compounds for their challenging structural elements and potential therapeutic properties.

9.1 Quinones: A Current Literature Quantitative Research Analysis

To give a current snapshot of the interest raised in international research, a search throughout the Scopus online database was carried out on 9 February 2022 by means of a string TITLE-ABS-KEY ("quinone*"). The search returned 57,981 documents and covered the time range from 1879 to 2022. The oldest document titled "On the action of organo-zinc compounds on quinones" was published by Japp, F.R. on 1879 in *Journal of the Chemical Society.*

Considering the documents up to 2022, some examples of most relevant documents are "Bio-inspired lanthanum-ortho-quinone catalysis for aerobic alcohol

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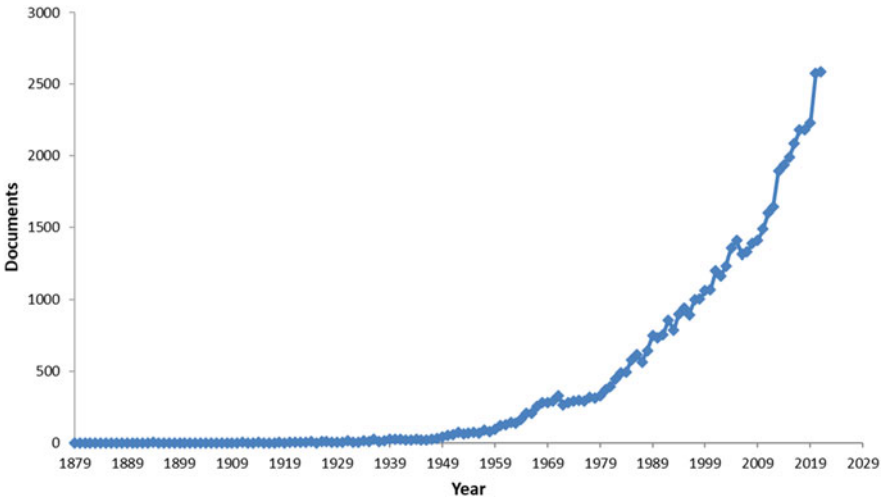

Fig. 9.1 Publication trends (1879–2021) on quinones search. (Based on data from Scopus)

oxidation" (Zhang et al. 2022) and "Antibacterial activity of quinone derivatives" (Sahoo et al. 2022).

Publications trends (1879–2021) concerning quinones are reported in Fig. 9.1.

The types of documents include mainly "Articles" about 90.3%, followed by "Review" at 5.2%, and "Conference paper" (2.0%) (Data from Scopus database). Among the "Book" category, it is worth mentioning the book published by Price and Johnson (2013) on "Occurrence, medicinal uses and physiological importance of quinones". Among the most relevant "Editorial" documents, some examples are: "Quinone binding and catalysis" (MacMillan, and Hunte, 2010); "Quinones and quinone enzymes" (Sies and Packer 2004a, b).

Figures 9.2 and 9.3 respectively report the most productive countries/territories and institutions. Regarding countries/territories, the USA ($n = 15{,}128$) was the most productive country, followed by China ($n = 8087$) and Japan ($n = 6182$).

The most productive institution was the Chinese Academy of Sciences ($n = 1095$). All the Top 10 institutions contributed with more than 379 publications.

The main subject areas covered are *Biochemistry, Genetics and Molecular Biology*, *Chemistry*, *Pharmacology, Toxicology and Pharmaceutics*, *Agricultural and Biological Sciences*, and *Medicine*.

As instance, in the *Biochemistry, Genetics and Molecular Biology* area the most cited document (4071 times) is published by Minnikin et al. (1984) on "An integrated procedure for the extraction of bacterial isoprenoid quinones and polar lipids."

Moreover, it can be observed that, among the top-recurring keywords, nonhumans, quinone derivative, unclassified drug, quinones, human, metabolism, chemistry, appear.

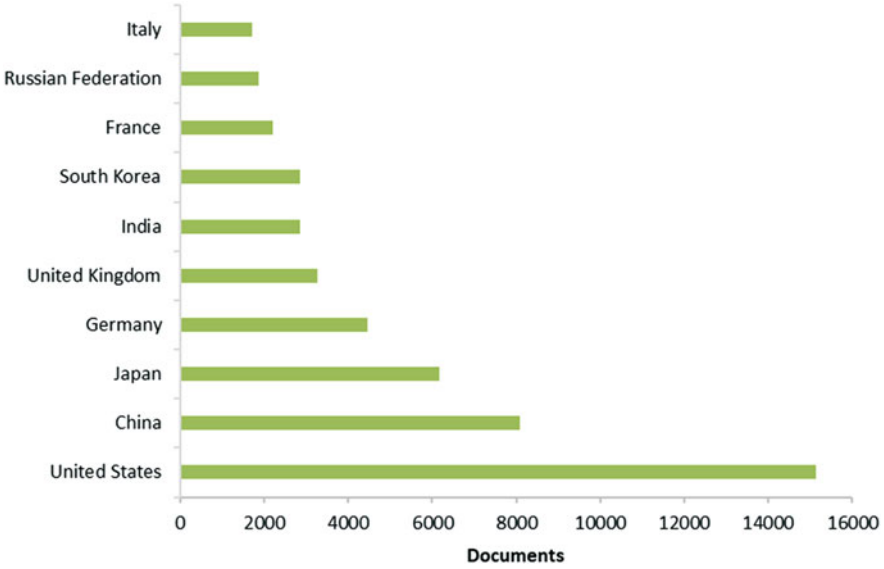

Fig. 9.2 Most productive countries/territories. (Based on data from Scopus)

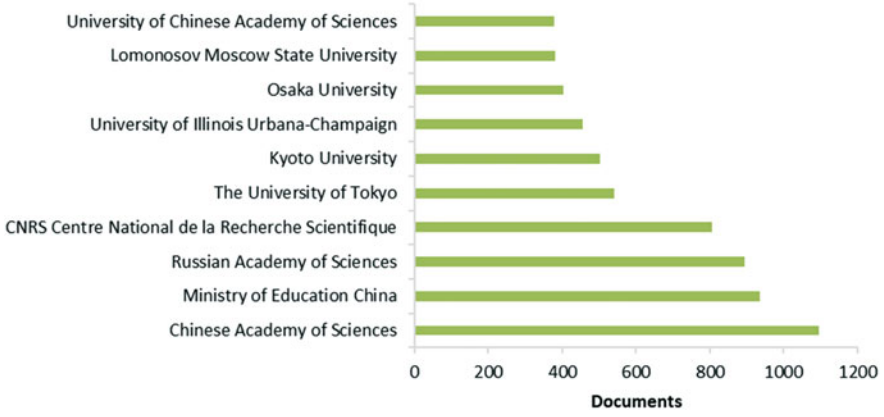

Fig. 9.3 Most productive institutions. (Based on data from Scopus)

9.2 Beyond the Chemistry: Anthra-, Benzo-, and Naphthoquinones

The structure and chemistry of bioactive compounds are the basis for their functionalities. Their structural skeleton makes them suitable as scaffolds in organic synthesis and versatile in different applications. They undergo a variety of interesting reactions. Over the past decade, a great number of new quinone-based compounds

were isolated from natural sources and quinones with diverse structural features were synthesized.

For anthraquinones, it is worth mentioning the work of Gartman and Tambar (2022) on recent total synthesis of anthraquinone-based natural products. Anthraquinones are generally classified into anthraquinone monomers, which can be subdivided into hydroxy anthraquinones, anthranones and anthranols, and bianthraquinones according to the structure of the mother nucleus (Zhao et al. 2016). Although their biosynthetic pathways are not yet fully clear, anthraquinones can be biosynthesized from the polyketide or shikimate pathway as described by Han et al. (2002). Anthraquinones usually occur in plants as glycosides: for example, the sennosides from senna are O-glycosides while aloins are C-glycosides. Anthraquinone compounds can have different chemical classification that relies on the nature and the position of the substituents on the basic structure such as hydroxyls, ketones, methyls, methoxyls, and carboxyls (Li and Jiang 2018).

Benzoquinone compounds can serve as reactive intermediate in organic synthesis for its own high electrophilic character related to special carbonyl structure: they can be reduced easily and be used in the process of chemical reaction and as versatile biological electron carriers. They represent a valuable tool for the synthesis of biologically active complex heterocyclic ligands (Harry et al. 2017; Radhika et al. 2019). It is worth mentioning the recent review of Radhika et al. (2019) on recent advances and prospects in the chemistry of o-benzoquinones. The recent paper of Asha and Suma (2022) described the synthesis, electrochemical, and antimicrobial study of 2,5-diamino benzoquinones.

1,4-Naphthoquinones are compounds derived from naphthalene, with different structures and biological activities. Kumagai et al. (2012) documented the chemical biology of naphthoquinones and their environmental implications. It is worth mentioning the review of Kamo et al. (2018) on recent topics in total syntheses of natural dimeric naphthoquinone derivatives.

Wu et al. (2021a) provide a new approach to generate novel molecules in the commonly used chassis cells *Saccharomyces cerevisiae* and *Escherichia coli*.

Agarwala et al. (2022) studied and reported the experimental and calculated infrared spectra of disubstituted naphthoquinones.

It is worth mentioning the research published by Zamora and Hidalgo (2021) in the *Food Chemistry* journal on formation of naphthoquinones and anthraquinones by carbonyl-hydroquinone/benzoquinone reactions as a potential route for the origin of 9,10-anthraquinone in tea.

9.3 Occurrence: Anthra-, Benzo-, and Naphthoquinones

Quinone-based compounds are widely found in nature. Several natural quinone derivatives have been isolated in different natural sources from different families of plants, fungi, and some animals. For anthaquinones approximately 700 of these pigments have been isolated. Of the 700 compounds discovered, 200 of these came

from plants with the remaining ones from fungi, lichens, and insects (Duval et al. 2016). Searching in the EuroFIR eBASIS (Bioactive Substances in Food Information Systems) database (Kiely et al. 2010; Plumb et al. 2017; EuroFIR eBASIS n.d.) considered as the first EU-harmonized food composition database containing composition data and biological effects of over 300 major European plant-derived foods organized in 24 classes of compounds, 200 data points are present for anthraquinones and 3 for other quinones.

Concerning anthraquinones, the review of Shakour and Farag (2021) is addressed on current status and future perspectives of diverse host-associated fungal systems as a dynamic source of novel bioactive anthraquinones in drug discovery. Luo et al. (2017) reported a new anthraquinone and a new naphthoquinone from the plant *Spermacoce latifolia*. Shi et al. (2016) reported a new anthraquinone from seeds of *Cassia obtusifolia*. Çiçek et al. (2019) described two-dimensional qNMR of anthraquinones in *Frangula alnus* (*Rhamnus frangula*) using surrogate standards and delay time adaption. Gecibesler et al. (2021) described the isolation of secondary metabolites from *Rheum ribes* L. and the synthesis of new semi-synthetic anthraquinones.

On benzoquinones, Yu et al. (2021) reported the distribution of 2-tert-butyl-1,4-benzoquinone and its precursor, tert-butylhydroquinone, in typical edible oils and oleaginous foods marketed in Hangzhou City, China.

Moving towards naphthoquinones, their interest comes from their prevalence as natural products and as environmental chemicals. Devi et al. (2016) discussed the applications of naphthoquinones and their biosynthesis in carnivorous plants. Annisa et al. (2020) analyzed 1,4-naphthoquinone in the Indonesian medical plant from extract *Eleutherine palmifolia* (L.) Merr by UHPLC. Wu et al. (2021b) determined the main naphthoquinones in *Onosma hookeri* Clarke. var. *longiforum* Duthie and its optimization of the ultrasound-assisted extraction using response surface methodology. Qin et al. (2022) proposed a novel extraction strategy combining microemulsion and high voltage electric discharge for naphthoquinones extraction from walnut (*Juglans mandshurica* Maxim) green husk, as a competitive option to extract hydrophobic compounds in a less-organics-consumption way.

9.4 Biological Properties and Potential Nutraceuticals/Pharmaceutical Applications: Anthra-, Benzo-, and Naphthoquinones

Quinones and their derivatives play an important role in pharmaceutical, medicinal, and environmental applications. Anthraquinone derivatives show a multifaceted pharmacological activity including and not limited to laxative, anti-inflammatory, antifungal, antibacterial, antiviral, and neuroprotective effects. Morgan et al. (2022) described in vitro anticancer screening and preliminary mechanistic study of A-ring substituted anthraquinone derivatives. For benzoquinones, the mini-review of Silakari et al. (2020) is addressed on p-benzoquinone as a privileged scaffold of

pharmacological significance: 1,4-Benzoquinone is the key structural motif of numerous biologically active synthetic and natural compounds.

Farooq et al. (2019) studied novel anti-aging benzoquinone derivatives from *Onosma bracteatum* Wall. Miao et al. (2020) studied benzoquinone derivatives with antioxidant activity to inhibit activated hepatic stellate cells and attenuate liver fibrosis in TAA (Thioacetamide) induced mice. Wróbel-Biedrawa et al. (2020) reported anti-melanoma potential of two benzoquinone homologues embelin and rapanone.

In terms of the new drugs from the venom of dangerous animals (spiders, snakes, scorpions, snails), Carcamo-Noriega et al. (2019) reported 1,4-benzoquinone antimicrobial agents against *Staphylococcus aureus* and *Mycobacterium tuberculosis* derived from scorpion venom. It is worth mentioning the current paper of Badary et al. (2021) studied the thymoquinone as a promising natural compound with potential benefits for COVID-19 prevention and cure.

Naphthoquinones with 1,4-naphthoquinone pharmacophore can be considered as privileged structures in medicinal chemistry. Several studies reported for naphthoquinones, antibacterial, antifungal, antiparasitic properties, anti-inflammatory, antimicrobial, cardioprotective, anti-ischemic, hepatoprotective, antitumor, and neuroprotective activities (Wellington 2015; Qiu et al. 2018; Ahmadi et al. 2020; Aminin and Polonik 2020); particularly, Aminin and Polonik (2020) well described some biological properties and application of 1,4-Naphthoquinones. Naphthoquinones are reported to inhibit the growth of proliferative cells and microbes. As instance, Kim and Lee (2021) reported antifungal and antiaflatoxigenic properties of naphthoquinones toward Aspergillus flavus and their mode of inhibitory action on aflatoxin biosynthesis. Aranda-López et al. (2021) reported the cysticidal effect of a pure naphthoquinone on *Taenia crassiceps cysticerci*.

Ferraris et al. (2021) reported pre-clinical activity of amino-alcohol dimeric naphthoquinones as potential therapeutics for Acute Myeloid Leukemia.

Hosseini et al. (2021) studied the synthesis, and in vitro biological evaluations of novel naphthoquinone conjugated to aryl triazole acetamide derivatives as potential anti-Alzheimer agents.

The application of nanotechnologies (Souto et al. 2020a, b; Yeung et al. 2020) is a promising approach to enhance the therapeutic potential of a drug. Rani et al. (2022) showed how gum-based nanocapsules comprising naphthoquinones enhance the apoptotic and trypanocidal activity against *Trypanosoma evansi*.

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Chapter 10
Non-Alkaloid Nitrogen Containing Compounds

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Abstract Plants have been the target of a growing interest by the scientific community in chemical and nutritional characterization studies, due to the presence of bioactive compounds and their potential industrial use, namely in food and pharmaceutical sectors. Different secondary metabolites found in plants ensure their survival and reproduction. Non-alkaloids compounds are an important group of secondary metabolites present in the plant kingdom, which have been widely studied and aimed at their industrial application, namely in the pharmaceutical area. This group of compounds has an important ecological function of protection; and in addiction an excellent bioactive performance has been highlighted and exploited for promising application in the pharmaceutical industry.

This chapter focuses on the characterization of different classes of non-alkaloid nitrogen compounds: non-protein amino acids, cyanogenic glycosides compounds, and glucosinolates. Its chemical and structural characteristics as well as its biosynthesis and presence in plants will be presented. The bioactivities presented by each of the classes will be equally focused as well as their applicability.

10.1 Non-Protein Amino Acids

10.1.1 Structure and Biosynthesis

Amino acids occur naturally and can be found in their free or combined form. They are divided into two groups: essential and non-essential amino acids (Fig. 10.1). The former also called natural or protein amino acids (PAAs) are universally distributed and incorporated into proteins during ribosomal synthesis. For this group, there are

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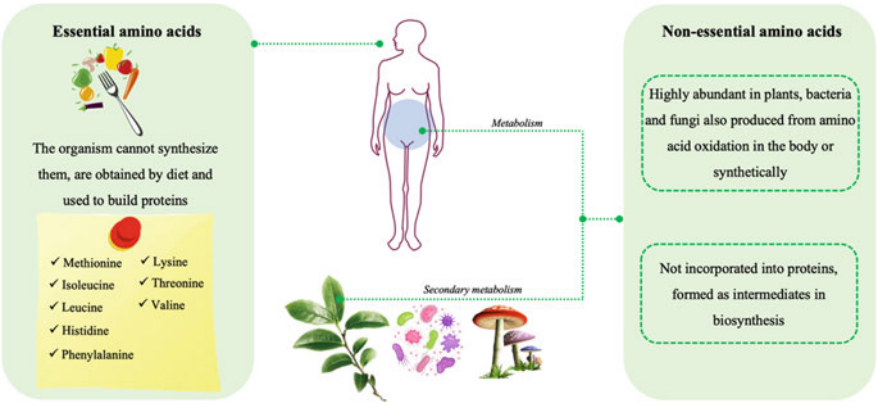

Fig. 10.1 The distinction between essential and non-essential amino acids and their origin. *Self-authored scheme*

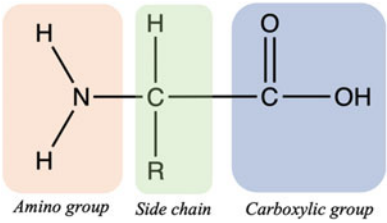

Fig. 10.2 Amino acid's general chemical structure. *Self-authored*

specific transfer RNAs and codon triplets (Bell et al. 2008). PAAs are commonly known, and most amino acid studies are related to this group as a result of their important role in protein synthesis. This group of amino acids corresponds to approximately 2% of the total existing amino acids. On the other hand, the group of non-essential amino acids comprises non-protein or non-proteinogenic amino acids (NPAAs) that do not have specific transfer RNAs or codon triplets. Although NPAAs are not naturally encoded in the human genetic code, organisms such as bacteria, fungi, and plants produce a wide variety of these compounds, which have several functions and properties (Ding et al. 2020; Vranova et al. 2011), they can also be produced from the oxidation of amino acids in the body and human metabolism (Bell et al. 2008; Jander et al. 2020; Parthasarathy et al. 2021). This section will focus on the NPAA group.

The general structure of amino acids consists of a central carbon, bonded to hydrogen (-H), a carboxylic group (-COOH), an amine group ($-NH_2$), and a side chain or radical (-R) that varies between the different amino acids (Fig. 10.2) (Bell et al. 2008; Maloy 2013). In PAAs, the amine group is linked to an α-carbon, while in NPAAs this group can be linked to a β-carbon (Selmar 2010).

More than 800 NPPAs occur naturally in different plant species. These have a diversified structure and functions (Ding et al. 2020), some of them are simple PAAs homologs, such as canavanine, which is an arginine analog (Selmar 2010). With the increase in the number of techniques and equipment available, as well as the

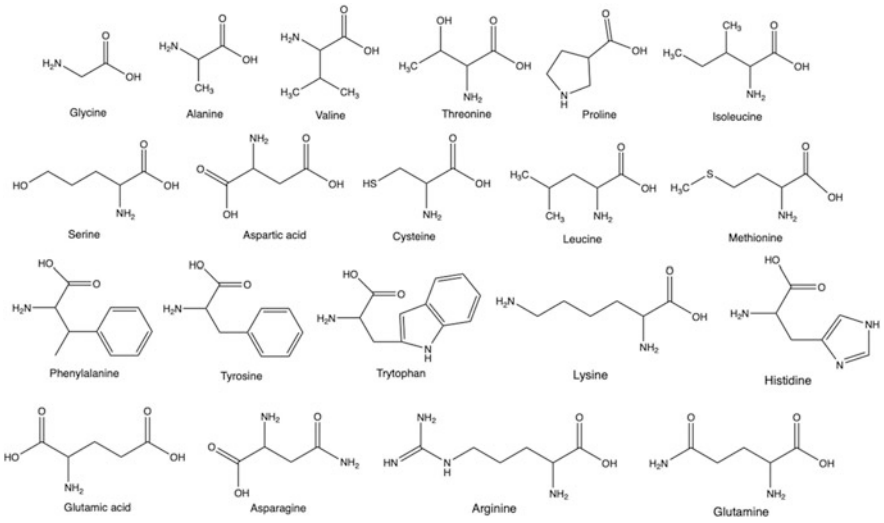

Fig. 10.3 Structures of some protein and non-proteinogenic amino acids (Self-authored)

associated sensitivity, this number is expected to increase (Bell et al. 2008). Although no generalized conclusions have been drawn about the structure/activity relationship of NPPAs, it appears that those with cyclic, heterocyclic, or aromatic skeletal structures play a structural role in plant cells, as well as a role as stress inducers, ion transport inhibitors, and of oxidation. In turn, those with an aliphatic structure perform signaling functions (Vranova et al. 2011). The chemical structures of some of the amino acids mentioned in this chapter are summarized in Fig. 10.3.

The biosynthesis of amino acid is associated with several biochemical pathways. The same amino acids can be synthesized by different metabolic pathways in different and/or the same organism (Bell et al. 2008). Contrary to what is observed for cyanogenic glycosides and glycosylates discussed in this chapter, the biology and biochemistry of NPAAs are poorly understood (Selmar 2010). While PPAs have a more complex biosynthetic process and most studies focus on their biosynthesis, it is known that NPPAs are synthesized by simpler pathways, mainly from glucose (alanine, glutamate, proline, serine, cysteine, aspartate), except for tyrosine which is synthesized from phenylalanine (Ayon 2020; Huang et al. 2011; Jander et al. 2020; Puigserver 2018; Selmar 2010). It is also known that the enzymes involved are the transaminases and that the carbon skeleton can be supplied as an intermediary in the glycolytic pathway and the citric acid cycle (or Krebs cycle) by the cells of the body (Litwack 2018).

Some NPAAs act as intermediaries for the biosynthesis of PAAs, such as *L*-ornithine, a metabolite resulting from arginine biosynthesis (Selmar 2010), or as a source of carbon and nitrogen, such as, for example, *L*-ornithine, *L*-homoserine, and *L*-adenosylmethionine (Huang et al. 2011; Jander et al. 2020). NPPAs may result from simple modifications of the PPAs' biosynthetic pathways through structural and

pathway product changes of existing PPAs, while others have their metabolic pathways (Bell et al. 2008). Pyruvic acid can give rise to alanine, and glutamic acid can give rise to arginine, while aspartic acid can give rise to asparagine, serine and finally threonine can give rise to glycine (Litwack 2018; Puigserver 2018). Glutamate, in turn, is synthesized by the reductive amination of α-ketoglutarate which, with the aid of glutamate dehydrogenase and followed by transamination, gives rise to α-ketoacid and NPAAs such as arginine, glutamine, proline, and histidine (Ayon 2020).

There are several biosynthetic mechanisms in which NPPAs are involved, playing a very important role in the central metabolism of plants. These compounds are found in different plant tissues, especially in leaves and seeds (Huang et al. 2011). They can be found in plants cultivated for food and forage purposes, in wild species to which animals have occasional access, as well as in toxic fungi, in algae consumed by humans, and in microalgae that can contaminate mollusks used in food (Bell 2003; Yamane et al. 2010). They are found in high concentrations in some species, including grasses and vegetables, namely in the families of Leguminosae, Sapindaceae, Aceraceae, Hippocastanaceae, and Cucurbitaceae, and are thought to be used as nitrogen storage compounds (Yamane et al. 2010). In *Dioclea megacarpa* Rolfe, its presence can correspond to approximately 8% of the dry weight of its seeds (Selmar 2010). The same occurs in several vegetables, which accumulate large amounts of canavanine, and other NPAAs, in their seeds as a form of nitrogen storage (Huang et al. 2011; Jander et al. 2020).

10.1.2 Presence and Function in Plants

The presence of NPAAs in plants is extremely important as a result of the many functions they perform, namely: anti-herbivorous, allelochemical, antimicrobial, fungicide, insecticide, signaling and protection against stress, nitrogen and carbon storage, as well as toxic effects against vertebrates and invertebrates (Bell 2003; Huang et al. 2011; Lea and Azevedo 2016; Parthasarathy et al. 2021; Vranova et al. 2011). These compounds provide to the plant the ability to protect and compete with other plants. Their similarity to PAAs may influence the primary metabolism (Selmar 2010). In addition to behaving as molecules with a protective function, these compounds allow increasing the species tolerance to stress factors such as temperature, salinity, and osmotic stress (da Rodrigues-Corrêa and Fett-Neto 2019). One of the examples is 1-aminocyclopropane carboxylate the direct precursor of ethylene, a hormone involved in different stress mechanisms and simultaneously a signaling molecule. Physiological processes such as cell wall biosynthesis, stomatal development, as well as responses to stress stimuli and pathogens are directly influenced by 1-aminocyclopropane carboxylate (Jander et al. 2020).

The presence of this class of compounds is often used with a competitive advantage for the plant in which they are found (Dunlop et al. 2015). Over the years, vegetable species have developed defense mechanisms with great sensitivity,

which allows them to evolve and protect themselves from invasive species, such as *Festuca rubra* subsp. *commutata* (commonly known as fescue herb), which deposits a large amount of meta-tyrosine in the surrounding soil (Dunlop et al. 2015). NPAAs are also found in floral nectar and are thought to affect the nervous system of insects, regulating nectar intake by altering muscle function (Nepi 2014). It is also described that gamma-aminobutyric acid (GABA) is essential in several physiological and developmental processes of plants, regulating plant metabolism, energy dissipation, fruit development, and plant immune responses. It is also involved in communication mechanisms, accumulating as a result of bacterial or fungal infections (Nepi 2014; Parthasarathy et al. 2021). *L*-canavanine is probably the most studied NPAA in plant defense against insects. It was first isolated from pig beans and is the main nitrogen storage compound in the seeds of several plants of the Leguminosae family (Huang et al. 2011). This NPAA is highly toxic, namely, against bacteria, yeasts, algae, fungi, plants, mammals, and insects (Huang et al. 2011). In turn, β-alanine is part of the constitution of vitamin B5, being a precursor of Coenzyme A and of the protein that transports carbon within the cell, is also involved in the biosynthesis of lignin, and in the composition of carnosine, a constituent of muscle tissue and cerebral (Parthasarathy et al. 2021). Although several NPPAs have been identified, the defensive functions they play and the biosynthetic mechanisms involved have not yet been elucidated (Dunlop et al. 2015).

10.1.3 Bioactivity

As previously mentioned, NPAAs have highly toxic effects, it is thought that they can present these effects through several mechanisms, namely in incorrect incorporation into proteins, or by interfering with both the primary metabolism and neurological processes of insects. However, some species have developed defense mechanisms against these compounds, which allow them to overcome their toxic properties through detoxification or changing the specificity for the toxin. One example is the beetle *Caryedes brasiliensis* [BRUCHIDAE], which feeds on the seeds of *D. megacarpa* that contain high amounts of canavanine and has developed a mechanism that allows it to discriminate against this amino acid (Huang et al. 2011; Yamane et al. 2010).

Although the production of NPAAs contributes to the defense and survival of some species, there are simultaneously those that are vulnerable and that develop undesirable effects, such as humans and animals. The literature reveals records of some toxic effects developed in some species when consumed in large amounts. For example, a study developed by Dunlop et al. (2015) revelead that the consumption of large quantities of *Lathyrus sativus* Linnaeus can cause skeletal abnormalities and neurological changes in humans, horses, and cattle. Ingestion of *Lathyrus odoratus* L. seeds is associated with cases of neurolathyrism, a neurodegenerative disease characterized by limb paralysis, muscle rigidity, as well as seizures, and emaciation (da Rodrigues-Corrêa and Fett-Neto 2019). Mimosine, present in the leaves and

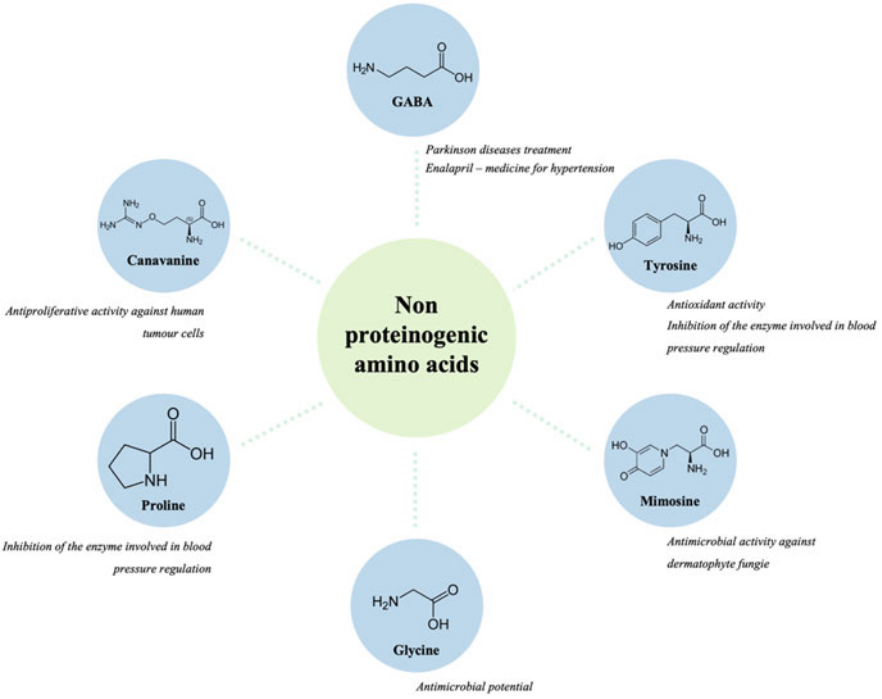

Fig. 10.4 Some of the bioactive properties associated with non-proteinogenic amino acids (Self-authored)

seeds of *Leucaena leucocephala* (Lam.), also presents toxic effects for cattle and sheep, its prolonged ingestion is associated with difficulty in gaining weight, hair loss, low fertility, and situations of abortion (da Rodrigues-Corrêa and Fett-Neto 2019). The consumption of *Macrozamia riedlei* (Gaudich.) leaves is also associated with the origin of "Zamia somersaults" in herds, as a result of the presence of the neurotoxin beta-methylamino-*L*-alanine (Dunlop et al. 2015).

Simultaneously, several studies refer to the biochemical potential of NPAAs, which have been showing varied potential (Fig. 10.4). GABA is an NPAA widely distributed in nature, found in animals, plants, and microorganisms. It is distributed throughout the human body and is involved in mechanisms that regulate blood pressure and heart rate (Sarasa et al. 2020). This amino acid is also the precursor of the neurotransmitter dopamine and is used for the treatment of Parkinson's disease (Dunlop et al. 2015; Kasperkiewicz et al. 2012) as well as in medications used to alleviate symptoms associated with hypertension, such as enalapril (Kasperkiewicz et al. 2012). Also, the presence of proline, or tyrosine at the *C*-terminal, was associated with the inhibition of the angiotensin-converting enzyme, involved in the regulation of blood pressure (de Castro and Sato 2015).

The study of the anti-proliferative effect of canavanine was tested and the anti-proliferative effect against blood mononucleocytes was verified (Bence et al. 2002).

Also, Vynnytska et al. (2011) demonstrated that canavanine can be used as a new therapy against several human cell lines, namely keratinocyte, cervical, hepatocellular, pancreatic, lung, and breast adenocarcinoma. Arginine deprivation in tumor cells triggers the activation of a set of mechanisms that cause cell apoptosis, its replacement by low doses of canavanine, its analog, significantly accelerated the occurrence of these mechanisms (Vynnytska et al. 2011).

Antibiotic resistance has become increasingly evident and worrying, which leads to an urgent need for the discovery of efficient molecules to combat these microorganisms. Given the ability of some plants to fight invading agents, some NPAAs have been tested. Mimosine, present in several *Fabaceae*, has a high potential against the dermatophyte's fungi *Trichophyton tonsurans* and *Trichophyton rubrum* as well as beta-(3-isoxazolin-5-on-2-yl)-alanine with potent activity against *Saccharomyces cerevisiae* (Narancic et al. 2019; Parthasarathy et al. 2021). Several studies are underway to improve the potential associated with this class of amino acids in bacterial biofilms (Ikeda et al. 2021). Sequences rich in glycine amino acids obtained from the cooking water of *Engraulis japonicus* are described as molecules with high antimicrobial potential (de Castro and Sato 2015; Tang et al. 2015). In turn, the presence of amino acid sequences such as tyrosine was described as potent antioxidants, namely in the reduction of Fe^{3+} to Fe^{2+}, as tyrosine can act as a proton donor and efficiently eliminate radicals with an electronic deficit (Duan et al. 2014).

10.1.4 Extraction, Purification, and Stability

As a result of the different bioactive properties associated with amino acids, several efforts have been made to determine and analyze the amino acid composition in different plant species. Although the total amino acid content can be determined by the colorimetric method of ninhydrin or 2,4-dinitrofluorobenzene, the chromatographic methodologies are quite limited. Studies describe the use of high-performance liquid chromatography (HPLC), anion exchange chromatography, as well as capillary electrophoresis. In some cases, when chromophore groups are present, it is necessary to proceed with a derivatization process in the pre-column or post-column, and use a suitable detector—by ultraviolet, fluorescence, or diodes. Although these methodologies are not efficient for all samples and amino acids, efforts continue to be made to improve, validate, and optimize methodologies. Regarding the extraction methodologies used, although there is very little information on this topic, solid-phase extraction is the most commonly used due to its fast, efficient, and economical nature (Shafaei et al. 2017). Due to the numerous properties associated with this class of compounds, their large-scale production has been explored. NPAAs can be obtained through different methodologies, namely through fermentation, enzymatic synthesis, as well as through chemical and/or biocatalytic processes.

The fermentation process consists of using microorganisms for the synthesis of amino acids that after synthesizing them accumulate in the cytoplasm. Its excretion

is caused by manipulating parameters such as cell membrane permeability and cell lysis provocation. This process takes place under aseptic conditions, temperature control, and pH. In turn, enzymatic synthesis involves microbial cells or the corresponding enzymes to convert the intermediate to the desired amino acid or its precursor. In addition to the numerous varieties of naturally produced NPAAs, these can also be obtained through synthetic methodologies. Most are synthesized based on the structure of naturally occurring amino acids and can be obtained from chemical, biocatalytic processes, or a combination of both. The main difficulties of its chemical synthesis are related to low yield, complexity, and the number of reaction steps, as well as stereoselectivity (Narancic et al. 2019). In turn, the biocatalytic process, despite having fewer steps than chemical synthesis, involves several enzymatic reactions and consequently a high cost of the cofactors used, especially when large-scale production is required (Ding et al. 2020). The main challenge is associated with dependence on cofactors, which make the process more expensive and often require a complex and less efficient approach. Furthermore, enzymes involved in synthesis may have stability problems or low activity, although this challenge can be overcome through protein engineering (Narancic et al. 2019).

10.1.5 Applications

In addition to their large-scale production being explored, NPPAs have also been used to produce peptide drugs. Its incorporation can improve pharmacokinetic aspects such as permeability, stability, bioavailability, specificity, half-life, as well as its potency since proteases recognize their substrates based on the amino acids involved in the site of cleavage (Ding et al. 2020; Kasperkiewicz et al. 2012). Furthermore, the incorporation of amino acid sequences with specific biochemical activity contributes to the enhancement of the demonstrated bioactive properties (de Castro and Sato 2015). Conversely, aspects such as immunogenicity and toxicity maybe some undesirable effects (Ding et al. 2020; Narancic et al. 2019). Several authors report that the incorporation of amino acid sequences with certain characteristics promotes bioactive potential.

10.2 Cyanogenic Glycosides Compounds

10.2.1 Structure and Biosynthesis

Cyanogenic glycosides (CG) are water-soluble secondary metabolites found in abundance in plants and classified as phytoanticipins. Although they are present in more than 2500 species, only 112 naturally occurring CG are described in the phytochemical literature. Structurally, this type of compounds is composed of an aglycone of the α-hydroxynitrile type attached to a sugar moiety with four possible

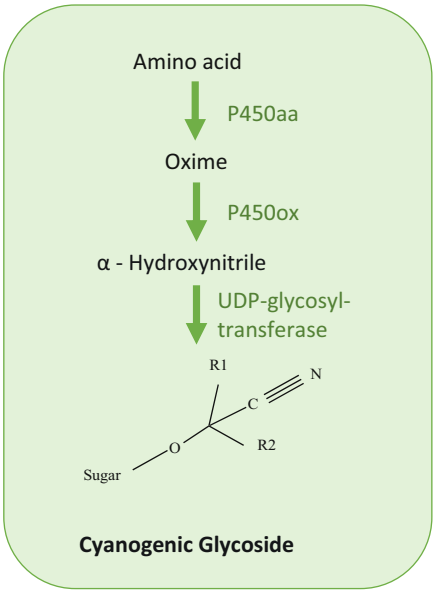

Fig. 10.5 Cyanogenic glycoside biosynthesis. Adapted from Gleadow and Møller (2014)

substituent attachment positions (Ganjewala et al. 2010; Sun et al. 2018; Yulvianti and Zidorn 2021). Aglycone, being constituted by a nitrile group linked to an aliphatic, cyclic, heterocyclic, or aromatic moiety, its biosynthesis occurs from aliphatic protein amino acids, *L*-valine, *L*-isoleucine, and *L*-leucine; from aromatic amino acids, *L*-phenylalanine and *L*-tyrosine; and the non-protein aliphatic amino acid, cyclopentenyl-glycine (Naveena et al. 2021; Yulvianti and Zidorn 2021). The most common sugar moiety in these compounds is glucose; however, the presence of allose, apiose, arabinose, rhamnose, and xylose in the molecular structure of CG is also reported (Yulvianti and Zidorn 2021).

The the formation process of CG in plants (Fig. 10.5) is mediated by cytochrome P450 monooxygenases and starts with the conversion of *L*-tyrosine (amino acid) into *p*-hydroxyphenylacetaldoxime through the CYP79A enzyme, followed by the formation of α-hydroxynitrile (cyanohydrin) from the oximes through of CYP71 or CYP736 enzymes after aldoxime decarboxylation and nitrile dehydration, finally cyanohydrin glycosylation occurs by soluble UDP-glycosyltransferase (Beran et al. 2019; Nyirenda 2020).

Of the 112 CG described in literature as occurring naturally, there are compounds that have been more studied, as they are present in economically important natural matrices. Thus, the most common compounds derived from tyrosine, phenylalanine, valine, and isoleucine are dhurrin, amygdalin, linamarin, and lotaustralin, respectively (Fig. 10.6) (Gleadow and Møller 2014).

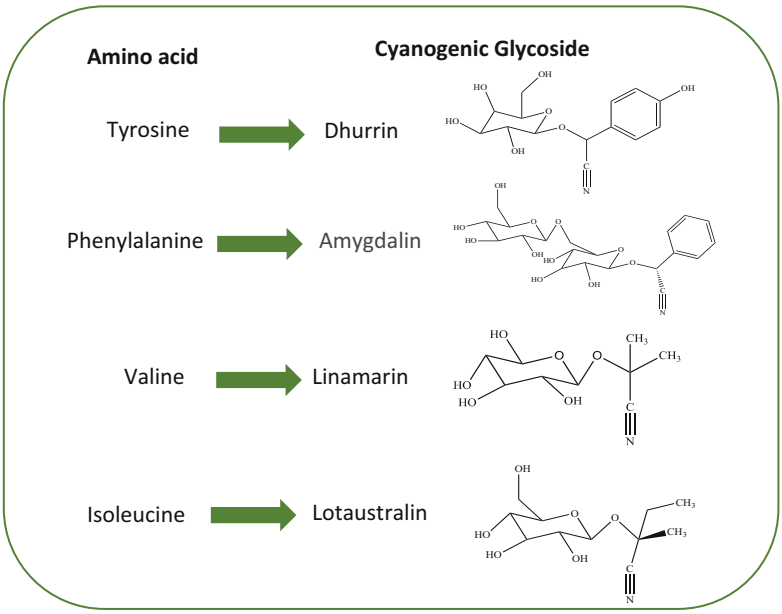

Fig. 10.6 Amino acids that give rise to the most common cyanogenic glycosides and their chemical structure. Self-authored scheme

10.2.2 Presence and Function in Plants

The CG are stable compounds (pH < 6) present in different plant families, including Euphorbiaceae, Fabaceae, Poaceae, Rosaceae, Asteraceae, Linaceae, and Passifloraceae (Table 10.1). When the hydrolysis of the β-glycosidic bond occurs through enzymatic action (β-glycosidase) the stability of these compounds is compromised, as this action results in the dissociation of labile cyanohydrin and the consequent release of hydrogen cyanide (toxic) (Fig. 10.7) (Beran et al. 2019; Gleadow and Møller 2014; Nyirenda 2020).

The biological function of CG is related with the regulation of the cell signaling, growth and defense of plants against various threats (microorganisms and predators), being particularly important in the initial stages of plant development. Thus, plants in vegetative states appear to present a higher concentration of these compounds mainly in young leaves, flowers, fruits, and seeds (Beran et al. 2019; Yulvianti and Zidorn 2021). Due to their chemical structure, CG can have other functions in the plant, namely in the storage, transport, and endogenous renewal of nitrogen in primary metabolism, that is, these compounds contribute to the management of nitrogen use by plants under variable environmental conditions; and they can also mitigate oxidative stress resulting from overproduction of reactive oxygen species (Cowan et al. 2021).

Table 10.1 Plants with cyanogenic glycoside

Cyanogenic glycosides	Plants families	Species example	Part of the plant	References
Linamarin	Euphorbiaceae	*Manihot esculenta* Crantz	Leaves, stem, root	Bolarinwa et al. (2016a), Yamane et al. (2010)
Lotaustralin	Fabaceae	*Lotus japonicus* L.	Flowers, leaves	Lai et al. (2015)
Dhurrin	Poaceae	*Sorghum bicolor* L. Moench	Leaves	Bolarinwa et al. (2016a), Yamane et al. (2010)
Amygdalin	Rosaceae	*Malus domestica* Borkh.	Seeds	Bolarinwa et al. (2016a), Yamane et al. (2010)
Prunasin methacrylate	Asteraceae	*Centaurea microcarpa* Coss. & Durieu ex Batt. & Trab.	Leaves, flowers	Baatouche et al. (2019), Yulvianti and Zidorn (2021)
Linustatin	Linaceae	*Linum usitatissimum* L.	Linseed	Harenčár (2021)
Passibiflorin	Passifloraceae	*Passiflora edulis* Sims	Leaves	Patel et al. (2011)

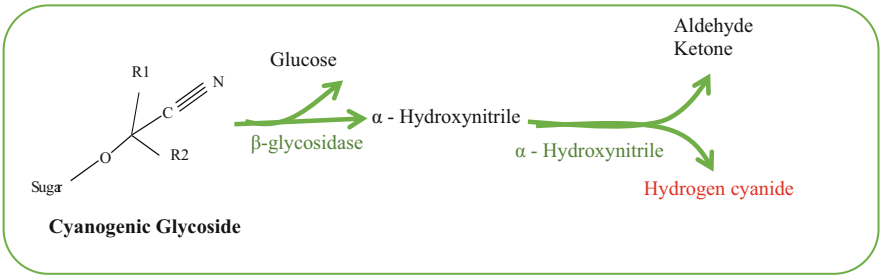

Fig. 10.7 Hydrolysis of the β-glycosidic. Adapted from Gleadow and Møller (2014)

It is important to emphasize that the hydrolysis of the glycosidic bond only occurs naturally when α-hydroxynitriles are in a medium with neutral or alkaline pH. CG are found in different parts of plants, they are stored in vacuoles and mostly in leaf tissue. In its turn β-glycosidases are found in the apoplastic space attached to the cell walls, in the vesicles, cytoplasm, or chloroplast and hydroxynitrile enzymes are present in the cytoplasm. However, the hydrolysis process can be triggered by external factors such as chewing, grinding, crushing and immersion or fermentation in presence of water (Bolarinwa et al. 2016a; Harenčár 2021).

Hydrogen cyanide is classified as a strong toxin, because when it binds to mitochondrial cytochrome oxidase it blocks cell respiration. Thus, the excessive consumption of matrices containing CG can lead to hydrogen cyanide poisoning causing milder pathologies. Accelerated breathing, dizziness, headaches, vomiting, stupor, mental confusion, and more serious pathologies such as irreversible paralytic

disorder, tropical ataxic neuropathy, optical atrophy, angular stomatitis, sensory gait ataxia, neurosensory deafness, goiter, and cretinism are some of the pathologies described in the literature (Gleadow and Møller 2014; Mosayyebi et al. 2020; Yulvianti and Zidorn 2021). In a review about CG in edible plants, Bolarinwa et al. (2016a), described that the toxicity of these compounds can occur with the ingestion of 0.5 and 3.5 mg of hydrogen cyanide per kilogram of body weight and adds, that it is difficult to estimate the optimal concentration of CG present in the diet, as its toxicity is estimated based on the concentration of free cyanide after hydrolysis.

CG are present in several food products, namely in almonds, stone fruit, pome fruit, cassava, bamboo shoots, linseed/flaxseed, lime beans, coco yam, chickpeas, cashews, and kirsch, as well as in the seeds of some fruits, such as apples, apricots, cherries, peaches, plums, and quinces. An uncontrolled ingestion of these foods can lead to hydrogen cyanide poisoning; however, the human body can tolerate a low amount of this toxin, managing to convert it into thiocyanate that is excreted in the urine (Bolarinwa et al. 2016a; Harenčár 2021). As many of these foods are part of the population's diet, the correct and sustainable detoxification of these products becomes a challenge for the food industry. Zhong et al. (2021) studied the elimination of CG and cyanide and hydrogen from cassava through an ultrasonic pre-treatment, and found that the applied treatment is more effective, faster, and safer in removing the compounds than the traditional immersion method, which takes days to remove the cassava cyanide.

10.2.3 Extraction, Purification, and Stability

A cyanogenic glycoside was isolated for the first time in 1830, from a vegetable source, namely, amygdalin obtained from *Prunus dulcis* (Mill.) D.Webb var. *amara* (DC.) H.Moore. Later in 2018, the last naturally occurring cyanogenic glycoside was isolated, namely the prunasin methacrylate was isolated from *Centaurea microcarpa* Coss. & Durieu ex Batt. & Trab. (Yulvianti and Zidorn 2021).

The extraction of CG from natural sources requires that the most adequate and sustainable methodology be selected. In addition, it is necessary to consider the polarity of these compounds when choosing the best extraction solvent and verify whether temperature or exposure to light can influence their degradation. Finally, it is also essential to select the most suitable extraction time to avoid solvent saturation (Rodríguez Madrera and Suárez Valles 2021; Shahwar et al. 2019). Different methodologies are described in the literature for the extraction of this type of compounds, from more conventional methodologies applying a solid/liquid extraction with hydroethanolic solvent (70%) at 30°C for 30 min (Senica et al. 2016a) to more innovative and sustainable methodologies such as using ultrasound-assisted extraction applying powers of 80% during 55 s (Rodríguez Madrera and Suárez Valles 2021).

Table 10.2 Examples of extraction techniques of cyanogenic glycosides from natural matrices

Cyanogenic glycosides	Natural matrices of extraction	Extraction techniques	References
Amygdalin	Seeds of peaches	The sample defatted with ethyl ether for 3 h and was reflux extracted with methanol at 60°C for 6 h.	Lee et al. (2017a)
Amygdalin	Cassava	Extraction with ethanol and the samples were boiled under reflux for 20 min.	Bolarinwa et al. (2016b)
Linustatin Neolinustatin Linamarin Lotaustralin	Flaxseeds	Extraction of whole seeds at room temperature on a shaker overnight. Crushing seeds in liquid nitrogen with hydroethanolic extraction (80%) in boiling. Hydroethanolic extraction (70%) at 7°C for 1 h.	Barthet and Bacala (2010)
Linamarin Lotaustralin Taxifilina	Cassava roots and bamboo shoots	Samples were ground in liquid nitrogen and the extraction was made with hydromethanolic solvent (80%) in an ultrasonic water bath (500 W) for 15 min.	Zhong et al. (2020)
Sambunigrin	Elderberry fruit	The sample was extracted with hydromethanolic solvent (70%) for 30 min at 30°C.	Senica et al. (2016b)

Regarding the choice of solvent, although CG contain at least one sugar molecule (high polarity–affinity for water), the literature describes the use of alcoholic and hydroalcoholic solvents to extract these compounds (Barthet and Bacala 2010; Zhao et al. 2019). Barthet and Bacala (2010) studied the optimization of CG from flaxseed (*Linum usitatissimum* L.) extraction methodologies and reported that there are several methodologies (Table 10.2), the most used being the extraction of ground seeds with 70% methanol in a water bath (30°C) with sonication during 1 h. Zhao et al. (2019) in his study on the quantification of glycosides in flaxseed report that CG (linustatin, neolinustatin, linamarin, lotaustralin) present in this matrix are conventionally extracted from defatted flaxseed using aqueous methanol solutions and ethanol. Regarding the temperature and extraction time applied to extract cyanogenic glycosides, methodologies that use different times and temperatures are described in the literature. In a study developed by Barthet and Bacala (2010) the optimization of CG extraction from flaxseeds was carried out at extraction times of 5, 15, 30, and 60 min and verified that although with 60 min they manage to extract a greater amount of compounds, the difference in yield between the extraction times is very low and they always applied the same extraction temperature of 40°C.

After the extraction of cyanogenic glycosides, it is important to carry out a purification step of the extract, to eliminate structural analogs compounds (bioisosteres, isomers, pathway intermediates, or degradation pathway metabolites) to obtain a pure extract with the desired pharmacological effects. Of the purification methods described, chromatographic is pointed out as the most accurate for the

purification of natural extracts, as is the case of column chromatography on silica gel (Gupta 2019; Samanthi et al. 2021). Samanthi et al. (2021) studied linamarin purification techniques from cassava, the compounds were separated by silica gel column chromatography and the compounds were eluted with 12% methanol in ethylacetate and their identification was made by thin-layer chromatography. In turn, Lee et al. (2013) studied the quantification of amygdalin in almonds (*Prunus dulcis* Mill.) and used a C18 solid-phase extraction (SPE) column to purify the compounds, taking advantage of the hydrophobicity.

Regarding the identification and quantification of these compounds, the literature describes several quantification methodologies such as high-performance liquid chromatography with refractive index detector (HPLC-RI), high-performance liquid chromatography with ultraviolet-visible detector (HPLC-UV-Vis), high-performance liquid chromatography with photo diode array detectors (HPLC-DAD), high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS), gas chromatography (GC), and thin-layer chromatography (TLC) (Rodríguez Madrera and Suárez Valles 2021; Zhao et al. 2019). HPLC and HPLC-MS/MS are described as the most viable methods for analyzing and detecting the cyanogenic glycosides. Li et al. (2019) in their study where they address the effects of germination on cyanogenic glycosides in flaxseed (*Linum usitatissimum* L.) identified and quantified four cyanogenic glycosides by HPLC-MS/MS.

After obtaining a pure extract, it is important to check its stability and define under which conditions the compounds maintain their activity. In particularly case of cyanogenic compounds, the stability is directly related to glycolysis, that is, glucose molecules (Mosayyebi et al. 2020; Szeja et al. 2016). Thus, stability through glycolysis is based on blocking the nucleophilic sites of the aglycone in order to reduce the interaction with cellular components (Mosayyebi et al. 2020). In practice, the stability of extracts of CG from flaxseed (*Linum usitatissimum*) was verified by Barthet and Bacala (2010) and concluded that the extract of linustatin and neolinustatin can be stored for one day at room temperature, but must be kept in refrigeration or freezing after 24 h. Also, Marian et al. (2018) studied the thermal behavior of amygdalin and verified that this cyanogenic glycoside in a nitrogen atmosphere is thermally stable up to 250°C. On the other hand, Castada et al. (2020) concluded that the hydrolysis of CG in macadamia is predominantly induced by changes in pH and not by heat, which indicates that CG present in this matrices are thermally stable compounds and are sensitive to changes in pH.

10.2.4 Bioactivity

There are several studies that report the bioactivity and therapeutic properties, namely, antioxidant, antibacterial, anti-inflammatory, anti-neuroinflammatory, healing and analgesic of CG (Senica et al. 2016a; Shi et al. 2019; Yang et al. 2021). These compounds are also associated with the treatment of cancer, atherosclerosis, gastric ulcer, psoriasis, arthritis, asthma, bronchitis, emphysema, leprosy,

and diabetes (Amaya-Salcedo et al. 2018; Pandey et al. 2020; Senica et al. 2016a). Of the most abundant cyanogenic glycosides, amygdalin is the compound that has shown the greatest therapeutic capacity. Hwang et al. (2008) studied the analgesic activity of amygdalin in rats and the results suggested that amygdalin is effective in relieving inflammatory pain and can be used as an analgesic with antinociceptive and anti-inflammatory activities. Also, Tang et al. (2019) carried out a study on the effect of amygdalin in acute liver injury in mice and concluded that this cyanogenic glycoside reduces lethality, improves histopathological changes in the liver, and significantly inhibits inflammatory responses.

Due to its chemical properties, amygdalin has been studied by several researchers, especially those dedicated to cancer treatment. Song and Xu (2014) described amygdalin as a potent antitumor, justifying with the fact that this cyanogenic glycoside has the ability to decompose cancerous substances in the body, killing cancer cells, blocking the source of nutrients for tumor cells and inhibiting the growth of cancer cells. Mamdouh et al. (2021) investigated the effect of amygdalin on hepatocellular carcinoma and concluded that this compound is a good cytotoxic agent in vitro, showing the ability to induce necrosis and interrupt the cell cycle.

The results of studies of the pharmacological properties of amygdalin are very promising; however, there are clinical studies that demonstrate that amygdalin metabolites can convert to hydrocyanic acid. The major concern centers on the fact that accumulation of hydrocyanic acid over the time, as mentioned above can produce adverse toxic effect on the human body, so it is important to develop targeted therapy methodologies for cancer (Shi et al. 2019). Suicide gene therapy, antibody-directed enzymatic prodrug therapy, and nanoporous imprinted polymers emerge as promising methodologies to reduce cyanide toxicity in healthy cells, while simultaneously, inducing antitumor effects in cancer cells (Mosayyebi et al. 2020).

10.2.5 Applications

Compounds extracted from natural matrices are increasingly used by several industries, namely food, pharmaceutical, cosmetics, and agricultural industries. Given that CG have some toxicity associated with their application in the food industry is very limited, so their main application has been in the pharmaceutical and agricultural industry. Park and Coats (2002) propose CG as an alternative to insecticides because, when present in plants, cyanohydrins can be used as an alternative fumigant and also as a soil fumigant.

Shi et al. (2019) in the review about the potential of amygdalin as an anticancer agent, demonstrating that this compound has an antitumor effect on lung, bladder, and renal cell carcinoma, as it affects the cell cycle in a way to induce apoptosis and cytotoxicity. Also, Halenár et al. (2013) summarize the positive effects of this compound in the treatment of some cancers, but emphasize the fact that the Food and Drug Administration (FDA) does not approve amygdalin as a treatment for

cancer, justifying itself with the lack of clinical evidence about its efficacy and the potential toxicity of these compounds for humans.

Although there are several studies that demonstrate the potential of CG for industries, as far as we know its commercialization and use is not allowed in the USA and Europe. For example, it is forbidden to sell amygdalin and laetrile (a less harmful amygdalin derivative with one subunit of glucose). However, there are laboratories and clinics in Mexico that are offering amygdalin preparations and therapies, as "Cyto Pharma De Mexico," which has been in the market for 40 years (Jaszczak-Wilke et al. 2021). It is considered that more studies are needed to obtain an extract rich in CG with industrial application (pharmaceutical) and without consequences for human health.

10.3 Glucosinolates

10.3.1 Structure, Localization, and Function

Glucosinolates (GSLs) are secondary metabolites found primarily in the Brassicales order. This order comprises several families, being Brassicaceae (also called Cruciferae), the most studied. This family contains more than 3000 species within 350 different genera, and so far, all the studied plants of this genus include GSLs (Fahey et al. 2001; Rodman et al. 1996).

GSLs are formed by a core structural element, the (Z)-thiohydroximate group. The core has a sulphate ester group at the oxygen position and a β-*D*-glucopyranose residue at the sulphur position. GSLs also present a variable side chain or R-group, which is described in the literature as the responsible for its biological effects (Fig. 10.8) (Blažević et al. 2020).

Several studies performed on *Arabidopsis* genus have elucidated amino acids as the precursors of these compounds, which define the subtype of GSLs to be synthesized. This synthesis consists of 3 steps: the first stage is the elongation of the amino acids present in the side chain through the insertion of methylene groups. As parent amino acids, Ala, Leu, Ile, Val, and Met lead to aliphatic GSLs; Tyr and Phe to aromatic GSLs, and Trp to indole GSLs (Ishida et al. 2014). Then the formation of the core structure of GSLs occurs during the second stage through a metabolic reconfiguration of the amino acid moiety. Finally, the parent GSLs formed can be subjected to secondary modifications of its R-group (Halkier and Gershenzon 2006).

10.3.2 Presence and Function in Plants

GSLs are responsible for the taste and odors of edible plants from the Cruciferous family (namely cauliflower, broccoli, and other similar vegetables) and of the

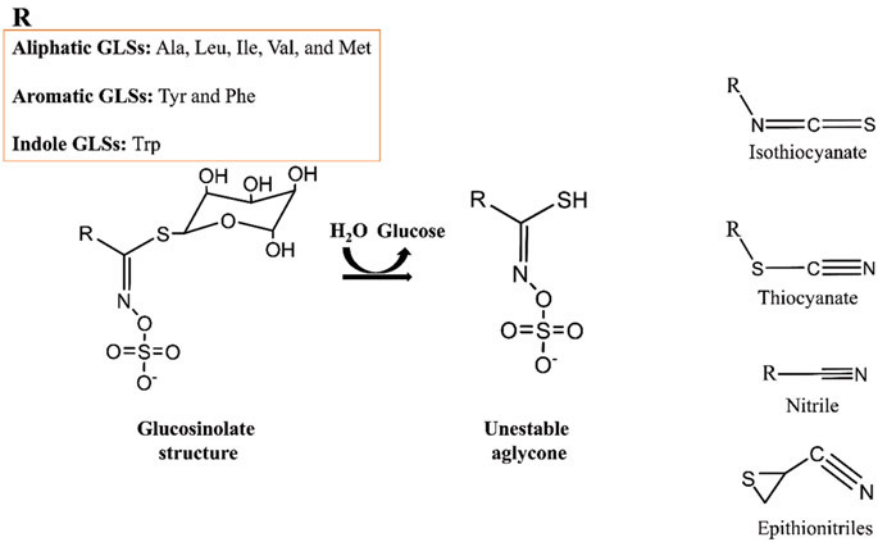

Fig. 10.8 Chemical structure of glucosinolates containing a side chain or R-group and a core structural element, which is formed by a (Z)-thiohydroximate group and a β-*D*-glucopyranose residue. In addition, the figure shows the different products formed during myrosinase hydrolysis. Image created with *ChemDraw, version 20.1.1.125*

condiments derived from these plants (mustard and wasabi, among others). These organoleptic characteristics are determined by their hydrolysis products (HPs) formed from the break of their thioglucoside linkage by enzymes known as myrosinases (thioglucoside glucohydrolases). These enzymatic reactions generate an unstable aglycone due to the loss of a glucose residue, which rearranges to form different breakdown products, including isothiocyanates, nitriles, epithionitriles, and thiocyanates (Fig. 10.8). The formation of several products depends on the previous structure of the GSLs side chain and the presence of additional proteins and cofactors. In addition, these HPs are also responsible for the important bioactive properties of GSLs, such as cytotoxicity against cancer cells or their use as weed biofumigation (Blažević et al. 2020; Engel et al. 2002; Halkier and Gershenzon 2006). In plants, the glucosinolate-myrosinase system is involved in the interaction with the environment during herbivore attacks. This system is regulated to prevent the continuous formation of HPs if the plant is not under attack through the physical separation of the enzymes from their substrate. Myrosinases are separated from GSLs by compartmentalization in different cell organelles or stored in "myrosin cells." Although GSLs and myrosinases are ubiquitous and scattered throughout all plant organs, GSLs are in cells with large vacuoles, while the enzyme is found within myrosin cells, which are in direct contact with the large vacuoles comprising GSLs. Thus, when herbivores damage the plant tissue, myrosinases' activity increases due to its interaction with GSLs, triggering intensive hydrolysis (Andréasson and Jørgensen 2003; Bones and Rossiter 2006; Sánchez-Pujante et al. 2017).

10.3.3 Extraction, Purification, and Stability

The extraction of GSLs and HPs has been widely studied for various purposes. Bioresidues obtained during oil extraction from seeds of the Brassicaceae family could be highly valuable for animal feed due to their content in protein and fiber, but their high concentration in GSLs makes them toxic for animals. Therefore, the detoxification of seeds used in animal feed has been extensively investigated through GSLs extraction. GSLs extraction has also been attracting attention due to its potential application in nutraceuticals, pharmaceuticals, pesticides, and biodiesel production (Deng et al. 2015).

The recovery of these compounds from natural matrices involves several steps, being the critical step the sample preparation. In the case of cruciferous crops, injuries, cutting, boiling, and fermentation should be avoided to prevent GSLs hydrolyzation by myrosinases. Freeze-drying of small plants or the use of liquid nitrogen on larger plants is recommended to prevent the reduction of these compounds (Śmiechowska et al. 2010).

Special attention must be paid to the extraction conditions when the compounds to be determined are the HPs. The pH is the most critical extraction condition; pH between 5 and 8 will produce higher hydrolysis of GSLs to isothiocyanate, while a more acidic pH will present a higher concentration of nitriles (Sivakumar et al. 2007). GSLs are water-soluble compounds usually extracted with water or aqueous organic solvents through solid–liquid extraction.

Conventional extraction methods such as maceration, percolation, or Soxhlet have been reported for GSLs extraction. Many modifications of these extraction were tested, being boiling water the most common methodology. However, these methodologies require long extraction times, large quantities of solvents, and high temperatures, which may imply a risk of compound degradation and represent high economic burdens and potential negative effects on the environment. Aiming to reduce these limitations by improving extraction efficiency, several non-conventional extraction techniques have been described. These innovative technologies include ultrasound, microwave, pressurized liquid extraction, pulsed electric field technology, and supercritical fluid extraction (Deng et al. 2015; Fomo et al. 2020; Roselló-Soto et al. 2016). Table 10.3 presents some examples of GSLs extraction methodologies.

The principal methods to determine these analytes concentrations in extracts are spectrophotometric and chromatographic.

The primary *spectrophotometric* technique to quantify the GSLs content in mature plants was developed and validated by Gallaher et al. (2012). Modified from a preliminary work published by Jezek et al. (1999), it is known as the ferricyanide assay and is based on the production of 1-thioglucose during the hydrolysis of GSLs. This compound is oxidized by ferricyanide, producing a chromogenic compound that can be measured spectrophotometrically. There are more specific methods that allow the colorimetric quantification of the indole GSLs, through its reaction with diazotised sulfanilic acid in the presence of o-phosphoric

Table 10.3 Conventional and non-conventional extraction methods used for GSLs and their HPs

Extraction Methodology	Plant material	Compounds	References
Conventional extraction methods			
Maceration	Roots, seedlings, and seeds of *Arabidopsis thaliana* Columbia-0	Glucosinolate profiles determined by HPLC	Wittstock et al. (2016)
	Leaves from *Brassica juncea, B. carinata, B. nigra, B. napus, B. campestris,* and *B. hirta*	Allyl isothiocyanate quantification by gas chromatography	Mayton et al. (1996)
Percolation	*Limnanthes alba* seeds	Isolation and structure determination of glucolimnanthin by HPCL and NMR	Stevens and Reed (2011)
Soxhlet	*Brassica oleracea* leaves	Glucosinolates content by colorimetric methods and determination of sulforaphane by HPLC	Pongmalai et al. (2015)
Non-conventional extraction methods			
Ultrasound	*Brassica napus* peel and pulp	Glucosinolates determination by HPLC-MS	Stefanucci et al. (2020)
	Brassica oleracea seedlings	Metabolomic determination including glucosinolates by EESI-MS analysis	Xue et al. (2021)
Microwave	*Brassica oleracea* (commercial cultivars Parthenon, Marathon, Nubia, Naxos, and Viola)	Glucosinolates determination by LC-DAD–ESI-MS	Ares et al. (2014)
	Eruca sativa seeds	HPLC analysis of desulfated GSLs	Omirou et al. (2009)
Pulsed electric field	*Brassica juncea* seeds	Glucosinolates quantification by HPLC	Hebert et al. (2020)
	Brassica oleracea from market	Sulforaphane quantification by HPLC	Mahn et al. (2021)
Pressurized liquid extraction	*Isatis tinctoria* leaves	Glucosinolates determination by HPLC-MS	Mohn et al. (2007)
	Broccoli leaves (*Brassica oleracea*, Parthenon, Nubia, and Naxos cultivars)	Glucosinolates determination by UHPLC–qTOF-MS	Ares et al. (2015)
Supercritical fluid extraction	*Eruca sativa* leaves	Glucosinolates determination by HPLC-MS	Solana et al. (2014)
	Sinapis alba seeds	Determination of 4-hydroxybenzylglucosinolate degradation products by HPLC and NMR	Buskov et al. (2000)

acid; or the total content of isothiocyanates by their ability to react with the compound 1,2-benzenedithiol (Thies 1990; Zhang et al. 1992).

Chromatographic techniques are most often used to determine GSLs and HPs, being liquid chromatography-tandem mass spectrometry (LC-MS) the most commonly applied (Thinh Nguyen et al. 2020). Analytical procedures based on this technique allow analyzing samples from appropriately extracts or juices of the plant material. The most widely technique employed is the High-Performance Liquid Chromatography (HPLC) with an inverted phase system. A preliminary step of enzymatic desulphation by a sulphohydrolase (sulphatase) may be needed (Śmiechowska et al. 2010).

Desulpho-GSL can be detected by an HPLC coupled to a DAD detector, while some HPs need an HPCL coupled to an evaporative light-scattering detector (ELSD). In addition, MS help to identify and explain the structures of the GSLs and their HPs, through different ionization methods. The most often used is the electrospray ionization with high sensitivity in determining these compounds (Moreno et al. 2006; Nakagawa et al. 2006). Nevertheless, electron-impact ionization (EI), chemical ionization (CI), ionization through fast-atom bombardment (FAB), chemical ionization under atmospheric pressure (APCI), and matrix-assisted laser desorption ionization (MALDI) are other methods also available (Bennett et al. 2004; Tian et al. 2005). In addition, nuclear magnetic resonance (NMR) can be used for the ultimate confirmation of GSLs structures (Olsen et al. 2016).

10.3.4 Bioactivity

The effects of GSLs and their HPs on the human body depend on the modifications and assimilation in the digestive tract. The hydrolysis by myrosinase enzymes is necessary to transform GSLs into their bioactive form, HPs. These enzymes are inactive in cooked plants; therefore, GSLs are hydrolyzed in the small intestine by gut bacterial and raw plant myrosinases (Barba et al. 2016; Hanschen et al. 2014). The biochemical transformation of GSLs in their HPs depends on the pH and the presence of different cofactors, which are specific proteins that modulate their transformation (Bernardi et al. 2000). Myrosinases are active at a wide pH range, from the stomach (2.0) to the small intestine (6.0) (Román et al. 2018). The major HPs, isothiocyanate, is formed through the Lossen rearrangement, where the aglucone of the GSLs decomposes into molecular sulfur and its corresponding nitrile. In addition, isothiocyanate may be formed in the presence of the epithiospecifier modifier protein (ESM). Other minor HPs are formed by different cofactors. The epithiospecifier protein (ESP), nitrile specifier proteins (NSPs), and thiocyanate-forming protein (TFP) favored the formation of the nitriles, through their interaction with the unstable aglucon. ESP may also stimulate the formation of epithionitriles from aliphatic GSLs, while TFP generates thiocyanates in the presence of 2 specific GSLs; 1, benzyl glucosinolate and 4-methylsulfanylbutyl

glucosinolate (Wentzell and Kliebenstein 2008; Williams et al. 2008; Zhang et al. 2006).

The glucosinolates HPs present significant bioactivities that benefit directly on human health, namely anti-inflammatory and cancer risk reduction, diminution of diabetes risk, prevention of osteoarthritis, neuroprotective effect, and antioxidant, antimicrobial, and antifungal properties. Chronic inflammatory diseases are associated with the onset of cancer development, especially concerning the digestive tract (Armstrong et al. 2018; Sturm and Wagner 2017). Several HPs have been correlated with the inhibition of inflammatory mediators. Sulforaphane has been associated with an improvement of colitis in mice by the modification of the expression levels of pro-inflammatory markers and Nrf2-regulated cytoprotective enzymes (Wagner et al. 2013). In addition, sinigrin (a GSL) showed high potential to reduce atherosclerosis, inhibit COX-2 expression, suppress the tumor necrosis factor (TNF)-α, and the production of interleukin (IL)-6 in macrophages. Broccoli metabolites also reduce several inflammatory factors, such as IL-6 and C-reactive protein (Jang et al. 2017; Lee et al. 2017a).

Regarding the prevention of the inflammatory disease osteoarthritis, which is characterized by inflammation, persistent pain, and neuropathic components; sulforaphane has been reported as an inhibitor of cartilage destruction and, additionally, high consumption of GSLs had a positive impact on the articular joint tissues (Davidson et al. 2013).

Several epidemiological studies reported a correlation between diet and cancer prevalence. Cruciferous vegetables are an interesting source of phytochemicals with anticarcinogenic properties, such as glucosinolates. The inclusion of these vegetables on a diet has been associated with preventing breast, stomach, colorectal, kidney, and prostate cancers (Sarıkamış 2011). Regarding the protective effect of isothiocyanates, its mechanism of action has been related to its ability to induce phase 1 and/or phase 2 detoxification enzymes, such as quinone reductase, glutathione-S-transferases, and UDP-glucuronosyl transferases (Munday and Munday 2004; Thinh Nguyen et al. 2020). Among them, sulforaphane has been extensively investigated as a potent activator of these enzymes in cell culture and animal studies. However, this compound has not yet been applied in therapies due to its short metabolic lifetime (2 h) (Zhou et al. 2018). Phenethyl isothiocyanate has also been pointed as an important compound in prostate cancer chemoprevention (Seo and Kim 2017).

Other significant examples of GSLs derivatives with proven anticancer effects are compounds that suppress the proliferation of tumor cells. Erucin causes discriminatory apoptosis in human leukemia cells, while indole-3-carbinol suppresses the proliferation of several cancer cell lines and prevents spontaneous tumorigenesis (Esfandiari et al. 2017; Weng et al. 2008). Thus, many studies have identified the factors that determine GSLs and their HPs anticancer bioactivity for many different cancer types. In addition, more than 30 proteins have been identified as targets for intracellular GSLs derivates interaction, and isothiocyanates have been reported as biochemicals with pleiotropic effects in all steps of chemoprevention (primary prevention during initiation carcinogenesis phase; secondary prevention during

promotion stage, and as adjunctive therapy during progression stage) (Hanschen et al. 2014; Mi et al. 2008, 2011).

Numerous studies have confirmed the efficacy of these compounds as neuroprotective. The mechanisms of action are related to those explained above for their anti-inflammatory and anticancer effects. These compounds regulate phase 2 enzymes, activating the response element to nuclear factor erythroid 2–related factor 2/antioxidant (Nrf2/ARE) in the central and the peripheral nervous systems (Jaafaru et al. 2018; Lafarga et al. 2018). In addition, sulforaphane is involved in the inflammatory pathways, apoptosis, and the reduction of the activation of cell death (Sikorska-Zimny and Beneduce 2020). The sulforaphane protective effect against neurodegenerative diseases was also evaluated in Alzheimer's disease mice models. These compounds improved cognitive impairment by reducing cholinergic neuron loss and significantly lessening the cholinergic system reactivity in mice's hippocampus and frontal cortex (Chen et al. 2011; Lee et al. 2014).

Obesity and diabetes are two major global disorders with a strong relationship, especially type II diabetes (Verma and Hussain 2017). Obesity is considered a low-grade chronic inflammation, and consequently, GSLs and derivates may help in the treatment/prevention of this metabolic disorder (Yuan et al. 2018). Glucoraphanin and its derivate sulforaphane have been reported to inhibit *in vitro* adipocyte differentiation and lipolysis (Martins et al. 2018). In fact, the consumption of broccoli sprouts, rich in these compounds, has been shown to increase genes related to lipolysis and considerable decrease inflammatory factors (López-Chillón et al. 2019; Raiola et al. 2017). Regarding their antidiabetogenic effect, glucotropaeolin derivates reduce hepatic glucose in mice, which plays an important role in preventing diabetes (Guzmán-Pérez et al. 2016). Moreover, glucoraphanin GSL produced an insulin-sensitizing impact in a US clinical trial with men and women (Ma et al. 2018).

In addition to the above-mentioned bioactive properties of GSLs and their derivates, they are also a potential natural agent for food preservation due to their antimicrobial effects (Saladino et al. 2017). Their biocidal properties have been associated with their HPs, being the isothiocyanates the most studied compounds in the food preservation and plant pathogen control field (Brandi et al. 2006).

Allyl isothiocyanate is the most studied HPs and has shown strong antimicrobial activity against a broad spectrum of bacteria. Its efficacy was tested to eliminate pathogenic bacteria from contaminated vegetable seeds. Allyl isothiocyanate reduced *Escherichia coli*, which can produce life-threatening hemorrhagic colitis, hemolytic uremic syndrome, and thrombotic thrombocytopenic purpura in young, old, and immunocompromised people (Park et al. 2000). Additionally, sulforaphane has also demonstrated a great potential to eradicate pathogenic microbes with high resistance to conventional agents. This compound enhanced the effect of several antibiotic combinations in the resistant gastric infection with *Helicobacter pylori* (Fahey et al. 2002; Haristoy et al. 2003).

The HPs of GSLs also present antifungal activity. Isothiocyanates showed *in vitro* inhibition of a broad range of fungi. Its efficacy against saprophytic and parasitic fungal species in culture medium, food products, and plant defense has been reported

and demonstrated in many works, being allyl isothiocyanate again, the most studied compound (Mayton et al. 1996; Saladino et al. 2017). Several analogs of this compound and 57 derivates of phenylethyl isothiocyanate exhibited a remarkable in vitro antifungal effect against 16 different strains. Additionally, this compound showed an antifungal impact in a low dose against fermentative yeast, mycotoxigenic molds, and mycotoxins in culture media and food systems (Drobnica et al. 1967; Manyes et al. 2015).

10.3.5 Applications

Beyond the bioactivities reported above, which can be used as future applications, some GSLs and their derivatives are either understudied or already commercialized. Some of these compounds are toxic for nematodes and insects, which are destructive pests for many crops. Thus, several works have studied the impact of their application as amendments into the soil or in a spray to apply in the aerial parts of plants (Pane et al. 2013; Zasada and Ferris 2004).

The most studied HP, allyl isothiocyanate, has been applied successfully to inhibit microorganisms' proliferation in tofu and different meats for food safety and control (Nadarajah et al. 2005a, b). Methods to extract rich-isothiocyanate oils from *Brassica* L. plants and generate several nutraceuticals, functional foods, and pharmaceuticals compositions have been developed. Teas, enriched oils, capsules, pills, drinks, supplements, additives, skin/hair, and agricultural are examples of the products containing interesting bioactive HPs on the market (Deng et al. 2015; Fahey 2005).

Regarding patents generated from GSLs compounds, a Google Patent search revealed over 3000 applications with 900 issued. There are some interesting purposes among the patents issued in the pharmaceutical field; one example is the development of a double topical formulation containing a GSLs precursor (specifically glucoraphanin, the precursor of sulforaphane) and a myrosinase as an activation agent. The precursor and the agent are both encapsulated to desirably treat and prevent skin ailments and other cancerous conditions due to their activation of phase II enzymes (Talalay et al. 2011)

10.4 Conclusions

Non-alkaloid compounds are secondary metabolites present in a wide range of plant matrices, which have been widely studied and characterized by the scientific community. These compounds have important defenses in plant metabolism, namely, protective functions. In addition, its bioactive characteristics have been highlighted and presented in several bibliographic studies. These characteristics have been explored due to their potential application in different industries, namely food,

agriculture, pharmaceuticals, and cosmetics. However, the toxicity of these compounds has limited their use, namely in the application of novel foods. Thus, despite the existence of many studies on the structure, and presence in different plant matrices, studies about the extraction optimization of these compounds are still very scarce. As other classes of these compounds have already been studied in more detail and there are already several patents for their extraction and application, essentially related to the application in pharmaceuticals and pesticides, it is considered that the scientific community still has a long way to go, to take full advantage of the bioactive potential of non-alkaloid compounds.

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Chapter 11
Sulfur-Containing Compounds from Plants

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Abstract Sulfur (S) is an essential nutrient assimilated through the diet and incorporated into organic structures such as amino acids, coenzymes, and other bioactive compounds. Plants' ability to regulate stress resistance via secondary metabolism

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has extended the interest in S-containing compounds, driven by their additional properties as bioactive molecules. Among plant families that produce S-compounds, the *Brassicaceae*, which includes broccoli, cabbage, and cauliflower, and the *Liliaceae* family, which includes garlic and onion, stand out. In recent years, the research has been focused on S-containing amino acids (mainly methionine and cysteine) and glucosinolates (GSLs) and their hydrolysis products like isothiocyanates but also in other S-containing compounds such as phytoalexins or cysteine sulfoxides. GSLs are becoming more popular because of their specific biological properties, including antioxidant, anti-inflammatory, or antimicrobial, among others. Accordingly, a diet rich in vegetables containing S-containing compounds has been associated with a lower risk of developing cancer, neurological diseases like Alzheimer's, inflammatory bowel disease, cardiovascular diseases, several skin disorders, and obesity. Hence, S-metabolites can therefore be used as therapeutic and preventative components in functional foods and nutraceuticals, as well as cosmeceutical products. This chapter aims to revise the most important features related to sulfur metabolism and S-containing compounds from plant sources, with emphasis on their involvement in secondary metabolism, natural sources, structural classification, biological functions, and applications in human nutrition and health.

Abbreviations

Generic

ATP	Adenosine triphosphate
APS	Adenosine 5′-phosphosulfate
PAPS	Phospoadenosine 5′-phosphosulfate
PAP	Adenosine 5′-phosphate
Cys	Cysteine
Met	Methionine
GSH	Reduced glutathione
GSSG	Oxidized glutathione
SAM	S-adenosylmethionine
SIR	Sulfite reductase
Cysta	Cystathionine
Hcy	Homocysteine
OAS	O-acetylserine
Glu	L-glutamate
Gly	Glycine
AMPS	Antimicrobial peptides
CRT	Chloroquine-resistance transporter
γ-EC	γ-Glutamylcysteine
TAAC	Paps/chloroplast thylakoid atp/adp carrier
SULTR	Sulfur transporters
SOT	Sulfotransferase
Ser	Serine

SMM	S-methylmethionine
NA	Nicotianamine

Compounds

MBIT	Methoxybenzyl isothiocyanate
SQDG	Sulfoquinovosyl diacylglycerol
AITC	allyl isothiocyanate,
SFN	Sulforaphane
PEITC	phenethyl isothiocyanate
MIC-1	4-[(α-L-Rhamnosyloxy) benzyl] isothiocyanate extracted from *Moringa oleifera*
7-MSI	7-methylsulfinylheptyl isothiocyanate
GSL	Glucosinolates
TSN	Thiosulfinate
ITC	Isothiocyanate
6-MITC	6-(methylsulfinyl)hexyl isothiocyanate
DNCB	2,4-dinitrochlorobenzene
DSS	Dextran sulfate sodium

Health and Diseases

AD	Alzheimer's disease
T2D	Type-2-diabetes
IL	Interleukin
NF-κB	Nuclear factor-κB
TNF-α	Tumor necrosis factor-α
Nrf2	Nuclear factor erythroid 2
TC	Total cholesterol
TG	Triglycerides
TrkB	Tropomyosin receptor kinase B
LDL-C	Low-density lipoprotein cholesterol
HDL-C	High-density lipoprotein cholesterol

11.1 Introduction

Sulfur (S) constitutes an essential nutrient for living beings, ranging from microorganisms to plants and animals, which present an assimilatory metabolism that enables the incorporation of this element to organic structures, such as biomolecules like amino acids, coenzymes, and bioactive compounds, among others (Fike et al. 2015). Regarding plant nutrition, sulfur is considered as the fourth major nutrient after the macronutrients nitrogen (N), potassium (K), and phosphorus (P), and it is involved in the biosynthesis of a plethora of different compounds, for instance: sulfolipids, antioxidants, cofactors, and secondary metabolites (Abadie and Tcherkez 2019; Prasad and Shivay 2018). Due to the relevance of these compounds, sulfur management is a crucial factor that regulates key physiological processes in

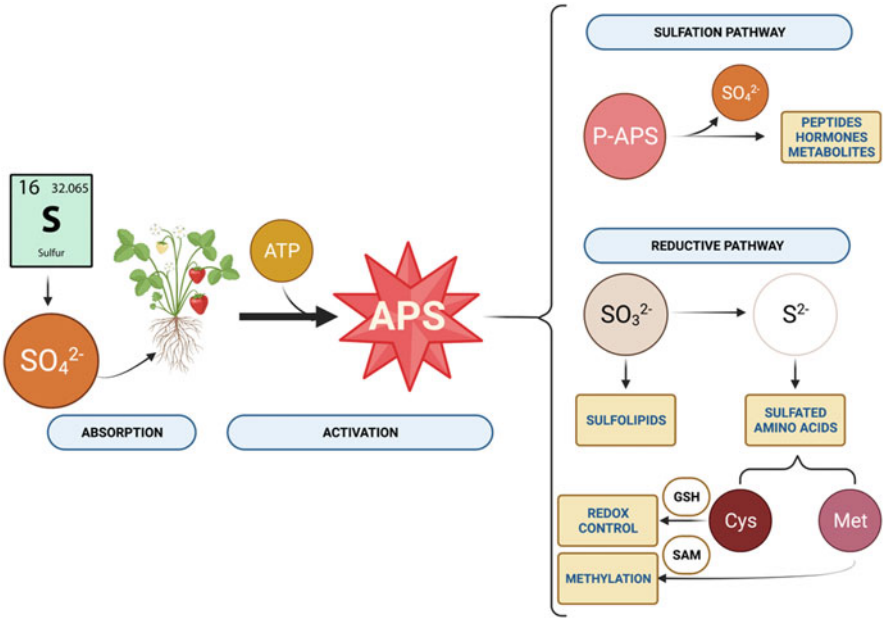

Fig. 11.1 General major sulfur assimilation pathways found on plants. Created with BioRender.com

plants, including photosynthetic performance and abiotic and biotic stress resistance (Capaldi et al. 2015). In this sense, the regulation of stress resistance via secondary metabolism has broadened the interest of S-containing compounds in the field of plant physiology, motivated by the additional properties of these phytochemicals as bioactive compounds.

Thus, concerning sulfur metabolism (Fig. 11.1), plants normally absorb it by roots in the form of sulfate (SO_4^{2-}, the principal mineralized S-containing ion present at soils), and this process can be enhanced by the presence of metallic cations, especially copper (Cu), selenium (Se) and zinc (Zn) (Na and Salt 2011). Once sulfate is uptake, it is further activated by the condensation with adenosine triphosphate (ATP) to form adenosine 5′-phosphosulfate (APS) via ATP sulfurylase. From that, two major pathways are followed (Prasad and Shivay 2018; Kopriva et al. 2019): on the one hand, the sulfation pathway involves the phosphorylation of APS to form phosphoadenosine 5′-phosphosulfate (PAPS), which is in charge of the donation of sulfate groups to relevant molecules, either from the primary metabolism and secondary metabolism, such as peptides and hormones. On the other hand, through the sulfate reductive pathway, sulfur is sequentially reduced by PAPS reductase to form sulfite (SO_3^{2-}), involved in the biosynthesis of sulfolipids, and sulfide (S^{2-}) by the action of sulfide reductase, required for the biosynthesis of sulfur-containing amino acids, cysteine (Cys), and methionine (Met). Afterwards, Cys may be further modified to synthesize glutathione (GSH), a molecular mediator

of redox cell status and responsible for cell chemical detoxification, whereas Met is considered as the precursor of S-adenosylmethionine (SAM), which constitutes a donor of methyl groups in different metabolic reactions, including the biosynthesis of ethylene.

With respect to S-containing peptides, these biomolecules play a definitive role in plant disease resistance against pathogenic microorganisms, as they are commonly known as pathogenesis-related antimicrobial peptides (AMPs), including different families, such as defensins and thionins, which are small Cys-rich peptides exhibiting potent bactericidal and fungicidal activities (Künstler et al. 2020). In the case of S-containing amino acids, Cys is a non-essential amino acid with a multifaceted catalytic mode of action, acting as metal ligands and participating in a series of post-translational modifications throughout the action of different thiol-oxidoreductases as thioredoxins and glutaredoxins (Couturier et al. 2013). As a result, due to the great variety of cysteine oxidation states, they are considered as cellular switches at a cellular level, modulating the biological activity of proteins and stress tolerance, via redox control (Couturier et al. 2013). In this sense, Cys is incorporated in the structure of GSH, together with L-glutamate (Glu) and glycine (Gly), which is recognized as a universal intracellular signaling mediator involved in key subcellular processes, such as reactive oxygen removal, DNA synthesis and protection, and signal transduction (Lv et al. 2019). Interestingly, recent reports have highlighted the potential role of GSH as an anticancer therapy, thanks to the promotion of the induction of programmed cell death (apoptosis and related processes) and autophagy of cancer cells (Lv et al. 2019). On the other hand, Met is one of the essential amino acids that cannot be synthesized by humans, being plants one of the major food sources. Due to its extreme sensitivity to oxidation, several oxidized forms of Met, commonly known as Met sulfoxides, can be observed in plant cells and tissues committed to the stress-related redox signaling (Rey and Tarrago 2018). Nevertheless, one of the key roles of Met in living beings is methylation, developed by the cofactor *S*-adenosyl methionine (SAM) throughout the action of methyltransferase on Met. In fact, SAM is considered as the second most used cofactor in nature (Zhang et al. 2021), motivated by the importance of methylation in different processes, ranging from natural product biosynthesis and regulation and the modulation of molecule stability and bioavailability, to epigenetics. On these bases, and keeping in mind all the functions associated with sulfur, it is important to note the deleterious impact of this essential nutrient in plant physiology, ranging from growth inhibition due to the impairment of nutrient uptake and the imbalance of photosynthetic performance (Aarabi et al. 2020; Zenda et al. 2021).

Moreover, sulfur has the ability of regulating the activity of phytohormones, as it is the case of abscisic acid, and polyamines, playing an effective indirect role in the tolerance against stress-related stimuli (Hasanuzzaman et al. 2018). In parallel, a wide range of S-containing secondary metabolites are isolated from plant sources, for instance: phytoalexins, such as camalexin or rapalexin A, glucosinolates (GSL) and their derivative isothiocyanates, and thiosulfinates, like allicin. All of them share a common feature, as these families are highly diverse low molecular weight antimicrobial compounds, whose efficiency has been proven by either in vitro and

in vivo models (Künstler et al. 2020). Among sulfolipids, one of the most important members of this family is sulfoquinovosyl diacylglycerol (SQDG), considered as one of the major constituents of thylakoid membranes within chloroplasts together with the glycolipids monogalactosyl diacylglycerol and digalactosyl diacylglycerol, which exhibits a definitive role in the biosynthesis of plant glycerolipids during cell membrane formation (Shimojima 2011).

Sulfur represents around 3% of the Earth's mass, however, at the Earth's crust it has been estimated to be under 0.1% being more abundant in the core (Walsh 2020). Concerning plant sources, different vegetable families stand out as the prominent rich sources of sulfur compounds. Thus, cruciferous vegetables belonging to Brassicaceae family, are considered the greatest sources of S-containing compounds, followed by species from other families like Liliaceae, Capparaceae, and Caricaceae (Aarabi et al. 2020). Among cruciferous, broccoli, cabbage, cauliflower, and related species constitute a great natural food source of GSL with associated immunoregulatory activity, whereas different *Allium* species (Liliaceae family), such as garlic and onion, are an excellent source of natural organosulfur compounds, like allicin, with associated beneficial potential for the treatment of cardiovascular diseases and cancer (Miekus et al. 2020). Therefore, the performance of S-containing compounds as bioactive molecules depends on their structural features, especially disulfide bonds, as they promote the antimicrobial effect of thiosulfinates, and they contribute to the redox chemistry in cellular systems (Abdalla and Mühling 2019).

In this chapter, a current overview of critical aspects related with sulfur metabolism and S-containing compounds from plant sources, with a special focus on their influence in secondary metabolism, natural sources, structural classification and their associated biological activities, and promising applications to be incorporated into the fields of human nutrition and health.

11.2 Sulfur Assimilation by Plants: Biosynthesis of S-containing Compounds

Sulfur sources commonly used by humans reveal different stable oxidation states of sulfur: H_2S (−2), Na_2S_2 (−1), elemental sulfur (S = 0), S_2Cl_2 (+1), SO_2, HSO_3^- (+4), and SF_6, H_2SO_4 (+6) (Walsh 2020). Regarding the abiotic chemical forms that plants use as natural sources of sulfur, the solid anionic sulfate (SO_4^{-2}) and the gaseous sulfur dioxide (SO_2) are the major ones. Sulfate is present in soils, while the sulfur dioxide form has been detected in the air of highly polluted industrial areas. The latter can be absorbed and assimilated by the aerial parts of the plant, mainly by leaves. Whereas roots are responsible for the assimilation of the sulfate form from soils, which is the most relevant pathway of sulfur transport (Leustek and Saito 1999).

Once sulfate has overpassed the cellular barrier, it represents an inorganic source of sulfur embedded into a biological system, therefore it has to be transformed into

an organic molecule (Leustek and Saito 1999). As abovementioned, in higher plants, two metabolic pathways, that share a common initial step, are the main responsible for this transformation (Fig. 11.1). Initially, the enzyme (Fike et al. 2015) ATP sulfurylase catalyzes the activation of the sulfate group by hydrolyzing the ATP bond between β- and γ-phosphates, later the sulfate group get transferred into the ATP γ-phosphate and yield APS. At this point, APS represents a branch in the biochemistry of S since it can get transformed into sulfite (SO_3^{-2}) or into 3′--phosphoadenosine-5′-phosphosulfate (PAPS) (Capaldi et al. 2015; Na and Salt 2011; Rausch and Wachter 2005; Koprivova and Kopriva 2014) (Fig. 11.2).

When APS acts as substrate, together with the reduced form of glutathione (GSH), of the (Abadie and Tcherkez 2019) APS reductase, it gets reduced into SO_3^{-2} (oxidation state +4) while GSH gets oxidized into oxidized GSH (GSSG). Then, the SO_3^{-2} becomes newly reduced into sulfide (S^{-2}, oxidation state −2) by the (Prasad and Shivay 2018) sulfite reductase (SIR) and supported by the oxidation of ferredoxin (Rausch and Wachter 2005) (Fig. 11.2). SIR (Prasad and Shivay 2018) has been demonstrated as a key enzyme for ensuring the normal growth in plants, its downregulation may cause a severe bottleneck in the assimilation of sulfate accompanied strong perturbations of the plant metabolism and deficient responses in adaptive and defense reactions (Khan et al. 2010). In the case, the expression of (Prasad and Shivay 2018) SIR gets downregulated, and SO_3^{-2} gets accumulated, the excess of SO_3^{-2} can be counteracted by its oxidation mediated by the (Capaldi et al. 2015) sulfite oxidase to produce sulfate and oxygen peroxide as defense mechanism (Fig. 11.2). The product of (Prasad and Shivay 2018) SIR, sulfide, serves, together with O-acetylserine (OAS), of substrate for the enzyme (Kopriva et al. 2019) O-acetyl-serine sulfhydrylase (or cysteine synthase) to eventually become cysteine (Cys) (Capaldi et al. 2015; Na and Salt 2011; Walsh 2020; Gigolashvili and Kopriva 2014). In further reactions Cys can follow two metabolic transformations. In a first metabolic route, Cys can get catalyzed by the (Künstler et al. 2020) cystathionine-γ-synthase into cystathionine (Cysta) which is later transformed by (Couturier et al. 2013) cystathionine-β-lyase into homocysteine (Hcy) to finally get catalyzed by (Lv et al. 2019) methionine synthase into methionine (Met) (Capaldi et al. 2015) (Fig. 11.2). At this point, Met can be embedded into the molecular structure of proteins where it would represent an important source of thiol groups (-SH), or it can continue suffering enzymatic transformations. Met can mainly follow two different transformation pathways, by one hand it can get transformed into S-adenosyl-methionine (SAM by (Rey and Tarrago 2018) SAM synthase) and then into nicotianamine (NA, by (Zhang et al. 2021) NA synthase) a metabolite involved in the transport of metals like iron, copper, or manganese (Capaldi et al. 2015; Na and Salt 2011). In the other hand, Met can also evolve by the action of the (Aarabi et al. 2020) methionine S-methyl transferase into S-methylmethionine (SMM), a very abundant metabolite in plants with unknown function apart from methyl donor (Capaldi et al. 2015). In a second metabolic route, Cys may represent the substrate of the (Zenda et al. 2021) γ-glutamylcysteine synthetase (also known as GSH 1) to form γ-glutamylcysteine (γ-EC), which after the catalysis exerted by the

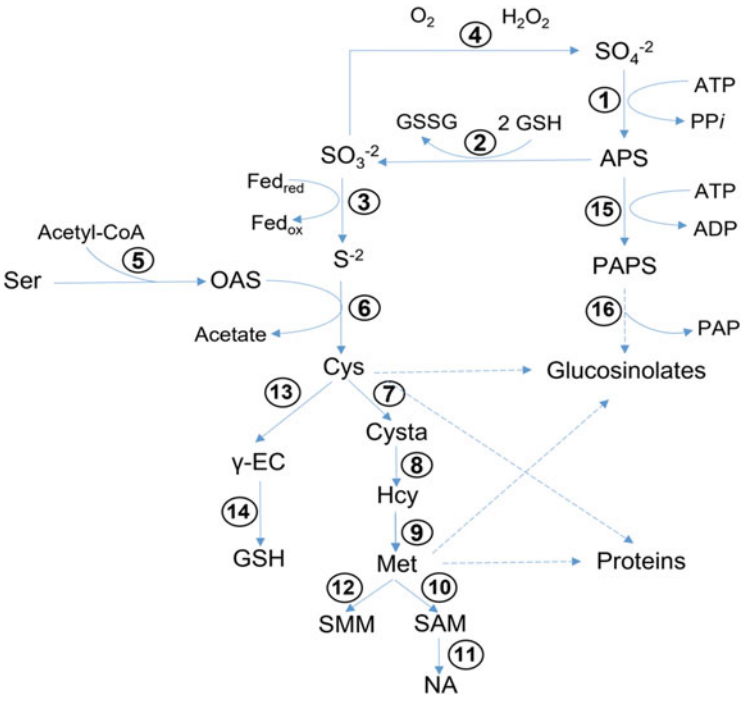

Fig. 11.2 Metabolic assimilation of inorganic sulfur and its biochemical transformation into organic forms in higher plants. Sulfate (SO_4^{-2}, oxidation state +6) get transformed by (Fike et al. 2015) ATP sulfurylase into APS. APS may be the substrate of (Abadie and Tcherkez 2019) APS reductase that by the oxidation of 2 molecules of GSH produces GSSG and sulfite (SO_3^{-2}, oxidation state +4). Sulfite gets reduced once more by (Prasad and Shivay 2018) SIR that requires the reductive power of Fedred and yields sulfide (S^{-2}, oxidation state −2) and Fedox. If sulfite gets accumulated, it can be alternative oxidized by (Capaldi et al. 2015) sulfite oxidase into sulfate and produce H2O2 in parallel. Sulfide together with OAS (obtained by the condensation of Ser and acetyl-CoA catalyzed by (Na and Salt 2011) OAS thyollyase) get condensed by (Kopriva et al. 2019) cysteine synthase to produced acetate and Cys. Cys can get transformed into Cysta by (Künstler et al. 2020) Cysta-γ-synthase; Cysta becomes Hcy by (Couturier et al. 2013) Cysta-β-lyase; Hcy is catalyzed by (Lv et al. 2019) Met synthase into Met. Met can become into SAM by (Rey and Tarrago 2018) SAM synthase, and then into NA by (Zhang et al. 2021) NA synthase or Met can become SMM by (Aarabi et al. 2020) Met S-methyl transferase. Cys can also get transformed into γ-EC by (Zenda et al. 2021) γ-glutamylcysteine synthetase and later γ-EC may become GSH by (Hasanuzzaman et al. 2018) glutathione synthetase. APS may be also the substrate of (Shimojima 2011) APS kinase to produce PAPS and ADP. PAPS serves as sulfate donor to (Walsh 2020) sulfotransferase that produces glucosinolates and recovers PAP. Compounds highlighted with bolds represent branches along the biochemical pathway. Abbreviations: Acetyl-CoA: acetyl coenzyme A; ADP: adenosine diphosphate; APS: 3′-adenosine-5′--phosphosulfate; ATP: adenosine triphosphate; Cys: cysteine; Cysta: cystathionine; γ-EC: glutamylcysteine; Fedred/ox: reduced or oxidized ferredoxin; GSH: reduced glutathione; GSSG: oxidized glutathione; Hcy: homocysteine; Met: methionine; NA: nicotianamine; SAM: S-adenosyl-methionine; Ser: serine; SMM: S-methylmethionine; OAS: O-acetylserine; PAP: 3′-phospho-adenosine 5′-phosphate; PAPS: 3′-phosphoadenosine-5′-phosphosulfate

(Hasanuzzaman et al. 2018) glutathione synthetase it will produce glutathione (GSH) (Na and Salt 2011) (Fig. 11.2).

The second biochemical pathway that APS may follow is as substrate of the (Shimojima 2011) APS kinase, which transfers it a phosphate group from ATP, so APS becomes PAPS (Walsh 2020; UNIPROT n.d.). This product, PAPS, serves of sulfate donor to the enzyme (Walsh 2020) sulfotransferase (SOT), which specifically transfers the sulfuryl into a hydroxyl group and so it also recovers a molecule of 3′-phospho-adenosine 5′-phosphate (PAP) (Leustek and Saito 1999; Hirschmann et al. 2014) (Fig. 11.2). The major products synthesized by (Walsh 2020) SOT are glucosinolates (GSLs), compounds rich in nitrogen and sulfur, recognized as major secondary metabolites whose cleavage has been related with protective roles in stressful contexts due to the presence of pathogens or herbivores (Hirschmann et al. 2014).

Regarding the transport of sulfur in plant tissues, the chemical molecule it has adopted will be tightly related with its carrier. We will focus on the transporters regarding sulfate and its principal products: PAPS, Cys and thiols (GSH and γEC). The initial assimilation of sulfate from soils is mediated by a group of proteins that act as co-transporters of protons, sodium ions and sulfate. This family of carriers named after SULTR (SULfur TRansporters) presents 12 transmembrane regions inserted in the plasmatic membrane of the cell and in the membrane of different organelles (Gigolashvili and Kopriva 2014). The carriers found in the membrane of the root cells, SULTR 1;1 and 1;2, permit to assimilate sulfate from soil and their expression is inducible by conditions of sulfate deprivation (Khan et al. 2010). However, further cellular transport has been disclosed to be facilitated by alternative proteins of the SULTR family. Among them, SULTR 4;1 and 4;2 are present in the vacuole membrane, while SULTR 3;1 has been identified in the chloroplasts membrane (Gigolashvili and Kopriva 2014). As exposed above, once sulfate has got transformed into some of the multiple products, its transport is mediated by different carriers. When sulfate becomes PAPS, the responsible of their transport between cytoplasm and chloroplasts has been identified as the PAPS/chloroplast thylakoid ATP/ADP carrier (TAAC) (Gigolashvili and Kopriva 2014). In the case of thiols (GSH and γEC), the carriers present in the chloroplastidic membranes have been identified to be chloroquine-resistance transporter (CRT)-like proteins or CRLs. When sulfate gets transformed into Cys, it is still unclear the molecular identity of their transporters. Even though, carriers involved in the Cys transport remain elusive to date, it has been suggested that may be involved both non-specific or general amino acid transporters and specific cysteine transporters that may modulate their specificity (Lee et al. 2014).

11.3 S-containing Amino Acid Occurrence and Functional Diversity

Sulfur (S) can be combined in numerous different chemical forms, such as S-containing amino acids, which possess valuable antioxidant activities. Several in vitro assays have demonstrated that the addition or supplementation of S-containing amino acids to food products or the human body could be useful to inhibit oxidative stress or decrease cellular damage (Kim et al. 2020). S-containing amino acids can be classified into essential and non-essential amino acids. The first group is formed by methionine and cysteine, which are elementary for protein biosynthesis, both are synthesized by microorganisms and plants from environmental S and they are the only ones that incorporate S into proteins (Kim et al. 2020; Yamazaki et al. 2020). The second group is formed by homocysteine, taurine, and glutathione-containing amino acids (Kim et al. 2020).

11.3.1 Essential Amino Acids

Cysteine ($HSCH_2CH(NH_2)COOH$) (Fig. 11.3) (Shamsipur and Chaichi 2005) is one of the most important S-containing amino acids, since it is responsible for different cellular activities, especially related to metabolism, protein folding (due to its ability to form disulfide bonds), or detoxification, among others. Therefore, its detection has been of great interest (Yadav et al. 2021). Its sulfhydryl group (-SH) plays an

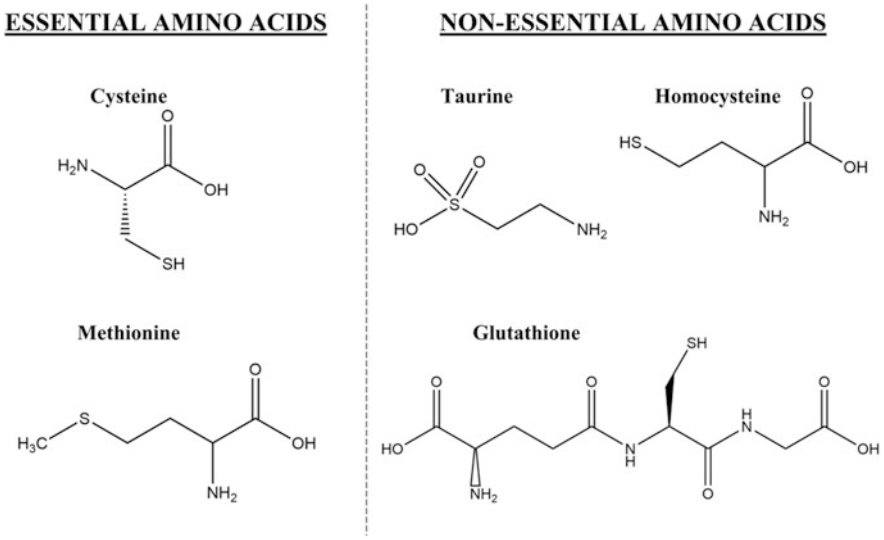

Fig. 11.3 Chemical structure of essential and non-essential S-containing amino acids

important role in the biological activity of proteins and enzymes (Shamsipur and Chaichi 2005). Moreover, cysteine is important for maintaining redox conditions and protecting cells from oxidative stress (Yamazaki et al. 2020). The metabolism of this amino acid starts with its activation to S-adenosylmethionine, which is a cofactor in the transfer of methyl groups, 59-deoxyadenosyl groups, synthesis of polyamines, ethylene synthesis in plants, among others. Therefore, cysteine can be converted into two other amino acids: glutathione and taurine. Among them, taurine has a higher concentration in most tissues (Brosnan and ME B. 2006).

Regarding the functional diversity of cysteine, the sulfur atom of this amino acid is subjected to various oxidative alterations in the cellular environment, which enables and regulates a wide variety of biological phenomena (catalysis, metal binding, protein turnover and signal transduction). The best-known oxoforms that cysteine can present are thiol and disulfide, but in turn, oxidized derivatives such as sulfenic (RSOH), sulfinic (RSO2H) and sulfonic (RSO3H) acid also appear in many proteins. This is possible because the thiol side chain of cysteine can occur in a variety of oxidation states and is one of the most sensitive to redox transformations (Reddie and Carroll 2008). These diverse properties make them an attractive target for future studies with potential for biotechnological applications. In an in silico study, 44 cysteine proteases from the carnivorous plant *Drosera capensis* were identified from molecular modeling and network analysis of the protein structures, resulting in proteases with different structural properties with a potential diversity of functional characteristics such as thermal stability or affinity for the substrate (Butts et al. 2016). A further understanding of the functional roles played by specific cysteine oxidation states could facilitate the development of different approaches to biochemical and cellular studies (Reddie and Carroll 2008).

Methionine ($CH_3S(CH_2)2CH(NH_2)$ COOH) (Fig. 11.3) is an essential amino acid which plays an important role in biological methylation (Shamsipur and Chaichi 2005). It is the amino acid that starts protein synthesis in most eukaryotic proteins (N-formylmethionine performs the same function but in prokaryotic proteins), and it is one of the most hydrophobic amino acids, so most of the methionine residues in globular proteins can be found in the hydrophobic inner core (Brosnan and ME B. 2006). Metabolization of methionine occurs via the transamination pathway. This pathway, (only significant at high methionine concentrations), can produce some toxic end products (Brosnan and ME B. 2006). Plants, unlike other eukaryotes, possess the molecular machinery for the *novo* synthesis of methionine. This is possible because this amino acid is a precursor of S-adenosyl-methionine (AdoMet), with several functions such as being the main donor of the methyl group in transmethylation reactions and an intermediate in the biosynthesis of polyamines and the phytohormone ethylene. In plants, it has a regulatory function and may be involved with plant growth hormones (cytokinins and auxins) and interactions with pathogens, although this is still under study (Ravanel et al. 1998).

The functional diversity of methionine is limited compared to other amino acids, its functionalization may affect protein function to a lesser extent and its abundance in proteins is around 2%. The main function of this amino acid is the protection against oxidative stress and it is often used as a substitute for

hydrocarbon-containing residues (Taylor et al. 2018). Moreover, it is widely used in food additives, flavoring compounds, and animal feed supplements (Yamazaki et al. 2020).

11.3.2 Non-essential Amino Acids

Taurine ($NH_2CH_2CH_2SO_3H$) (Fig. 11.3) is an amino acid that, although it is not incorporated into proteins, is involved in the regulation of cell volume, provides substrate for the formation of bile salts and is also one of the most abundant amino acids in different parts of the body such as the brain, the ocular retina and others (Ripps and Shen 2012). However, on rare occasions, it has been possible to find it in plant material, probably due to the complications of determining its concentration since it can overlap with other ionized compounds (Lähdesmäki 1986). A study evaluated the efficacy of exogenous taurine in mediating plant defense responses to B and Cr toxicity, showing that this amino acid improved growth, photosynthetic pigments synthesis, and nutrient uptake against the detrimental effects of these two pollutants by regulating the production of nitric oxide, hydrogen sulfide, glutathione, and phenolic compounds.

Homocysteine ($HS\text{-}CH_2\text{-}CH_2\text{-}CH(NH_2\text{-}COOH)$) (Fig. 11.3) is a precursor of methionine and vice versa (Miller 2012). It is generated during the metabolism of methionine and can be toxic to yeast, bacteria, and animal cells in high amounts (Arasimowicz-Jelonek et al. 2013). In addition, high levels of this amino acid have been related to cardiovascular diseases, which are currently on the rise (Atazadegan et al. 2021). In plants, it can be synthesized through two different pathways, from sulfate (with the formation of cysteine) or as a by-product in cellular methylation reactions (Arasimowicz-Jelonek et al. 2013). There is not much information about the relationship between biological properties with respect to this amino acid, but one study has investigated this correlation. The study was performed *in vivo* with garlic, cinnamon, black and green tea, curcumin, resveratrol, ginger, and soybean extracts resulting in lower levels of homocysteine but the same study suggested that more studies are needed (Atazadegan et al. 2021). The relationship of this amino acid with the response of plants to stress situations is also not well known. In a study, the homocysteine disposition in the leaves of the plant *Solanum tuberosum* L., commonly known as potato plant, was observed by means of an immunohistochemical method. In the most susceptible genotype of potato, there was an overproduction of this metabolite, thus affirming that it could be involved in a pathophysiological mechanism (Arasimowicz-Jelonek et al. 2013).

Glutathione ($C_{10}H_{17}O_6N_3S$) (Fig. 11.3) is a fundamental tripeptide in both the animal and plant kingdoms consisting of the amino acids: glutamate, cysteine, and glycine. It has various functions, but the most notable is redox-homeostatic buffering. Its state depends on its oxidation and on nutritional factors that influence both the activity and the structure of proteins by modifications in the thiol-disulfide balance (Noctor et al. 2011). In relation to plants, glutathione performs certain

functions that influence their development and cannot be replaced by other thiols (Noctor et al. 2012). Glutathione is highlighted because of its properties as antioxidant: it detoxifies reactive oxygen species (from the ascorbate-glutathione cycle); it has the ability to express defense genes and to signal stress (Zechmann and Müller 2010); it is involved in the detoxification of both herbicides and heavy metals; redox homeostasis and antioxidant biochemistry (Noctor et al. 2012). A study of the subcellular distribution of glutathione in various parts of *Arabidopsis thaliana*, *Cucurbita pepo* and *Nicotiana tabacum* plants provided more detailed information on the defense reactions under environmental stress situations in plants and the relevant protective functions of glutathione (Zechmann and Müller 2010).

11.4 Glucosinolates and Their Hydrolysis Products: Occurrence and Diversity

11.4.1 Glucosinolates (GSLs)

Glucosinolates (GSLs) are a class of secondary metabolites containing sulfur and nitrogen. Their chemical structure presents a β-glucopyrano unit (glucose), thiohydroximate-*O*-sulfonate group (linked to glucose), and a side chain (R) (Fig. 11.4). The side chain (R) is derived from one out of the following eight amino acids and can be three classes, namely: aliphatic (alanine, leucine, isoleucine, methionine, or valine), aromatic or benzenic (phenylalanine or tyrosine) and indole (tryptophan) (Redovnikovic et al. 2008). Glucoraphanin, gluconastrutiin, and glucobrassicin are examples of GSLs derived from aliphatic R (4-methysulfinylbutyl), aromatic R (2-phenylethyl) and indole R (indol-3-ylmethyl), respectively (Fig. 11.4). Nowadays, there are around 200 well-known structures of GSLs; however, only 5 GSLs are associated with human diet, namely: glucobrassicin (GBS), sinigrin (SIN), glucoraphasatin, glucoraphanin (GRA), and glucoiberin (GIB) (Redovnikovic et al. 2008).

GSLs are mostly present in plants that belong to the family *Brassicaceae* which contains around 3000 species and most consumed edible plants (Prieto et al. 2019; Holst and Williamson 2004). However, the distribution of GSLs in the plant is not equitable, since their highest levels are found in young leaves, reproductive tissues and roots, whereas shoots and mature leaves contain the lowest levels (Agerbirk and Olsen 2012). In addition, within the same family, there is a great diversity in relation to the composition of GSLs. For example, there are differences among kale, cabbage, broccoli, and Brussels sprouts, all belonging to *Brassica oleracea* L. In the case of kale, the most abundant GSL is sinigrin (SIN), while in cabbage, glucoiberin (GIB) is the most prominent. Around 50% of total GSLs of broccoli is glucoraphanin (GRA); however, SIN, progoitrin (PRO) and glucobrassicin (GBS) are the major GSLs in Brussel sprouts (Padilla et al. 2007).

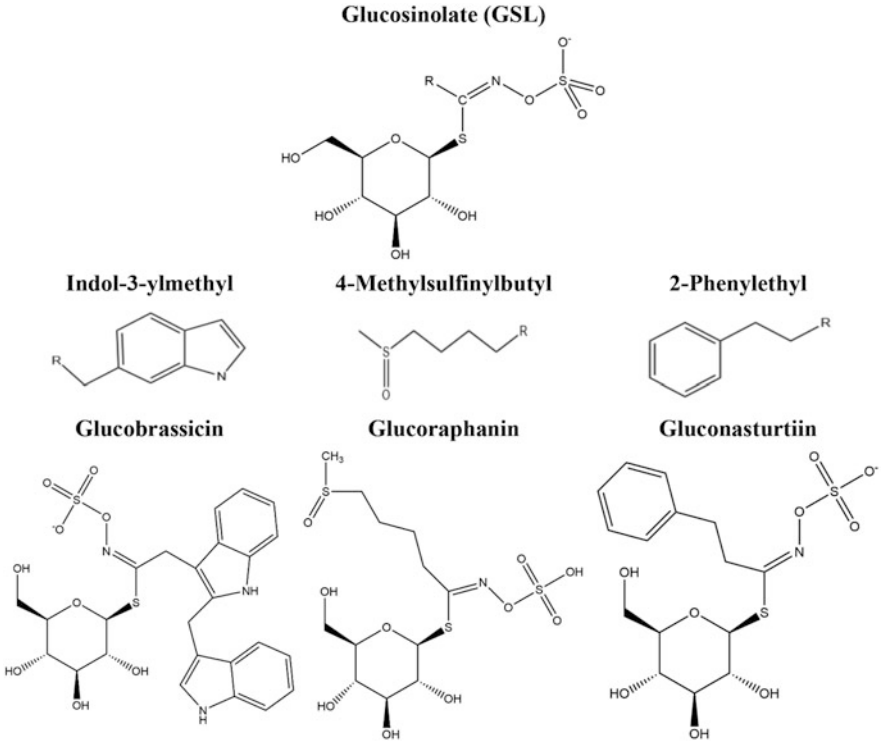

Fig. 11.4 Chemical structure of glucosinolates (GSLs) and examples depending on the side chain (R)

The biosynthesis of GSLs includes three main phases (Wittstock and Halkier 2002): *i)* elongation of the side chain, resulting in the production of the R group from amino acids (only occurs if the amino acids are Met or phenylalanine); *ii)* conversion of amino acid moiety to the GSL core structure by the addition of glucose and sulfur, and *iii)* successive secondary modifications of the side chain, producing different derivatives. These modifications are the responsible for the identification of around 200 known GSL structures in the literature (Redovnikovic et al. 2008). Furthermore, all possible modifications of the GSL R chain are important since both physicochemical properties and biological activities of GSL degradation products are originated from the structure of the R chain. The synthesis process is usually carried out in the cytosol and includes a specific number of genes that codify the necessary information for the biosynthesis chain, enzymes involved in the different modifications, and all transcription factors for each group of GSLs (aliphatic, aromatic, and indole) (Sønderby et al. 2010).

11.4.2 Glucosinolates Hydrolysis Products (GHPs)

GSLs are stable molecules, generally non-toxic compounds found in plant cells. When plant tissues are subjected to certain stress factors such as heat, cutting, insect attacks, or chewing, (Fig. 11.5) the β-thioglucosidase enzyme, known as myrosinase, is released-activated. Myrosinase is usually present in the external surface of the plant cell wall and especially in the vacuoles of myrosin cells (Wittstock and Halkier 2002). When GSLs get in contact with this enzyme, myrosinase breakdowns GSLs producing β-D-glucose and thiohydroximate-*O*-sulfonate (unstable aglycones) through catalysis and hydrolysis. Then, aglycones are restructured into biologically active and/or toxic molecules, this is, they rearrange to form degradation products, known as GSL hydrolysis products (GHPs). These active molecules are used by the plants as defense and protection system against stressful conditions such as herbivores, pathogens, or environmental changes (Barba et al. 2016). However, GHPs can exert beneficial activities for the human health such as antioxidant, anticancer, and anti-inflammatory properties. For instance, Cohen et al. (2000) showed that men consuming between 3 and 5 cruciferous portions per week had up to 41% less chance of suffering from prostate cancer (Cohen et al. 2000). In addition, other authors have studied the biocidal effects of GHPs, such as sulforaphane (SFN) (Fig. 11.6), extracted from broccoli, that was able to treat gastritis, caused by *Helicobacter pylori* bacteria (Wu et al. 2016).

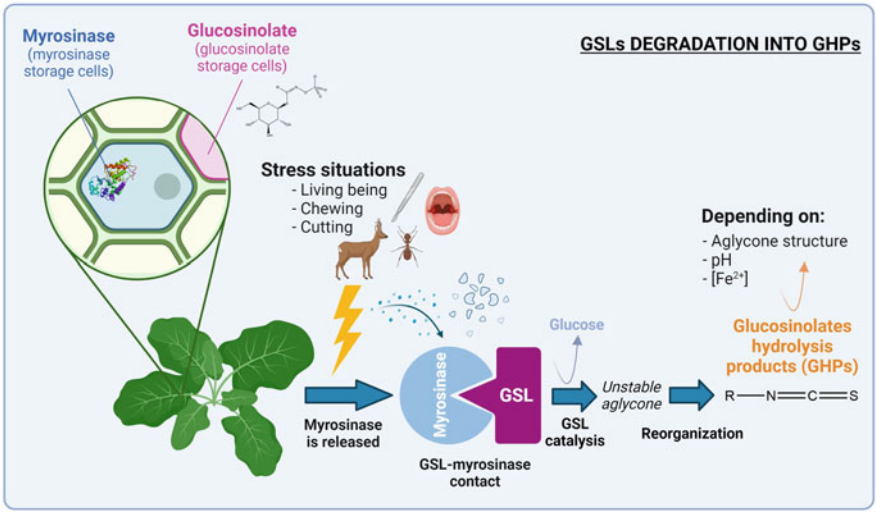

Fig. 11.5 Process of glucosinolates (GSLs) degradation and the formation of glucosinolates hydrolysis products (GHPs). When the plant is subjected to stress situations, myrosinase is released and gets in contact with GSLs, being responsible for the catalysis and hydrolysis of GSLs, resulting in the formation of unstable aglycone which are then restructured in different GHPs. The formation of each GHP depends on certain factors such as the structure of aglycone, pH or concentration of Fe^{2+}, among others. Created with BioRender.com

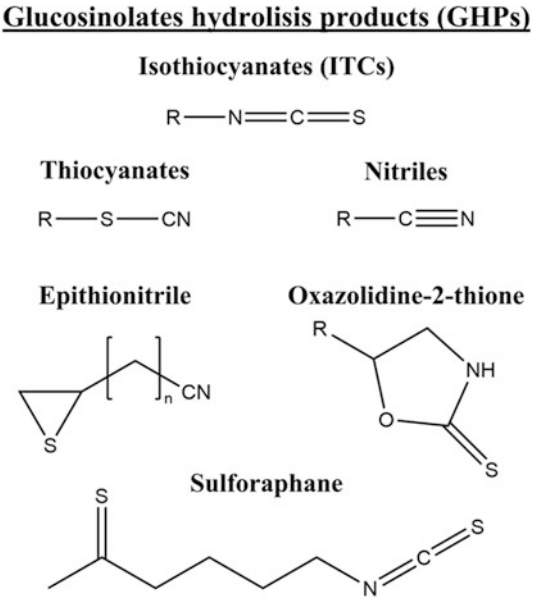

Fig. 11.6 Chemical structure of glucosinolates hydrolysis products (GHPs)

Among GHPs, isothiocyanates (ITCs), nitriles, epithionitriles, and thiocyanates are the most common (Fig. 11.5). The determination of their final products depends on the aglycone structure, the genotype, the presence of myrosinase-interacting proteins such as epithiospecifier proteins (EPSs), and chemical factors that modify the action of myrosinase such as pH, availability of ferrous ions (Fe^{2+}), drying method or time and temperature of plant storage, among others (MDC et al. 2013; Wittstock and Burow 2010; Kamal et al. 2022). For instance, unstable aglycones are reorganized to ITCs forms if the medium pH is neutral, while, nitriles and EPS nitriles are formed at acid pH (Barba et al. 2016). Epithionitriles are formed when there is a terminal double bond in the side chain, whereas oxazolidine-2-thione is formed if the carbon 3 of the side chain is hydrolyzed.

ITCs (general structure, R-N=C=S) are the most studied GHPs and the most hydrolytic product. They are characterized by including molecules where R is an alkyl or aryl group (Kamal et al. 2022). The type of ITCs will depend on the type of GSL they are derived from so, SIN is the precursor to allyl ITCs; glucotropaeolin is the precursor to benzyl ITCs; phenethyl ITCs are formed from the degradation of gluconasturtiin; and SFN from glucoraphanin. The role of ITCs in vegetables is related to plant protection against the attack of herbivores and other pathogens, whereas in humans are used to ameliorate inflammation processes (Prieto et al. 2019). Studies showed that ITCs contain important biological properties such as chemopreventive, anti-inflammatory, anti-mutagenic, and biocidal effects, therefore they can be used as possible therapeutic tools. Among ITCs, SFN (Fig. 11.6) is the most studied because of its health-promoting effects (Traka 2016). Young broccoli sprouts and cauliflower are rich in SFN, but it can also be found in cabbage and kale

(Ishida et al. 2014). Studies showed that SFN reduce oxidative stress and inflammation by the activation of Nrf2 (Nuclear factor erythroid 2-related factor 2) under stressful conditions and the inactivation of NFkβ (nuclear factor kappa-light-chain-enhancer of activated B cells), reducing TNF, Cox-2, or IL-6 levels responsible for the inflammation and proliferation (Mangla et al. 2021; Corssac et al. 2018). Another study concluded that SFN reduced apoptosis by the deceleration of Phase I and activation of Phase II via enzymatic (Fernandes et al. 2016). However, the consumption of elevated amounts of GSLs has been associated with toxic effects such as enlarged thyroid, reduced plasma thyroid hormone levels, certain organ abnormalities, decreased growth or even mortality in the most serious cases (Prieto et al. 2019). A study demonstrated that the very high intake of allyl ITCs can cause important damages in the gastrointestinal tract, resulting in abdominal pain in ruminants and colic in horses (Taljaard 1993). Another study showed that the prolongated ingestion of vegetables containing ITCs caused thyroid hypertrophy in poultry and fish (Burel et al. 2000).

11.4.3 Occurrence and Variability

The concentration of GSLs and GHPs will vary depending on various factors, such as thermal treatments. GSLs and the enzyme myrosinase are thermolabile thus, the concentration of GHPs is higher when consuming raw vegetables than after being processed by heating when myrosinase is inactivated (Hanschen et al. 2012). For instance, authors concluded that the cooking process, boiling, can cause GSLs losses of 5–75% depending on GSL structure and vegetable (Nugrahedi et al. 2015; Verkerk et al. 2009). Nevertheless, sometimes, certain vegetables (*e.g.,* broccoli or cauliflower) need to be cooked. Studies concluded that boiling is the most effective cooking process in reducing the levels of GSLs. Martinez-Hernandez et al. (2013) showed that boiling broccoli for 3.5 min reduced upon 80% of total GSLs, or 100% of total GSLs in the case of boiling red cabbage for 5 min (Martínez-Hernández et al. 2013). Therefore, avoiding boiling vegetables may increase the bioavailability of both GSLs and GHPs. Other cooking processes such as steaming, microwaving, or stir-frying showed small changes in the quantity of GSLs (Hwang and Hwang 2015).

Abiotic stress also affects the levels of GSLs and GHPs in plants, such as salinity, drought, extreme temperatures and light, or nutritional deficiencies. Salinity is the factor that most affects plant physiology (Zhu 2001). Studies showed that the concentration of GSLs in broccoli is increased when salinity stress is found above the tolerance levels; however, the amount of GSLs is reduced when salinity levels are very high (around 80 mM) (López-Berenguer et al. 2008). This might be due to the primary metabolism being restricted, while the secondary metabolism is not (López-Berenguer et al. 2009). Regarding drought, authors observed that the water stress leaded to an increase in the levels of GSLs in certain *Brassica* species (Jensen et al. 1996; Zhang et al. 2008; Schreiner et al. 2009). This increase can be because the drought allows plants to reduce some vegetative growth parameters, resulting in

an accumulation of secondary metabolites, although there is a lack of primary metabolism (Jones and Hartley 2016). In the case of the extreme temperatures and light, it has been observed that the concentration of GSLs in plants varies depending on the temperature and light quality (Engelen-Eigles et al. 2006). Studies showed that the seasons can modify the GSLs content in *Brassica* species such as radish, oilseed, or cabbage, being more accumulated in the spring season conditions (moderate temperatures, high light intensity and low humidity) than in winter season conditions (Padilla et al. 2007; Charron and Sams 2004; Schreiner et al. 2002). Finally, a lack of certain nutrients such nitrogen (N) or sulfur (S) can produce some physiological disorders (Coulombe et al. 1998), an excess of N supply can lead to a decrease in total GSLs (Velasco et al. 2007). However, authors have observed that broccoli plants containing deficit N supply can increase their total GSLs if S fertilization is abundant (Schonhof et al. 2007). Regarding S supply, a study showed that the increase in amounts of S leads to higher levels of total GSLs in *Brassica rapa* (Li et al. 2007).

On the other hand, the conversion of GSLs to GHPs can also occur in the digestive system of mammals, including humans. Unlike cruciferous plants, mammal tissues do not contain myrosinase, so the hydrolytic products are produced thanks to the action of the intestinal flora present in the colon (Dinkova-Kostova and Kostov 2012). When vegetables are ingested, the absorption of intact GSLs can occur in the stomach. However, most GSLs go to the small intestine, where they can also be absorbed (Angelino and Jeffery 2014). The non-absorbed GSLs can be hydrolyzed to ITCs by the action of myrosinase (already present in the vegetable) or by intestinal flora. In the first case, the process is conducted in the proximal gut, i.e., in the stomach and the small intestine, whereas the latter is carried out in the colon. The generated ITCs can be absorbed or/and excreted forming part of the feces (Cartea and Velasco 2008). Nowadays, most studies show the degradation of GSLs in ITCs by intestinal microbiota. Nonetheless, due to the wide diversity of intestinal bacteria, the production of other GHPs such as nitriles or epithionitriles is very probable, but more investigation is needed (Barba et al. 2016).

11.5 Other S-containing Metabolites

S-derived secondary metabolites comprise several families of low molecular weight compounds mainly correlated with the defense mechanisms in the Plant Kingdom. As previously referred, GLSs and ITCs stand out as the most abundant. However, additional organosulfur compounds such as cysteine sulphoxides and phytoalexins play a significant role in the innate immune system in plants while promoting health benefits after human consumption. Their metabolism, occurrence, and structural diversity were researched over the following decades and will be summarized in this section as follows.

11.5.1 Phytoalexins

Phytoalexins are S-containing secondary metabolites firstly identified in potatoes infected with *Phytophthora infestans* (Müller et al. 1939). Briefly, potato tubers infected with a non-virulent strain of pathogen-induced resistance to a further virulent strain. This finding suggested that potato tubers produced substances (phytoalexins) with antimicrobial properties, protecting the tissue against later infection by additional virulent strains of the pathogen. Phytoalexins are synthesized from various interlinked biosynthesis pathways. They can derive from the phenylpropanoid pathway, through the mevalonic acid pathway and/or the Tr pathway (Fig. 11.7) (Ahmed and Kovinich 2021).

Recent findings suggest that phytoalexins biosynthesis shares a common transcription factor network, which could be engineered to enhance their biosynthesis in plants (Großkinsky et al. 2012). However, there is still a gap in the fully metabolic mapping of phytoalexins, which difficult the development of bioengineering strategies to obtain these compounds (Ahmed and Kovinich 2021). Despite their diverse biosynthesis, they commonly mediate a broad antimicrobial activity towards different pathogens from *Botrytis (*Murata et al. 2020*;* Pedras et al. 2010*)*, *Pseudomonas* (Abdalla and Mühling 2019; Murata et al. 2020), *Plasmidiophora* (Pedras et al. 2008; Zhang et al. 2016), *Phytophthora* (Abdalla and Mühling 2019; Ahmed and Kovinich 2021; Long et al. 2021), *Albugo* (Pedras and Zheng 2010), or *Alternaria* (Long et al. 2021) genera among others. Besides the biotic response, phytoalexins have been identified in plants in response to UV light and depending on agricultural

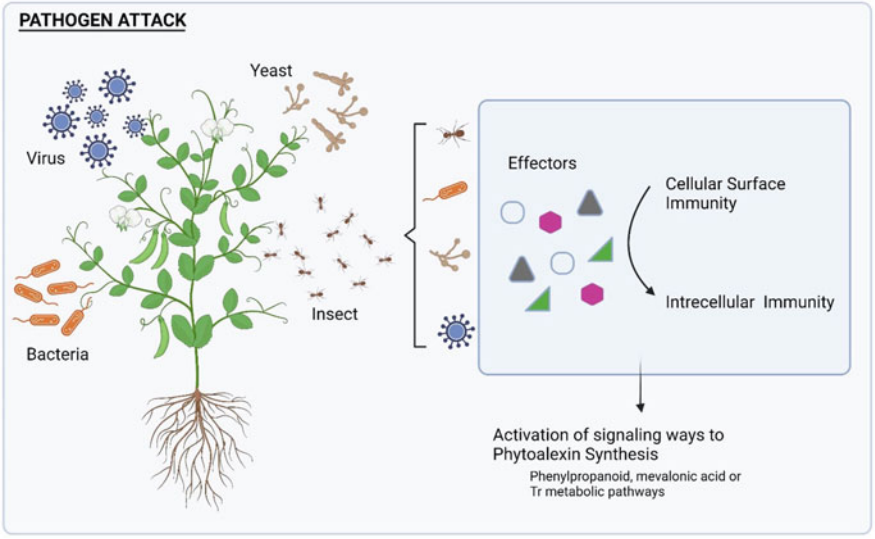

Fig. 11.7 Schematic representation of phytoalexins production in plants. Created with BioRender.com

practices such as Cu fertilization, irrigation processes, or soil salinity (Abdalla and Mühling 2019). The structural characterization of these secondary metabolites is challenging work because the accumulation of phytoalexins is restricted to the infection site or physical damage, and they are rapidly catabolized after successful pathogen defense and/or plant healing. Thus, little information is available regarding phytoalexins' structural characterization. To date, several phytoalexins structures were identified in Gramineae, Oryzeae, Asteraceae, Alliaceae, and Brassicaceae families (Table 11.2).

Phytoalexins show a great heterogeneity and structural diversity. They can be divided into Sesquiterpenoids, Isoflavonoids, Isocoumalins, Diterpenoids, and Furanoacetylenes (Umezawa and Shin 1999). Nevertheless, some similarities have been found in plant foods from the same family. Indeed, stilbene-type phytoalexins are usually found in grapes, peanuts, and pine. However, leguminous family mainly synthetize isoflavonoid derivatives (80%) while Solaceae family have not report the synthesis of isoflavonoid phytoalexins (Umezawa and Shin 1999).

11.5.2 Cysteine Sulfoxides

Cysteine sulfoxides are organosulfur compounds usually found in *Allium* species that have been widely studied as non-volatile flavor precursors. Briefly, *Allium* species (onion and garlic) store a large amount of sulfur in the form of amino acids like S-alk(en)ylcysteine sulfoxides, which are further transformed into unstable intermediate compounds. Indeed, S-allyl-cysteine sulfoxide (alliin) was the first precursor of cysteine sulfoxides. Alliin attaches to pyridoxal-phosphate (pxy-p) and converts to salk(en)yl-cysteine sulfoxide and volatile compounds in *Allium (*Feizabad 2019*)*.

When plant tissues are subjected to damage, the enzyme alliinase catalyzes the conversion of S-alk(en)yl cysteine sulfoxides into their respective thiosulfinates (TSNs) or propanethial-S-oxide. Different amounts of alliinase can determine the presence and the number of cysteine sulfoxides. Depending on the *Allium* species and the specific conditions, allicin (product of the catalyzation of alliin) and TSNs can decompose to form additional S constituents including diallyl, methyl allyl, and diethyl mono-, di-, tri-, tetra-, penta-, and hexasulfides, the dithiins, vinyldithiins and (E)- and (Z)-ajoene. The 4 main compounds of cysteine sulfoxides are: (i) alliin or (+)-S-C2-(2-propenyl)-L-cysteine sulfoxide, (ii) methiin (MCSO) or (+)-S-methyl-cysteine sulfoxide, (iii) isoalliin (TPCSO) or S-(1-propenyl)-L-cysteine sulfoxide; and (iv) propiin (PCSO) or (+)-S-propyl-L-cysteine sulfoxide. Particularly, S-Methyl-L-cysteine sulfoxide (methiin) is the most widely distributed compound that appears in varying amounts in the healthy tissues of *Allium* species, mainly in *A. sativum, A. cepa, A. porrum*, and *A. urisinum* (Musah et al. 2009).

Cysteine sulfoxides are taste precursors, and the higher the concentration of these bioactive compounds, the higher the severity of taste. Cysteine sulfoxides are also the compounds responsible for the lachrymatory response after onion chopping.

Indeed, isoalliin, a main source of the lachrymatory factor reference, is the major cysteine sulfoxide from different *Allium cepa* and other onions such as *A. schoenoprasum* and *A. x proliferum* (Fritsch and Keusgen 2006).

11.6 Biological Activities and Potential Applications of S-containing Metabolites

Plant-based diets are well known to have a direct impact on human health. Apart from supplying fiber and micronutrients, edible plants, such as fruit and vegetables, contain secondary metabolites that can exert pharmacological effects on the human body (Liou et al. 2020). Several epidemiological studies have shown a relationship between diets rich in cruciferous vegetables and health benefits, due to their content in bioactive compounds such as S-containing metabolites (Richman et al. 2012). S-containing metabolites are bioactive molecules, as previously mentioned, being their major representatives and bioactive groups the GSL and their derivatives: ITC and TSN (Sahebi et al. 2017). The biological activities of several S-containing metabolites and their effect in different diseases or disorders as preventive agent and their evaluation as promising alternative treatments for these systemic conditions will be further explored (Table 11.1).

11.6.1 Anticancer Activity

It is widely recognized that cancer is one of the major causes of death in the world, and many natural products have been proven to possess anticancer properties, including berries, cruciferous vegetables, tomatoes, ginger, or garlic, among others (Raiola et al. 2015; Kim et al. 2017a; Pan et al. 2018; Bray et al. 2018; Rajakumar et al. 2018; Tafakh et al. 2018). Studies conducted over the past decade have revealed that S-containing metabolites, namely ITCs such as allyl isothiocyanate (AITC), sulforaphane (SFN), and phenethyl isothiocyanate (PEITC), and TSNs, such as allicin, can prevent a variety of cancers through different mechanisms of action.

4-[(α-L-Rhamnosyloxy) benzyl] isothiocyanate (MIC-1) extracted from *Moringa oleifera* seeds exerts the strongest growth inhibition effects on renal carcinoma cells without causing toxicity in normal kidney cells. Furthermore, MIC-1 also induces apoptosis and cell cycle arrest. Mice xenograft tumors growth was inhibited by MIC-1 in in vivo experiments, and MIC-1 significantly increased the Bax/Bcl-2 ratio in tumor tissues, supporting that MIC-1 may be an effective, natural, non-toxic dietary supplement for preventing and treating renal cancer (Xie et al. 2022). Besides, on bladder cancer cell lines, AITC has been shown to modulate gene expression according to TP53 genotypes as a potential antiproliferative compound

Table 11.1 Bioactivities of several S-containing metabolites in different diseases and/or disorders and their potential application for their prevention or alternative treatment

Disease/ disorder	Group	Compound	Bioactivities	Ref.
Anticancer activity				
Breast cancer	ITC	AITC	Reduction of mammary tumorigenesis.	Rajakumar et al. (2018)
Renal cancer	ITC	MIC	Inhibition of growth and migration of renal cancer cells.	Xie et al. (2022)
Colon cancer	ITC	GSL	Antiproliferative effects on colon cancer cells. Reduction in cell proliferation and viability.	Cuellar-Núñez et al. (2020, Radziejewska-Kubzdela et al. (2019)
Bladder cancer	ITC	AITC	Inhibition of bladder cancer cell proliferation.	Sávio et al. (2015)
Colorectal cancer	ITC	SFN	Anticancer effects are associated with antiproliferative, antiangiogenic, and antimetastatic activities.	Tafakh et al. (2018)
		PEITC	Cancer-preventive effect through tumor microenvironment regulation.	Shin et al. (2021)
Hepatocellular carcinoma	ITC	GSL	Anticancer properties of glucosinolates.	Lenzi et al. (2021)
Skin cancer	ITC	SFN	Regulation of signaling pathways contributing to chemopreventive effects in UVB-induced skin carcinogenesis.	Li et al. (2020a)
Neurodegenerative prevention				
Alzheimer's disease (AD)	ITC	SFN	Inhibition of AD-associated biomarkers levels of synaptic damage and neurodegeneration. Inhibition of Aβ-induced neuronal cell death.	Masci et al. (2015, Kim et al. (2017b), Lee et al. (2018), Zhang et al. (2017)
		6-MITC	Amelioration of $A\beta_{1-42}$-induced memory impairments. Re-establishment of physiological oxidative status, by decreasing apoptosis and neuroinflammation, contributing to behavioral recovery.	Morroni et al. (2018)
Traumatic brain injury	ITC	AITC	Attenuation of oxidative stress and inflammation.	Caglayan et al. (2018)
Nephrotoxicity	TSN	Allicin	Nephroprotective effects against cisplatin-induced	Abdel-Daim et al. (2019)

(continued)

Table 11.1 (continued)

Disease/ disorder	Group	Compound	Bioactivities	Ref.
			mediated by antioxidant and anti-inflammatory activities.	
Anti-inflammatory activity				
Mastitis	TSN	Allicin	Anti-inflammatory effects against *S. Aureus*-induced mastitis.	Chen et al. (2018)
	ITC	PEITC	Amelioration of inflammatory reactions.	Han et al. (2018)
Inflammatory bowel disease	ITC	6-MITC	Alleviation of inflammatory bowel disease.	(122)l
Sepsis and inflammation	ITC	MIC	Reduced the expression of inflammatory markers (Tnf-α, Ifn-α, IL-1β, IL-6) in the liver, kidney, spleen, and colon.	Sailaja et al. (2021)
Chronic inflammatory diseases	ITC	PEITC	Beneficial effects of PEITC on chronic inflammatory diseases.	Park et al. (2013)
Inflammation-related carcinogenesis	ITC	BITC	Prevented inflammation-related carcinogenesis.	Miyoshi et al. (2004)
Skin inflammation	ITC	PEITC	Inhibition of inflammatory responses *in vivo* and *in vitro*.	Lee et al. (2011)
		BITC	Attenuation of oxidative damage and leukocyte clearance at the inflamed region.	Miyoshi et al. (2004)
Skin protection: anti-inflammatory and anti-aging				
Atopic dermatitis	ITC	SFN	Alleviation of atopic dermatitis through inhibition of oxidative stress, DNA damage, inflammation, apoptosis.	Alyoussef (2021)
Radiation-induced skin injury (RISI)	ITC	SFN	Mitigation of RISI through upregulation of antioxidant enzymes, and suppression of ROS production.	Wei et al. (2021)
Photoaging	ITC	SFN	Mitigation of premature skin aging by suppressing melanogenesis and maintaining collagen homeostasis.	Ko et al. (2020)
		MBITC3	Anti-photocarcinogenic and anti-photoaging properties in the skin microenvironment.	Carpenter et al. (2018)
		SFN, PEITC	Protection of skin against UVR-induced oxidative stress and apoptosis.	Kleszczyński et al. (2013)

(continued)

Table 11.1 (continued)

Disease/ disorder	Group	Compound	Bioactivities	Ref.
		7-MSI	Inhibition of melanin synthesis by suppressing melanogenesis and autophagy activation.	(158)p
Skin aging	ITC	SFN	SFN ameliorates skin aging.	Petkovic et al. (2021)
Antimicrobial activity				
Skin infections	TSN	Allicin	Topical treatment with allicin improve skin infection caused by methicillin-resistant *S. aureus* (MRSA).	Sharifi-Rad et al. (2014)
Fungal infections	TSN	Allicin	Inhibition of bacteria and fungi proliferation, including antibiotic-resistant strains like MRSA.	Shadkchan et al. (2004)
General infections	TSN	Allicin	Prevention of drug resistance through the inhibition of biofilm formation due to its antimicrobial activity against both Gram-positive and Gram-negative bacteria.	Wallock-Richards et al. (2014), Wu et al. (2015), Loi et al. (2019)
Lung infections	TSN	Allicin	Antimicrobial and antibiofilm activity against lung pathogenic bacteria.	Reiter et al. (2017)
Denture stomatitis	TSN	Allicin	Antimicrobial and antibiofilm activities against *Candida albicans* and *Staphylococcus aureus.*	Zainal et al. (2021)
Glucose and lipid regulation in diabetes and cardiovascular disorders				
Diabetes	ITC	PEITC	Beneficial effects on the carbohydrate metabolism.	Chiba et al. (2019)
		SFN	Reduction of blood glucose levels in human patients with T2D and glucose production in hepatoma cell lines.	Axelsson Annika et al. (2017)
			Protective effects on T2D-induced cardiomyopathy.	Sun et al. (2020)
		MIC	Anti-obesity and anti-diabetic properties through inhibiting rate-limiting steps in liver gluconeogenesis resulting in a direct or indirect increase in insulin signaling and sensitivity.	Waterman et al. (2015)
		AITC	Possesses anti-diabetic, antioxidant, and anti-inflammatory activities.	Sahin et al. (2019)

(continued)

Table 11.1 (continued)

Disease/ disorder	Group	Compound	Bioactivities	Ref.
Fatty liver disease	ITC	AITC	Amelioration of lipid accumulation and inflammation.	Li et al. (2019)
	TSN	Allicin	Attenuates liver oxidative stress and inflammation.	Panyod et al. (2016)
Obesity	ITC	PEITC	Improvement of lipid metabolism and inflammation in the adipose tissue.	Gwon and Yun (2021)
		SFN	Anti-obesity effect by normalizing the expression of genes related to lipid metabolism.	Ranaweera et al. (2022)
Obesity glomerulopathy	ITC	SFN	Decreases-induced damage.	Lu et al. (2020)
Lipid metabolism disorder	TSN	Allicin	Alleviated lipid metabolism disorder.	Lu et al. (2017)
Preventive agent in cardiovascular-related disorders				
Atherosclerosis	ITC	SFN	Protection against angiotensin II-mediated injury by reducing oxidative stress and improving mitochondrial injury.	Zhang et al. (2020)
Chronic heart failure	ITC	SFN	Improvement of cardiac function and remodeling by inhibiting oxidative stress and inflammation.	Ma et al. (2018)
Cardiovascular disorders	TSN	Allicin	Antihypertensive properties due to vasoactivity effects that lower the blood pressure.	Benavides et al. (2007), Dubey et al. (2017)
			Decreases myocardial ischemia-reperfusion through inhibition of inflammation and oxidative stress.	Liu et al. (2019)
			Lowered blood pressure and triglyceride levels.	Elkayam et al. (2013)
			Anti-arrhythmic effect.	Huang et al. (2013)

Abbreviations: TSN: thiosulfinate; AITC: allyl isothiocyanate; ITC: isothiocyanate; GSL: glucosinolates; MIC: 4-(α-L-rhamnosyloxy)-benzyl isothiocyanate extracted from *Moringa oleifera*; MBITC3: methoxybenzyl isothiocyanate; 6-MITC: 6-(Methylsulfinyl) hexyl Isothiocyanate; 7-MSI: 7-(Methylsulfinyl) heptyl isothiocyanate; PEITC: phenethyl isothiocyanate; SFN: sulforaphane; T2D: type-2-diabetes

(Sávio et al. 2015). Moreover, through modulating the p53 signaling pathway, allicin induced apoptosis and regulated biomarker expression in breast cancer cell lines, suggesting that allicin is a valuable anticancer compound that targets p53 protein (Maitisha et al. 2021). PEITC has been also demonstrated to have anticancer properties both in *in vitro* and *in vivo* models. In cell cultures it induced apoptosis

Table 11.2 Phytoalexins identified in some plant foods in response to biotic or abiotic stressors

Plant Food	Phytoalexins	Ref.
Rice (*Oryza sativa*)	Oryzalexins, (Solenoids) momilactone-A, momilactone-B, (Phytocassanes) phytocassane-A, D, or E, phytocassane-B and phytocassane-C and the flavonoid sakuranetin (5,4′-dihydroxy-7-methoxyflavanone).	Arruda et al. (2016, Komkleow et al. (2021)
Cabbage (*Brassica oleracea* and/or *B. campestris*)	Brassinin, 1-Methoxybrassinin, Cyclobrassinin, 4-Methoxybrassinin, Brassicanal C, Isalexin, Spirobrassinin, Caulilexin A, Caulilexin B, Cyclobrassinone, (R)-1-Methoxy-Spirobrassinin, Pirobrassinin.	Abdalla and Mühling (2019)
Mustard (*Sinapis alba*)	Sinalbin A, Sinalbin B, Sinalexin.	Pedras and Zaharia (2000)
Wasabi (*Wasabia japonica*)	Wasalexin A, Wasalexin B.	Pedras (1999)
Maize (*Colletotrichum graminicola*)	Phytoalexin A1, Phytoalexin A2, kauralexins and zealexins.	Lim et al. (1970), Poloni and Schirawski (2014), Vaughan et al. (2015)
Sorghum (*Sorghum* spp.)	Apigeninidin (2-(4-hydroxyphenyl) benzopyrilium chloride) and luteolinidin (2-(3,4-dihydroxyphenyl)-chromenylium 5,7-diol).	Poloni and Schirawski (2014)
Chili pepper (*Capsicum annuum*)	Capsidiol.	Umezawa and Shin (1999)
Grape (*Vitis vinifera*)	α-Viniferin.	
Potato	Phytuberin.	
Soybean	Glyceollin.	
Beetroot	Betavulgarin.	
Chickpea (*Cicer arietinum*)	Medicarpin, pisatin and Maackiain.	

that were associated with cancer-chemopreventive effects, and it also was demonstrated to retard the proliferation of cancerous cells by inducing death receptor signaling. Regarding in vivo results, the intraperitoneal administration of PEITC inhibited tumor growth in xenograft glioblastoma cells but it also was demonstrated to delay the development of the colorectal cancer in a xenograft mouse model. In this experiment, PEITC was administered 2 weeks before the inoculation of cancer stem cells. This preventive administration reduced the tumor progression and the expression of pluripotent markers. Besides it also downregulated 12 genes associated to inflammation and immune response, such as CXCL 1–3 directly involved in cancer metastasis (Shin et al. 2021).

Other S-containing metabolites were also used to develop new therapies against different types of cancer. For example, the GSL-rich hydrolyzed extract obtained from *M. oleifera* had antiproliferative effects against colon cancer cells (Cuellar-

Núñez et al. 2020), SFN revealed anticancer effects in colorectal cancer cells through antiangiogenic, antimetastatic, and antiangiogenic effects (Tafakh et al. 2018), and regulated several signaling pathways including p53 signaling and cell cycle arrest in skin cancer (Li et al. 2020a). Hence, S-containing metabolites could contribute to the development of nutrition- and herbal-based therapies for the treatment and prevention of cancer.

11.6.2 Neurodegenerative Prevention

Neurodegenerative diseases prevalence is estimated to double over the next 30 years, due to the steady increase in life expectancy. Neurological disorders need to be prevented to reduce age-relative cognitive decline and the associated health care costs (Wimo et al. 2003). Hence, the search for therapeutic tools to counter the neurodegenerative processes is ongoing with clinical trials that look for demonstrating the efficacy of several drugs or reverse disease progression in most cases. One alternative strategy recognized as preventive tool targets lifestyle factors, such as physical activity and nutritional habits (Masci et al. 2015). In this sense, the antioxidant and anti-inflammatory properties of S-metabolites may be able to reduce age-related oxidative stress and inflammation, which may prevent neurodegenerative processes. SFN, found in cruciferous vegetables like broccoli, bok choy, and cabbage, has been shown to reduce inflammatory biomarkers and main pathological factors found in Alzheimer's disease (AD). For instance, several studies reported that SFN has the potential to prevent neuronal disorders by epigenetically enhancing neuronal brain-derived neurotrophic factor (BDNF) expression and its tropomyosin receptor kinase B (TrkB) signaling pathways (Kim et al. 2017b), decreasing the accumulation of Aβ and tau (two abnormal structures, amyloid plaques and neurofibrillary tangles whose accumulation is directly related with AD development) (Lee et al. 2018), and ameliorate neurobehavioral deficits (Zhang et al. 2017). Moreover, other ITCs showed beneficial effects related to neurological disorders, specifically allicin which revealed nephroprotective effects against cisplatin-induced nephrotoxicity (Abdel-Daim et al. 2019), and application of AITC immediately after traumatic brain injury revealed beneficial effects through attenuation of oxidative stress and inflammation (Caglayan et al. 2018). Considering these findings, S-containing metabolites should be further evaluated as candidates for neurodegenerative diseases therapy and prevention.

11.6.3 Anti-Inflammatory and Antimicrobial Capacity

Inflammation plays a crucial role in the pathogenesis of multiple chronic and communicable diseases and acute disorders, such as arthritis, inflammatory bowel disease, cancer, mastitis, injury, infections, and sepsis (Chen et al. 2018). In

traditional medicine, plants have been used to treat these conditions for thousands of years. Over the past century, natural products such as S-containing metabolites with strong anti-inflammatory properties have been developed. In in vivo experiments where dextran sulfate sodium (DSS) was used to induced colitis in murine model, 6-(methylsulfinyl)hexyl isothiocyanate (6-MITC) extracted from *Wasabia japonica* showed potential therapeutic effects against inflammatory bowel disease, through inhibition of NF-kB signaling, ameliorated fecal blood, colonic alterations, and DSS-induced weight loss indirectly indicating reduced intestinal stress (Lohning et al. 2021). S-containing metabolites have also been successfully tested in different skin diseases, such as skin cancer, atopic dermatitis, skin aging, and psoriasis, related to inflammations processes. SFN, for example, reduced the number of scratches, the severity of the dermatitis score, and the thickness of mice ears with 2,4-dinitrochlorobenzene (DNCB)-induced atopic dermatitis. Moreover, significant reductions in gene expression and protein levels related to atopic dermatitis were achieved, in addition to reducing DNA damage, inflammation, and apoptosis (Alyoussef 2021). Recent studies with SFN also revealed that mice fed with SFN for 3 months (442.55 mg/kg) had improved collagen deposition, thus slowing skin aging via activation of the Nrf2 pathway (Petkovic et al. 2021). Furthermore, another ITC, specifically 7-methylsulfinylheptyl isothiocyanate (7-MSI) can inhibit melanin synthesis in B16-F1 fibroblast cells by suppressing melanogenesis and autophagy activation (Kim et al. 2021). Other S-containing metabolites with active properties in inflammatory processes induced by microorganisms are allicin and PEITC. Both exhibited beneficial effects against mastitis through decreasing IL-1β and TNF-α production as well as inhibiting NF-κB and mitogen-activated protein kinase pathway, thus exerting anti-inflammatory effects against *S. aureus*-induced mastitis (Chen et al. 2019), and amelioration of inflammatory reactions by regulating proliferation of mast cells (Han et al. 2018), respectively. In this sense, microbial infections are tightly related to inflammatory processes, which can be counteracted by the double anti-inflammatory and antibacterial action of S-containing molecules. Besides, increasingly, the spread of drug-resistant bacteria associated with chronic infections has become a serious global concern that requires the urgent search for innovative and efficient treatments (Nakamoto et al. 2019). Among S-containing metabolites, allicin has broad antibacterial activity against Gram-positive and Gram-negative bacteria, as well as multidrug-resistant pathogens namely methicillin-resistant *Staphylococcus aureus* (MRSA) (Shadkchan et al. 2004; Sharifi-Rad et al. 2014), and biofilm formation against lung pathogenic bacteria (Reiter et al. 2017) and *Candida albicans* and *S. aureus (*Zainal et al. 2021*)*, demonstrating allicin's potential for development of therapeutic agents to treat several infections caused by multi-resistant bacteria.

Therefore, evidence suggests that S-containing metabolites can be a promising therapeutic approach in the treatment of several inflammation-related disorders, supporting their consumption as a preventive dietary supplement and their incorporation as active ingredients into cosmetic products, where they can preserve skin health and/or act as anti-inflammatory agents in some skin diseases.

11.6.4 Glucose and Lipid Regulation in Diabetes and Cardiovascular Disorders

Some of the most numerous health disorders have been associated with diets characterized for its high caloric content and its poor nutritional value. Regarding the potential factors involved in the development of these disorders can be highlighted as hereditary factors, food additives, environmental obesogens, or toxins that have been introduced into the food chain (Lu et al. 2017; Arzuaga et al. 2009). Among these illnesses, diabetes, lipid metabolism disorders and cardiovascular diseases are increasing their prevalence (Benjamin et al. 2018) and are tightly related. Indeed, several systemic conditions, such as metabolic syndrome, nonalcoholic fatty liver disease, type 2 diabetes (T2D), hypothyroidism, primary hepatocellular carcinoma, chronic kidney disease, non-cardiac vascular diseases, and obesity can be responsible of lipid accumulation which it may cause further cardiovascular fails (Panyod et al. 2016; Gao et al. 2017; Li et al. 2019; Gwon and Yun 2021). Regarding T2D, it is expected that by 2040, at least, 640 million people will suffer it. Poorly-controlled T2D will probably develop sequential complications that increase its associated mortality and morbidity (Boer et al. 2016; Mortada 2017). Some patients cannot be successfully treated with the current existing drugs; therefore, alternative solutions are being evaluated. Among them, several S-containing compounds have been studied in the context of diseases related to glucose metabolism (Li et al. 2020b). Supplementation of SFN from broccoli sprout reduces blood glucose levels in human patients with T2D and decreases glucose production in hepatoma cell lines (Axelsson Annika et al. 2017). Additionally, there is some evidence that supplementation of *Moringa oleifera* isothiocyanates in diabetic and obese mice has anti-obesity and anti-diabetic action, increasing insulin signaling and sensitivity (Waterman et al. 2015). Moreover, subcutaneous injection of SFN in diabetic rats revealed protective effects on T2D-induced cardiomyopathy (Sun et al. 2020). In this sense, other S-containing metabolites represent natural products with potential capacity to prevent cardiovascular illnesses, mainly those found in alliums such as garlic and onion, as well as some ITCs, as the previously mentioned SFN, are attractive candidates (Vazquez-Prieto and Miatello 2010). In fact, SFN has been described to be capable of protecting against angiotensin II-mediated injury in *in vitro* assays (in human umbilical vein endothelial cells), reducing atherosclerosis, which is one cause of coronary heart disease (Zhang et al. 2020). Furthermore, in in vivo experiments, rabbits with chronic heart failure treated with subcutaneous SFN revealed an improvement of cardiac function through inhibition of oxidative stress and inflammation (Radziejewska-Kubzdela et al. 2019). Some other studies have demonstrated beneficial properties of allicin on cardiovascular health, including antihypertensive (Benavides et al. 2007; Dubey et al. 2017), anti-arrhythmic effect (Huang et al. 2013), decrease of myocardial ischemia (Liu et al. 2019) and reduction of triglyceride levels (Elkayam et al. 2013). In this last line of research, S-containing metabolites have been proven to have positive effect in the prevention of lipid metabolism disorders, characterized by abnormalities in the concentration or

composition of lipoproteins, in particular total cholesterol (TC), triglycerides (TG), and low-density lipoprotein cholesterol (LDL-C), or deficiencies of high-density lipoprotein cholesterol (HDL-C) (Lu et al. 2017). Diet supplementation of ITCs and TSNs, namely AITC and allicin respectively, ameliorated lipid accumulation and inflammation in nonalcoholic fatty liver disease, in mice (Panyod et al. 2016; Li et al. 2019). Moreover, SFN supplementation decreased obesity-related glomerulopathy, a kidney disease associated with obesity, in mice (Lu et al. 2020). Finally, supplementation with PEITC and SFN also revealed promising applications in obesity through the improvement of lipid metabolism and inflammation (Gwon and Yun 2021), and regulation of expression of lipid metabolism-related genes (Ranaweera et al. 2022) in mice, respectively, thus corroborating the beneficial effects of its consumption in the control of lipid metabolism-related disorders.

Therefore, S-containing metabolites have been revealed to possess beneficial effects in the prevention of different lipid metabolism disorders, cardiovascular diseases, and diabetes control. Even though further research is required, they may represent a promising therapeutic approach for the alternative treatment of these systemic conditions.

11.7 Conclusions and Future Perspectives

Living organisms require sulfur (S) as a nutrient and undergo an assimilation process mainly through the diet that allows the incorporation of this element into organic structures, such as amino acids, coenzymes, and bioactive compounds, among others. Regarding plants as sources of S-compounds, different vegetable families stand out. Some examples are *Brassicaceae* family vegetables such as broccoli, cabbage, and cauliflower, as well as *Liliaceae,* comprising garlic and onion. As a result of their biological functions, such as antioxidant, anti-inflammatory, biocidal, antimicrobial, cytotoxic, among others, glucosinolates and hydrolysis products isothiocyanates are becoming increasingly popular. Consequently, a diet rich in vegetables containing molecules rich in S was associated with a lower risk of developing several types of cancer, neurological disorders like Alzheimer's, and reduced inflammatory bowel disease, mastitis, photoaging, diabetes, cardiovascular disorders, fatty liver disease, and obesity. Therefore, functional foods and nutraceuticals containing S-metabolites have great potential for the prevention and treatment of several illnesses.

However, a deeper understanding of mechanisms of action regarding the effectiveness and interactions of S-metabolites should be conducted in the future. Besides, to develop new innovative products and therapeutic approaches, more biological functions of S-metabolites and processing effects such as cooking and other heat treatments must be investigated, since they could affect the biological functions and safety of these compounds. Finally, it would also be beneficial to carry out more clinical trials to confirm the health benefits of S-containing metabolites on

humans and to pay special attention to the potential side effects and establish safe doses.

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Chapter 12
Influence of Genetics on the Secondary Metabolites of Plants

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Abstract Secondary metabolites are natural products and have been attributed with the diverse roles in plants such as survival, interaction with environment, protection from biotic/abiotic stress conditions and as volatile signalling molecules. Secondary metabolites are broadly classified into chemical classes such as terpenoids, alkaloids, phenylpropanoids, steroids etc., and are known to be modulated by various factors, including physiological, genotypic, and environmental factors. These secondary metabolite compounds are biosynthesized via different pathways specific to each class of chemical entities produced in plants. The biosynthetic pathway operates in a linear or branched fashion where any alteration/modulation in the pathway or single gene or group of gene influences the production and accumulation of these metabolites. It has been well established that up or down regulation of concerned genes brought about by natural or artificial means subsequently leads to alteration in enzymatic activities responsible for secondary metabolite synthesis. There has been considerable progress in literature on recombinant expression of pathway genes and its association in enhancement of secondary metabolites in plants. Equally interesting is to understand the genetic impact of secondary metabolites in plants. It has been shown in some earlier studies that genetics play a key role in defining composition as well overall yield of secondary metabolites in plants. There is impact of genotype and environment interaction in secondary metabolite levels that will determine the overall expression of a distinct chemotype/genotype-based concept. A large number of genotypes with characteristic composition have been identified to be developed as key varieties suited for defined metabolites. Such examples exist in

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plants such as *Artemisia* annua, *Mentha* species, *Ocimum* species, *Withania somnifera* and several others, where valuable metabolites were shown to be genetically associated. The aim of the current article is to comprehend this less characterized area of secondary metabolism in plants and develop a knowledge resource involving its biosynthesis, accumulation, regulation with reference to their genetic background which in future could be utilized for commercial and pharmacological applications in aroma and drug industries.

12.1 Introduction

Plant secondary metabolites (PSMs) are one of the most diverse group of compounds as they have tremendous diversity in their structure and functionality. Plants produce a plethora of low molecular weight organic compounds, being classified as primary and secondary on the basis of their direct involvement in growth and development of plants. Earlier PSMs are considered less important for the growth and development, being termed as "secondary." Progressive research in this area revealed their role in interaction with the environment and in defense mechanism, so the term "specialized" will be more appropriate instead of "secondary" (Qari and Tarbiyyah 2021). Overall there is no boundary to distinguish the plant metabolite as primary or secondary because current understanding of plant metabolic engineering extends their area of functionalization (Sangwan et al. 2018; Erb and Kliebenstein 2020). These metabolites are of economically as well as ecological importance, therefore, special attention on their production is needed (Sangwan and Sangwan 2014; Sangwan et al. 2010). Moreover, there is no straight pathway to define their synthesis and degradation and it is often observed that these metabolites are restricted in their distribution and it may be limited up to plant family, genera, or species level and even at the tissue specificity (Fang et al. 2012; Jadaun et al. 2020; Kushwaha et al. 2013a). In some plants, they accumulate in special structures like trichome, vacuole, or specialized glands (Yadav et al. 2014; Yu et al. 2018; Maurya et al. 2019). These metabolites are not only crucial for plant life but also play significant role for humans. Pharmacological activities of these compounds made them attractive for industrial applications. Various biotechnological approaches are followed to enhance their production (Fierascu et al. 2020; Srivastava and Sangwan 2020; Narnoliya et al. 2021).

Secondary metabolites from plants are governed by several factors such as physiological, metabolic, environmental, and genomic (Sangwan et al. 2001a, 2017; Farooqi et al. 2000; Sangwan and Sangwan 2000; Singh et al. 2015; Srivastava et al. 2015; Shukla et al. 2003; Yadav et al. 2015). Conclusively, metabolism of SMs is the result of interaction between gene (G) and the environment (E) (Padilla-González et al. 2019). Generally, these metabolites are produced by plants in substantial amounts but variation in quantity and quality is noticed under fluctuation of biotic and abiotic factors (Sangwan et al. 2001a; Akula and Ravishankar 2011; Mishra et al. 2020). Another major factor responsible for

accumulation pattern of SMs is the genotypic constitution (Maurya and Sangwan 2020). Polyploidy has gained the interest of numerous researchers due to various reasons: induction of polyploidy can be utilized to create genetic diversity in targeted crops, thus it has the potential to create new species. The proliferation of new species and genomes and the possible benefits of compatibility may have an impact on species evolution.

Genetic diversity of plants plays critical role in regulation of their metabolism either its primary or secondary. Quantitative trait locus (QTL) analysis is useful statistical method to employ the relationship between genotype and phenotypes (Miles, C. & Wayne). Metabolic quantitative trait loci (mQTLs) plays an important role to decode the involvement of gene in the production of a metabolite (Alseekh et al. 2015). There are different kinds of molecular markers which are available to record the genetic diversity in medicinal plants such as Simple Sequence Repeats (SSR), DNA polymorphism like Random Amplified Polymorphic DNA, and more specified ones such as Restriction Fragment Length Polymorphism, Amplified Fragment Length Polymorphism, and Single-Nucleotide Polymorphism (Sarwat et al. 2012) (Table 12.1). Now days Expressed Sequence Tag (EST) database is utilized to design the markers (Narnoliya et al. 2017).

In this chapter, we will discuss various factors contributing towards the accumulation of secondary metabolites in plants and also the influence of the genetic variability with respect to their association with the secondary metabolite production.

12.2 Influence of Genetics on Secondary Metabolites

Secondary metabolites are classified into three categories: alkaloids, terpenoids, and phenyl propanoids. We will discuss these compounds in detail with special reference to the effect of genetics on the secondary metabolites using examples of leading and important medicinal and aromatic plants (Table 12.2).

12.2.1 Withania Species *(Solanaceae)*

The genus *Withania* belongs to Solanaceae family and it is used in traditional medicines since ancient times. It has immense importance as an ingredient in different medicinal systems like Ayurveda, Unani, and Siddha medicinal practices. It shows health beneficial effects by controlling the aging process through its rejuvenating properties. Genus *Withania* comprises approximately 23 species, but only few species get attention for their usage and popularity. *W. somnifera and W. coagulans* are two of most studied species (Kushwaha et al. 2013b; Sangwan et al. 2014). Research is carried out significantly on *W. somnifera* followed by *Withania coagulans* (Mishra et al. 2013; Mishra et al. 2016; Jadaun et al. 2017a,

Table 12.1 List of selected plants showing the assessment of genetic diversity by using molecular markers

Plant	Family	Constituent	Marker	Application	Reference
Dioscorea pentaphylla L	Dioscoreaceae	Diosgenin	Start codon targeted	Clonal fidelity in tissue culture raised plants	Manokari et al. 2022
Ocimum spp	Lamiaceae	Citral	RAPD	Genetic diversity	Vieira et al. 2003
O. basilicum	Lamiaceae	Linalool, eugenol, cineole	AFLP	DNA genotyping, authentication	Labra et al. 2004
O. gratissimum	Lamiaceae	Eugenol, thymol, geraniol, xantomicrol, cirsimaritin	RAPD	Genetic diversity	Vieira et al. 2001
O. gratissimum	Lamiaceae	Eugenol, thymol	ISSR	Genetic diversity, Species identification	Kumar et al. 2019
Ocimum spp	Lamiaceae	Aliphatic acids, eugenol, thymol	RAPD	Genetic diversity	Chowdhury et al. 2017
Ocimum spp	Lamiaceae	Caryophyllene, alpha-caryophyllene, and linalool	EST-SSR	Diversity analysis, tagging of traits	Mahajan et al. 2015
O. basilicum and O. tenuiflorum.	Lamiaceae	Ursolic acid and oleanolic acid	duplex PCR assay	Authentication	Travadi et al. 2021
O. africanum	Lamiaceae	–	ISSR	Genetic variability	Makmur et al. 2020
W. somnifera	Solanaceae	withaferin A, withanone, withanolide D or withanolide A	RAPD	Genetic diversity	Chaurasiya et al. 2009
W. somnifera	Solanaceae	withanolides and withaferin A	AFLP	Genetic analysis	Dhar et al. 2006
Withania somnifera	Solanaceae	–	ISSR	Genetic diversity	Hiremath et al. 2021

Withania somnifera	Solanaceae	–	RAPD and ISSR	clonal fidelity, Genetic diversity	Nayak et al. 2013; Tripathi et al. 2012; Khan and Shah 2016
Withania somnifera	Solanaceae	–	EST	Genetic diversity	Parita et al. 2018
Artemisia annua *Artemisia* spp.	Asteraceae	Artemisinin	OPGMA-RAPD	Analysis of artemisinin and chemotypic variants	Sangwan et al. 1999
A. parviflora, *A. vulgaris L.,* *A. myriantha and* *A. nilagarica*	Asteraceae	Artemisinin	RAPD, ISSR, and IRAP	Analysis of artemisinin and chemotypic variants	Nganthoi and Sanatombi (2019)
Boerahvia diffusa	Nyctaginaceae	Rotanoids, punarnavin, steroids	RAPD	Mutant characterization, Genetic diversity	Shukla et al. 2001, 2003
M. cervina	Lamiaceae	Pulegone	ISSR	Morphological traits, EO constituents profile, and chemotypic variants	Rodrigues et al. 2013
Mentha spicata, *M. piperita, M. suaveolens,* *M. longifolia, M. aquatica,* *M. x piperita, M. arvensis* *subsp. Arvensis, M. spicata* *var. crispa,*	Lamiaceae	–	RAPD	Genetic diversity	Momeni et al. 2006; Virginia et al. 2021
M. spicata accessions (C30 and C33, *M. arvensis* accessions (C17 and C18), interspecific hybrid	Lamiaceae	–	RAPD and AFLP	Genetic diversity	Shasany et al. 2005
Mentha species	Lamiaceae	–	RAPD	Genetic diversity	Shinwari et al. 2011

(continued)

Table 12.1 (continued)

Plant	Family	Constituent	Marker	Application	Reference
Bupleurum spp	Apiaceae	Bupleurumol, saikosides	rDNA ITS	Species identification	Xie et al. 2009
Catharanthus roseus	Apocynaceae	vincristine and vinblastine	RAPD, ISSR, EST-SSR	Genetic linkage map	Gupta et al. 2007
Cymbopogon species	Poaceae	Citral, trans, geraniol	RAPD	Genetic diversity, Clonal fidelity	Sangwan et al. 2001, 2003;
Coscinium fenestratum	Menispermaceae	Berberine	18S r-RNA; PCR-RFLP	Authentication	Watthanachaiyingcharoen et al. 2010
Cynara cardunculus	Asteraceae	Cynarin, apigenin, luteolin	AFLP, SSR	Genetic diversity	Mauro et al. 2009
Datura spp	Solanaceae	Atropine, hyoscyamine and scopolamine	sequencing; microarray	Authentication	Carles et al. 2005
Echinacea laevigata Echinacea	Asteraceae	Cichoric acid and tetraene	AFLP	Analysis of population genetic structure and mating system	Peters et al. 2009
Euphorbia spp.	Euphorbiaceae	Jatrophanediterpenes	sequencing	Authentication	Xue et al. 2007
Fragaria vesca	Rosaceae	Biflavansabscisic acid	SCAR	Detection of seasonal control of flowering	Albani et al. 2004
Matricaria chamomilla	Asteraceae	Chamomillol, Chlorogenic acid, Chrysoeriol	RAPD	Genetic variation	Solouki et al. 2008
Origanum spp	Lamiaceae	carvacrol, thymol, limonene, pinene, ocimene, and caryophyllene	RAPD	Quality control	Marieschi et al. 2009
Salvia miltiorrhiza	Lamiaceae	Salvinorin	ISSR, SRAP	Genetic diversity	Song et al. 2010

Tribulu sterrestris	Zygophyllaceae	Harman	AFLP, SAMPL, ISSR, RAPD	Genetic diversity	Sarwat et al. 2008
Vitex rotundifolia	Lamiaceae	Vitexfolin A, B, and C	ISSR-PCR	Genetic variation and quality control	Hu et al. 2007
Withania somnifera	Solanaceae	Withaniolide	AFLP	Genetic variation and relationship	Negi et al. 2000
Trollius spp.	Ranuculaceae	orientin, vitexinand quercetin-3-*O*neohesperidoside	RAPD	Genetic diversity	Li and Ding, 2010

Table 12.2 Effect of polyploidy on secondary metabolites production

Plant Name	Common name	Family	Classification/ Clade	Ploidy	Name of Molecules	Molecule conc.	Reference
Agastache foeniculum	Blue giant hyssop	Lamiaceae	Angiosperms	Tetraploid	Essential oil (Methyl chavicols)	50% enhancements	Madani et al. 2021; Talebi et al. 2017; Talebi et al. 2016
Arabidopsis thaliana	Arabidopsis	Brassicales	Angiosperms	Polyploid (autotetraploid, allotetraploid)	γ-aminobutyric acid (GABA)	–	Soltis et al. 2015; Madani et al. 2021; Tavan et al. 2022; Van de Peer et al. 2021; Iannicelli et al. 2020
Artemisia annua	Annual wormwood	Asteraceae	Angiosperms	Polyploid (tetraploid)	Artemisinin (Sesquiterpene); essential oil	38% enhancements; 35.6% enhancements	Madani et al. 2021; Yunus et al. 2018; Yadav et al. 2015; Iannicelli et al. 2020
Atropa belladonna	Deadly nightshade	Solanaceae	Angiosperms	Polyploid (allohexaploid)	Tropane alkaloids	68% enhancements	Madani et al. 2021; Volkov et al. 2017; Lavania 2005
Avena sativa	Oats	Poaceae	Angiosperm	Polyploid (allotetraploid)	Avenacins, avenanthramides	–	Tate et al. 2005; Iannicelli et al. 2020; Kennedy et al. 2020
Cichorium intybus	Chicory	Asteraceae	Angiosperms	Tetraploid	Phenolic compound, Chlorogenic acid	10 times	Madani et al. 2021; Street et al. 2013
Coffea arabica	Coffee	Rubiaceae	Angiosperms	Polyploid (tetraploid)	Caffeine	Decrease	Tate et al. 2005; Iannicelli et al. 2020; Lavania 2005
Cymbopogon species	Lemongrass	Poaceae	Angiosperm	Diploid, tetraploid	Citral, geraniol, gernial, geranyl acetate	enhancement, New compositions	Sangwan et al. 2000; Farooqi et al. 2000

Dracephalummoldavica	Moldavian dragonhead	Lamiaceae	Angiosperms	Tetraploid	Essential oil	27.5% enhancements	Madani et al. 2021; Rauf et al. 2021
Galium mollugo	Hedge bedstraw	Rubiaceae	Angiosperm	Autopolyploid	Flavinoids, phenolic acid	–	TATE et al. 2005; Csepregi et al. 2016
Gossypium hirsutum	Cotton	Malvaceae	Angiosperm	Polyploid	Terpenoid aldehyde	–	Tate et al. 2005; Iannicelli et al. 2020; Park et al. 2019
Glycyrrhizia glabra	Licorice	Fabaceae	Angiosperm	Polyploid (tetraploid)	Glycyrrhizic acid, anthocyanins	Upto 12% enhancements	Iannicelli et al. 2020; Moghbel et al. 2015
Hyoscyamus muticus	Egyptian henbane	Solanaceae	Angiosperm	Autotetraploid	Scopolamine, tropane alkaloid,	200% enhancements, 36% enhancements	Madani et al. 2021; Dehghan et al. 2012; Lavania 2005; Iannicelli et al. 2020
Mentha arvensis	Field corn	Lamiaceae	Angiosperm	Autotetraploid	Essential oil	30% enhancements	Madani et al. 2021
Nicotiana tabacum	Tobacco	Solanaceae	Angiosperm	Polyploid	Pyridine alkaloids	–	Tate et al. 2005; Ghasemi et al. 2021
Oenothera lamarckiana	Evening primrose	Onagraceae	Angiosperm	Tetraploid	Ellagitannin	–	TATE et al. 2005; Greiner and Köhl 2014
Panax ginseng	Ginseng	Araliaceae	Angiosperm	Polyploid(tetraploid, octaploid)	Ginseosides (triterpenoid saponin)	22% enhancements	Iannicelli et al. 2020; Kim et al. 2004
Papaver somniferum	Opium poppy	Papaveraceae	Angiosperm	Polyploid (triploid, tetraploid)	Morphine	Upto 50% enhancements	Madani et al. 2021; Iannicelli et al. 2020; Lavania 2005
Salvia miltiorrhiza	Danshen	Lmaamiaceae	Angiosperm	Tetaploid	Tanshinone ⍰, tanshinone⍰⍰ A, Cryptotanshinone	33% enhancements; 25% enhancements; 162% enhancements	Madani et al. 2021; Iannicelli et al. 2020

(continued)

Table 12.2 (continued)

Plant Name	Common name	Family	Classification/ Clade	Ploidy	Name of Molecules	Molecule conc.	Reference
Sequoia sempervirens	Coastal redwood	Taxodiaceae	Gymnosperm	Hexaploid	Essential oil (Terpine-4-ol, Eugenol & γ-Terpine)	–	Tate et al. 2005; Youssef et al. 2021
Solanum tuberosum	Potato	Solanaceae	Angiosperm	Tetraploid (allotetraploid, autotetraploid)	Sesquiterpenes	22 folds enhancements	Gantait and Mukherjee 2021; Trojak-Goluch et al. 2021; Tavan et al. 2022; Iannicelli et al. 2020
Tanacetum parthenium	Feverfew	Asteraceae	Angiosperm	Tetraploid	Essential oil	32% enhancements	Madani et al. 2021; Ghasemi et al. 2021
Tolmiea menziesii	Piggyback palnt	Saxifragaceae	Angiosperm	Autotetraploid	–	–	Tate et al. 2005; Nezhadi et al. 2021
Triticum aestivum	Wheat	Poaceae	Angiosperm	Polyploid (Hexaploid)	Lutin	10.8 fold enhancements	TATE et al. 2005; Van de Peer et al. 2021; Iannicelli et al. 2020; Cuttriss et al. 2011
Vaccinium uliginosum	Western blueberry	Ericaceae	Angiosperm	Autopolyploid (tetraploid, hexaploid)	Flavonoids (anthocyanin), phenolic acid	2–17 fold variations	Tate et al. 2005; Lyrene2021; Mengist et al. 2020
Zingiber officinale	Ginger	Zingiberaceae	Angiosperm	Tetraploid	Gingerols, Carotenoids	No change, 1.375 folds enhancements	Iannicelli et al. 2020; Gantait and Mukherjee 2021

2017b, 2020). Although both species are very well studied for their ethnopharmacology and a number of phytomolecules have been isolated and identified from the species. Most of the pharmacologically active compounds belong to the terpenoid category of secondary metabolites and collectively this group of compound is termed withanolides.

Withania somnifera, Ashwagandha, the popular Indian ginseng or gooseberry, is a perennial, evergreen small shrub that is distributed globally in wide geographical regions. Although the whole plant possesses medicinal properties, the roots are preferably used for extraction of "Rasayana" (Sangwan et al. 2007). Many pharmacological activities have been attributed due to this Rasayana like adaptogenic, antisedative, anticonvulsion, anti-inflammatory, immunomodulatory activities (Mukherjee et al. 2021). Interestingly, it is effective against several brain and nervous system-related disorders like Parkinson's disease, Alzheimer's disease, and Huntington's disease (Dar and Ahmad 2020). The neuroprotective activity of *W. somnifera* is mediated through anti- oxidative stress mechanism. Withanolide A, withanolide D, β-sitosterol and stigmasterol are the major metabolites against Alzheimer's diseases (Hannan et al. 2020). There is transcriptomic data of *W. somnifera* available, which also reflects the tissue-specific accumulation of different withanolides (Gupta et al. 2013; Sangwan et al. 2014). There is a repository of transcription factors which are involved in regulation of secondary metabolites metabolism (Tripathi et al. 2017). There is variation in ploidy level of *W. somnifera*. Plants may have diploid ($2n = 24$), tetraploid ($2n = 48$), and hexaploid ($2n = 72$) levels, although the predominating ploidy is diploid ($2n = 24$). *W. somnifera* shows significant variation in morphogenetic characters. By using the EST-SSR markers, genetic diversity in 36 genotypes of *W. somnifera* is accessed (Parita et al. 2018). Application of ISSR marker showed genetic diversity in *W. somnifera* species collected from diverse geographical locations (Hiremath et al. 2021). Sangwan and his coworkers have carried out several experiments to explore more and more about the genetic and chemotypic diversity of *W. somnifera* plants collected from different locations in India (Sangwan et al. 2004). Different types of chemotypes (NMITLI-101, 110, 133, 104, 128, 141, 108, 109, 118, 135, and 144) have been identified (Tuli and Sangwan, 2009). The phytochemical analysis includes polypeptide polymorphism, isoenzymes, along with withanolide profiles. RAPD analysis reveals that they cluster together on the basis of genetic similarity. In another study, thirty cultivars are selected from eight different states and cultivated for approaching their stability and adaptability. Out of thirty, cultivars W 20, W 1 (cv. Pratap), W 2, W 3 (cv. Chetak), W 4 and W 6 were found to be highly stable for the root yield (Lal 2015). Five different varieties of *W. somnifera* (Jawahar, Nimithli, Chetak, Pratap, Poshita and) were examined for the withanolide content. There is a significant variation in all the varieties, with the highest content of alkaloids (withaferin A and withanolide A) found in Poshita, followed by Jawahar-20 (Singh et al. 2018; Mishra et al. 2020). In another study performed in Sri Lanka, withaferin A content was estimated in two varieties of *W. somnifera*, LC1 and FR1, these varieties were grown under the same climatic conditions. Different tissues (leaf, bark stem and root) of both varieties are screened for alkaloid content and results showed highest

content of withaferin A in leaves of LC1, with minimum content found in the root of FR1 (Siriwardane et al. 2013). Srivastava et al. (2018) reported genetic variability in 53 genotypes of *W. somnifera* collected from different geographical locations. According to pathway coefficient analysis, there is positive correlation in fresh root weight, total alkaloid (%) in leaf and deoxywithastramonolide in the root (%). Effect of genotype-environment interaction on withanolide content was studied on 16 genotypes of *W. somnifera*. A total of 12 characters were considered along with alkaloid content and a variation was observed in characters of all the genotypes grown under different locations (Kumar et al. 2020). Mirjalili et al. (2009) reported the genetic (by using the RAPD anlysis) and withaferin A diversity in Iranian natural populations of *W. somnifera* and *W. coagulans*.

12.2.2 Artemisia (Asteraceae)

Artemisia is a rich genus of aromatic plants with around 500 species including both herbs and shrubs (Valles and Arthur 2001). Several *Artemisia* species are perennial. However, few *Artemisia* species are annual or biennial (Vallès et al. 2003) because of its greater population and morphological complexities, the taxonomy of genus *Artemisia* is not clear (Hayat et al. 2009). Based on recent molecular studies, the genus *Artemisia* has been divided into five deferent subgenera. These subgenera are *Absinthium, Artemisia, Dracunculus, Tridentata,* and *Pacifca* (Vallès et al. 2011). The diversity of *Artemisia* is mainly found in Asia, Europe, and North America. China (around 150 accession) and Europe (174 accession) are main regions of *Artemisia* diversity. Japan (50 species), Pakistan (38 species), and Iran (35 species) also add diversity to the genus (Hayat et al. 2009). Only few *Artemisia* species (*Artemisia* annua, *Artemisia Absinthium*, and *Artemisia* vulgaris) are commonly utilized as traditional medicine in Asia and Europe from ancient time.

Artemisia annua (sweet wormwood or qinghao in China) is the most popular among *Artemisia species* due to its antimalarial properties from ancient times (Hong et al. 2015). *Artemisia annua* is native to Asian, North American, and European countries. The antimalarial properties of *Artemisia annua* are due to an important compound, artemisinin. The acceptance of artemisinin as antimalarial drug was gained due to the emergence of chloroquine resistance in the 1950s (Ma et al. 2020). Artemisinin, the sesquiterpenoid-based therapy has so far proved efficient in the treatment of millions of people in developing countries (Ma et al. 2020). The Chinese scientist Youyou Tu was honored with the 2015 Nobel Prize in Physiology or Medicine for her discovery of artemisinin, which became a promising drug (Liu and Liu 2016). Recently, artemisinin-derived compounds such as artesunate and artemether are basically used in medicine preparation for malarial drugs (Tu et al. 1981; Wright 2002).

The main chemical constituents of *Artemisia* species are polyphenols and essential oils, but also coumarins, acetylenes, sesquiterpene-lactones, alcohols,

flavonoids, monoterpenes, and sesquiterpene derivatives. The chemical diversity of constituents attributes *Artemisia* species with different pharmacological bioactivities including antibacterial, antimalarial, anticancer etc. (Padalia et al. 2016; Nigam et al. 2019; Sangwan et al. 2010). Vast research is ongoing with *A. annua* for the development of drugs to treat COVID-19 (Bisht et al. 2021).

Artemisinin, the endoperoxide sesquiterpene-lactone synthesized in the eight-celled trichome glands of *A. annua*, is an active ingredient in the artemisinin combination therapy-based treatment of malaria. The content of artemisinin varies among the varieties of *A. annua* from different origins. Even Indian population of *A. annua* plant exhibited very high level of genetic variation in RAPD analysis (Sangwan et al. 1999). Advances in genetics and metabolic engineering approaches widen the path to *in planta* artemisinin production. Microbial-based metabolic engineering of artemisinic acid (the precursor of artemisinin) had demonstrated some potential, but the further chemical synthesis of artemisinin are costly (Ro et al. 2006). Thus, the agricultural production of artemisinin is the only viable source for ACT. *A. annua* is an undeveloped crop and plant-based production of artemisinin is a challenging task. Development of improved varieties for artemisinin production purposes is the realistic target. Different approaches have been applied to improve *in planta* artemisinin yield.

Several plant breeding-based techniques are used to develop improved variety of *A. annua* in terms of higher artemisinin yield. Conventional breeding approach includes identification of superior parental lines with desired traits and crossing them to develop hybrid lines with improved artemisinin yield (Delabays et al. 2001; Cockram et al. 2012; Townsend et al. 2013). Artemisinin biosynthesis in *A. annua* is controlled by genetic factors (Ferreira et al. 1995b; Delabays et al. 2001, 2002). The vegetative phase of *Artemisia* plant can be maintained under long photoperiods, while flowering can be induced under short days. Flowering time of different varieties varies under field conditions, but can be induced in greenhouse. Delabays et al. (1992) had crossed artemisinin (1.1%, w/w) rich, late-flowering Chinese clone with European plants and developed new variety with 0.64% to 0.95% artemisinin content and dry leaf yields between 14 to 21 t/ha (Delabays et al. 1992). Banyai et al. (2010) have developed the tetraploid cultivar of *A. annua* by doubling the chromosomes number with higher level of artemisinin content. Graham et al. (2010) studied genomics of *A. annua* where all the genes of the pathway are shown in genetic linkage with marker traits. Artemis (1.4% artemisinin) is commercially important variety of *A. annua* developed by cross between two genetically different heterozygous parental genotypes C4 and C1 (Delabays et al. 2001). Graham et al. (2010) have developed genetic linkage and QTL maps for Artemis and validated positive QTL for artemisinin yield. 34,419 SNPs with mean SNP frequency of 1 in 104 base pairs were identified from the five EST databases of Artemis F1 hybrid material (Graham et al. 2010). Further, polymorphism confirmation with 19 AFLP primer combinations revealed 322 polymorphic markers. 49 SSR markers which are segregated in the Artemis F1 population were also identified through *in silico* approach (Graham et al. 2010). The mapping population of the Artemis F1 exhibited variation in plant phenotype, artemisinin content (0.93 to 20.65 mg/mg dry weight), glandular

trichome density (4.89 to 19.11 mm^2), leaf area (508.76 to 4696.08 mm^2) and plant fresh weight (160 to 4440 g). Graham et al. (2010) found strong segregation distortion of the advantageous alleles QTL on C4LG1 in favor of artemisinin yield trait (a product of artemisinin concentration and plant fresh weight). Although, artemisinin is present in *A. annua* plant leaf and stem tissues, but artemisinin yield depends on plant genetic potential (Ferreira et al. 1995a). Heterozygous nature of *A. annua* is also a challenge for plant breeders because developed plant exhibit varying degrees of artemisinin content (Delabays et al. 2001; Graham et al. 2010; Larson et al. 2013).

Besides *A. annua*, several other *Artemisia* species such as *A. absinthium, A. aff. Tangutica, A. apiacea, A. bushriences, A. campestris, A. cina, A. diffusa, A. dracunculus, A. dubia, A. indica, A. japonica, A. lancea, A. moorcroftiana, A. parviflora, A. roxburghiana, A. scoparia, A. sieberi, A. sieversiana, A. spicigeria,* and *A. vulgaris* are reported to have artemisinin (0.05–0.034%) in their aerial parts (Mannan et al. 2010; Singh and Sarin 2010; Rashmi et al. 2014). Nganthoi and Sanatombi (2019) had studied artemisinin contents in four *Artemisia* species (*viz., A. parviflora, A. vulgaris L., A. myriantha, and A. nilagarica*) of Manipur region in India and also established the genetic relationship among the four *Artemisia* species by using RAPD (15), ISSR (11) as well as IRAP (3) markers. Out of total 267, 203 and 58 reproducible fragments, 240, 187 and 51 were polymorphic with high average polymorphism (89.88% for RAPD, 92.5% for ISSR and 87.93% for IRAP) (Nganthoi and Sanatombi 2019). The highest similarity index in RAPD (0.5), ISSR (0.5) as well as IRAP (0.79) markers was found between *A. nilagarica* and *A. myriantha* (both belongs to Sub-genus *Artemisia*) with similar artemisinin content per gram DW in leaf (0.031%–0.044%), young flower (0.042%–0.052%), mature flower (0.031%–0.047%) and stem (0.008%–0.01%) (Nganthoi and Sanatombi 2019). While *A. parviflora* (belongs to the Sub-genus Dracunculus) depicted least similarity index with rest three *Artemisia* species and high level of artemisinin content in leaf (0.078%–0.087%). *A. nilagarica* and *A. myriantha* had depicted low genetic variability and have a closer evolutionary relationship, while *A. parviflora* had depicted higher genetic variability.

12.2.3 Ocimum *(Lamiaceae)*

Ocimum genus belongs to the Lamiaceae family and holds immense importance in the plant word due to its medicinal and aromatic properties. The plant is well adapted to survive under tropical warm and temperate conditions and is an inhabitant of tropical Africa, tropical Asia, and tropical America (Paton et al. 2004). The genus contains approximately 150 species out of which 66 species have been reported online (Chowdhury et al. 2017; Gurav et al. 2021). Several Ocimum species are studied in great details for their pharmaceutical constituents being the major ones *O. tenuiflorum, O. basilicum*, *O. Americanum*, O. africanum, *O. gratissimum*, *O. kilimandscharicum*, and *O. citriodorum* (Gupta et al. 2018; Gurav et al. 2021;

Maurya et al. 2019). In India, nine species, including three exotic species namely *O. adscendens*, *O. basilicum*, O. canum, *O. gratissimum*, *O. kilimandscharicum*, O. tenuiflorum, *O. americanum* L., *O. minimum* L., and *O. africanum* Lour have been reported (Chowdhury et al. 2017). Ocimum is rich in chemical diversity which makes it extremely useful in culinary, fragrance, therapeutic, and the cosmetic industry (Pandey et al. 2016). Ocimum is a repertoire of compounds like terpenoids, alkaloids, phenols, tannins, anthocyanins, lignins, quinones, saponins, flavonoids etc. Essential oils present in Ocimum contain linalool, chavicol, methyl chavicol, eugenol, camphor, 1,8-cineole, α-terpineol 6.5%, β-pinene, camphene, sabinene, cis-ocimene, trans-ocimene, ß-caryophyllene, geraniol, limonene, 10-Heptadecen-8-ynoic acid; Cyclopentane, thymol, etc. (Maurya et al. 2019; Enegide and Charles 2021). These compounds impart hepatoprotective, anti-diabetic, antioxidant, neuroprotective, anti-inflammatory, antiseptic, antidiarrheal, antispasmodic, and antimicrobial properties. They also play a role in the treatment of skin, respiratory tract infections, stomach ache, kidney malfunctions etc. (Prakash and Gupta 2005; Dhama et al. 2021; Enegide and Charles 2021). Basil seeds are a rich source of proteins (11.4–22.5 g/100 g), carbohydrates (40–63.8 g/100 g), dietry fiber (7.1–22.6 g/100 g) along with calcium, magnesium, and phenolic compounds (orientine, vicentine, and rosmarinic acid) (Calderón Bravo et al. 2021). Microencapsulation of essential oil from *O. basilicum* was conducted to develop a packaging material which could control the bacterial growth in packaged food and also increase the shelf life of food by inhibiting change in pH (Amor et al. 2021). Similarly, seed mucilage from *O. basilicum* was used with montmorillonite (MMT) as nanofiller to synthesize bionanocomposite films to be used as packaging material (Rohini et al. 2020). Its use as a seasoning, sauces, salads is well accounted for. These traits of Ocimum makes its essential oil very important in the national and international market and thereby an 8% compound annual growth rate is expected from 2019 to 2023 (Gurav et al. 2021).

A number of different approaches have been used to increase secondary metabolites in terms of concentration and components. With the aim of incorporating desired traits and increase the genetic stock; plant breeding has been practiced for many decades (Ahmar et al. 2020). In *Ocimum basilicum var. glabratum* Benth chemotypes which were morphologically similar but contained different chemical compounds, i.e., eugenol, methylchavicol, and camphor were established. It was assumed that the variation in accumulation of compounds with different origins (eugenol, methylchavicol are phenylpropanoids while camphor is terpenoid) was due to the presence of a multi-allelic gene which expresses differentially (Gupta et al. 2005). Similarly, a eugenol rich and methyl chavicol rich line had also been developed by breeding (Gang et al. 2001). In another study, a cross between basil varieties "Perrie" (rich in eugenol) and "Cardinal" (rich in methyl chavicol) on segregation generated eugenol chemotype (23%–25%), methyl chavicol chemotype (23%–25%, and an intermediate mixture of the two compounds (~50%). The segregation followed 1:2:1 pattern, thereby suggesting involvement of a single bi-allelic gene with incomplete dominance (Dudai et al. 2018). Other than these a large number of chemotypes have been developed by conventional breeding such as

CIM ayu; eugenol rich variety of *O. sanctum*, CIM Angana; high yielding variety of *O. sanctum* (Shyam tulsi); CIM Saumya; methyl chavicol and linalool rich short duration variety of *O. basilicum* (Lai 2003; Lal et al. 2004, 2008). Higher ploidy level is associated with higher essential oil content (Lavania 2005). Attempts were made to induce autotetraploidy by colchicine treatment at different plant stages and at different concentrations. Polyploidy resulted in large stomata, pollen grains and increased chloroplast number (Omidbaigi et al. 2010).

12.2.4 Mentha *Species*

The *Mentha* species (Lamiaceae) plants evolved through natural hybridization and selection, consisting of about 25 to 30 species (Brickell and Zuk 1997). *Mentha* essential oil is important for pharmaceutical, cosmetics, food, confectionery, liquor, and pesticide industries (Sangwan et al. 2000). *Mentha* is a rich source of plant secondary metabolites such as terpenes (Sangwan et al. 2000). Phylogenetic relationships and genetic diversity among *Mentha* species have been widely studied mainly by using molecular markers such as RAPD and amplified fragment length polymorphism (AFLP) primers-based genetic analyses (Gobert et al. 2002; Shinwari et al. 2011; Rodrigues et al. 2013; Virginia et al. 2021). Rodrigues et al. (2013) had studied the morphological, phytochemical, and genetic variation in 12 populations of *Mentha cervina* L. (Portuguese endangered medicinal plant) to access the level of diversity through ISSR markers. One hundred and twenty-one individuals of ISSR amplification gave a total of 175 bands (average: 82.4 fragments per individual) with 97.7% (171 band) of them being polymorphic. Out of twelve, four populations clustered together in morphological parameters as well as molecular studies, but the secondary metabolites, i.e., essential oils basis created different clustering (Rodrigues et al. 2013). Rodrigues et al. (2013) interpreted the observation as essential oils evolve more rapidly than the morphological traits, so the morphological traits were found more correlated with the genetic variation. Shinwari et al. (2011) studied the RAPD profile based similarity and diversity of two mint species (*Mentha royleana and Mentha spicata*) including 15 accessions of each. The polymorphism percentage observed in all the 15 accessions of *M. royleana* was 27.44% while in *M. spicata,* it was 47.2%. A high level of genetic polymorphism was noticeable among population, thereby indicating genetic richness and heterozygosity (Shinwari et al. 2011). RAPD profiles of different mint species/accessions *Mentha arvensis* L., two of *Mentha spicata* L., one each of *Mentha spicata* L. cv. viridis, *Mentha* × *piperita* L., *Mentha* × *piperita* L. cv. citrata and *Mentha* × *gracilis* Sole cv. RAPD markers could clearly identify various Mentha genotypes, accessions as well as hybrids. Fenwick and Ward (2001) identified 17 accessions of *Mentha* (three species) belonging to different geographical origins from the USA by using RAPD-based profiling. Momeni et al. (2006) had studied the genetic variation and taxonomic relationship between 17 accessions out of four *Mentha* species *viz., M. spicata, M. piperita, M. suaveolens,* and *M. longifolia* by RAPD fingerprinting.

In another study, Virginia et al. (2021) had established the genetic variations between ten varieties of *Mentha* (*Mentha longifolia, Mentha aquatica, Mentha x piperita, Mentha arvensis subsp. arvensis and Mentha spicata var. crispa* and five commercial varieties) by using eight arbitrary RAPD primer based profiling and recorded 100% polymorphism. Hundred percent polymorphism was interpreted due to out-breeding and the wide dispersal of seed and pollen grains (Virginia et al. 2021).

12.2.5 Geranium sp.

Geranium (family geraniaceae) is a very popular aromatic crop of commercial importance. Essential oil of geranium is used in a variety of formulations of commercial, pharmaceutical, and agricultural importance (Gallardo et al. 2012; Narnoliya et al. 2019). Transcriptome information of *P. graveolens* proposed the pathways involved in primary and secondary metabolite biosynthesis (Narnoliya et al. 2017, 2018). Geranium essential oil has several terpenes as constituents majorly geraniol, citronellal, linalool, geranyl acetate, limonene, pulegone, citronellyl format, citronellylacetate, menthone isomenthone, 10-epi-γ-eudesmol, etc. (Rana et al. 2002; Jadaun et al. 2017a, 2017b). A large number of species diversity (~ 200 speices) is reported in the *Geranium* genera in reference to morphological features and essential oil composition (Tyagi et al. 2003; Yi et al. 2018). Commercially, four species: *graveolens: odoratissimum: radens, and P. capitatum* were grown for harvesting the essential oil. On the basis of variation in oil yield and oil constituents, different chemotypes of *Pelargonium capitatum* are reported (Demarne et al. 1993). Production of somaclonal variations may be a good source of induction of the genetic variability in plants. Kulkarni et al. (1997) reported about the intra clonal variations in *Pelargonium* sp. as they differ significantly in essential oil composition (isomenthone is major constituent instead of geraniol and linalool). Somaclonal variations were studied in an Indian cultivar, "Bourbon" of rose-scented geranium (*Pelargonium graveolens*), they differ in the phenotype (height, leaf shape, leaf size, leaf dentation) and phytochemical composition (Ravindra et al. 2004). Glass house grown somaclones, highly dentated leaves (HDL) and less dentated leaves (LDL) show variability in leaf dentation pattern, herb yield as well as variability in the oil yield also (Saxena et al. 2008; Tyagi et al. 2003).

For identification of genetic diversity in *Geranium* species different types of DNA markers are also used. RAPD and ISSR analysis in cutting (diploid) and polyploidy seedlings revealed geraniol, geranyl formate, and linalool concentration was quite higher in polyploid plants (Yi et al. 2018). RAPD analysis was performed to trace the phylogenetic relationship among the following species of Geranium: *G. dissectum* L. (sec. Dissecta); G. persicum Schönb.-Tem., *G. tuberosum* L., G. kotschyi Boiss., *G. platypetalum* Fisch. & C. A. Mey., G. gracile Ledeb. Ex Nordm., *G. ibericum* Cav. (sec. Tuberosa subsect. Mediterranea R. Knuth). *G. stepporum* P.H.Davis (sec. Tuberosa subsect. Tuberosa (Boiss.) Yeo);

G. columbinum L., *G. rotundifolium* L., *G. sylvaticum* L., *G. collinum* Stephan ex Willd, G. pratense (Yin et al. 2021).

12.2.5.1 Molecular Markers in Association to Secondary Metabolism

Molecular markers have been used extensively in characterization of accessions, germplasm as well as mutant lines. Along with the progress that has markedly taken place during past two to three decades in molecular techniques in developing markers such as RAPD, RFLP, ISSR, microsatellites etc., the markers have been associated with various traits of interest. The plants with secondary metabolites have also been studied, and the co-relation with various markers to assist the analysis related to association between secondary metabolites and markers. RAPD markers were quite popular owing to ease in establishment and also not requiring genomic information (Sangwan et al. 1999, Shukla et al. 2005). Medicinally important plant punarnava (*Boerhavia diffusa*) which is used in herbal and medicinal puoposes. The plant grows in wild and majorly two varieties, white flowered and red flowered are in the trade (Shukla et al. 2005). The attempts to develop better varieties through conventional breeding approaches and also mutant generation through mutagens of physical and chemical nature were attempted. The genetic differences were well differentiated by the RAPD marker as well as by isozymic pattern analysis. The performance of RAPD markers in associating the trait was much better as compared to isozymes. Also the mutant which had unique characters, including flower color, exhibited a unique RAPD pattern.

Similarly, in Cymbopogon species, the fragrant members of Poaceae family were examined with RAPD, isozyme and protein polymorphic markers basis. Cymbopogons are aromatic species and differ in their ploidy status as well. *Cymbopogon martinii* is one of the most prominent species preferred by the farmers for cultivation owing to its rose like aroma. The essential oil contains geraniol and geranyl acetate in elite varieties of sofia and motia. The genetic score, gene diversity, heterozygosity, etc., were computed. Such genetic marker analysis could distinguish elite genotypes of Cymbopogon species (Sangwan et al. 2001a, 2007; Sangwan Neelam et al. 2003). Furthermore, information related to the genetic basis of wild and collected germplasm as well as requirement for trait introgression can be envisaged from the diverse growing plant types.

Similarly in another study, important trade type of Cymbopogon species were studied using RAPD marker to distinguish diversity of trade type varieties and species differing substantially in their oil types (Sangwan et al. 2001a, 2003). The study correlated essential oil metabolic diversity with the genetic diversity parameters. Markers also clustered together *Cymbopogon flexuosus*, *C. pendulus* and *C. khasianus* in one cluster, owing to the similarity in their essential oil constituent profile. Diagnostic markers and stand-alone markers were identified and associated with constituents (Sangwan et al. 2001a, 2003). In another study, genetic variation was created through somaclonal variation in Cymbopogon hybrid Jamrosa. Large numbers of growth parameters were impacted including essential oil content (Nayak

et al. 2003). Gross genetic variations in RAPD pattern of parent and somaclones of Jamrosa indicated the extent of variation in several physiological parameters as well secondary metabolites (Nayak et al. 2003). Superior clones with respect to higher terpinol geraniol content were evaluated in field trials for consistency in performance and characterization through RAPD molecular marker (Nayak et al. 2003).

Withania somnifera is one of the traditional resources of medicinal herb. Molecular markers including RAPDs, and SSRs have been used in studying the genetic diversity and characterizing the genotypes in metabolic core groups (Chaurasiya et al. 2009; Sangwan et al. 2017). Such markers provided clues in establishing genetic diversity of collection at intra- and interspecies levels. The levels of secondary metabolites such as wihanolidal diversity in terms of qualitative as well as quantitative variations were also elucidated. In vitro clonal plants were also established for their clonal stability and also evaluation for distinctness of secondary phytochemicals (Sabir et al. 2007). High throughput analytical techniques of metabolomics and a combination of markers together further substantiated large numbers of core collections for metabolites in an integrated manner (Dhar et al. 2006; Chaurasiya et al. 2009).

12.3 Status of Polyploidy and Its Impact on Secondary Metabolites

Polyploidy is a phenomenon of two processes, i.e., endomitosis and endoreduplication. Other processes like fusion of nuclei, ineffective mitosis, or the emergence of multinucleated cells are also involved in creating polyploid cells. Unlike normal mitosis, the endomitosis is a process in which the cell membrane is not destroyed, and mitosis takes place within the nucleus, chromosome numbers double and sister chromatids are likely to separate and return to the interphase mode, except those chromosomal spindles are not created and as a result sister chromatids are not stretched to the side of the cell and polyploidy cells are not created. The endometriosis process mostly occurs in animal cells and is rare in angiosperms (flowering plants). The endoreduplication nucleus may replicate DNA without going through the mitotic process, resulting in 4n, 8n, 16n, and so on (Ghasemi et al. 2021). Diploidy refers to the presence of two complete set of chromosomes in somatic cells, one from each parent, whereas polyploidy refers to the presence of three or more complete sets of chromosomes in somatic cells (Park et al. 2021). Each organism has a specific number of chromosomes that are divided into different groups. Ploidy refers to the number of chromosomal groupings. In this case, the number of chromosomes may alter. Euploidy and aneuploidy are the two types of alterations that occur. Polyploidy is formed by two mechanisms: somatic doubling and the creation of unreduced gametes (2n).

According to their origin, polyploidies can be classified as auto-polyploidies and allopolyploids (Ghasemi et al. 2021). Polyploidy can arise as a result of

autopolyploidy, which describes the occurrence of polyploidy inside a species, or allopolyploidy, which is described as the development of polyploidy as a result of hybridization between two different species (Park et al. 2021). Polyploidy refers to the duplication of the same genome in autopolyploids or the duplication of separate genomes in allopolyploids (Pradhan et al. 2018). The growth of chromosomes in one kind of diploid results in two (or more) homologous chromosomal pairs, resulting in auto-polyploidies. Allopolyploids are the result of chromosomal growth following the hybridization of two distinct species. In general, the terms euploid and aneuploid are used to refer to organisms with different ploidy a status, which is discussed below. In euploidy, the total number of chromosomes is the exact multiple of the base chromosome series. Euploids can be divided into three groups: monoploids, diploids, and polyploids. But most euploids have two sets of chromosomes and are diploid. However, some euploid species have more than two chromosomal classes and are polyploidy (Ghasemi et al. 2021). According to Talebi et al. (2016) autotetraploidy polyploidization increases the number of chromosomes, gene dosage, concentration, and activity of several enzymes and amino acids. An increased number of genes elevates phenylalanine and tyrosine levels, which are further required for the biosynthesis of phenylpropanoids. It is worth mentioning that geneticists use the letter x to denote the number of base chromosomes, which are collections of single chromosomes that combine to form a full set. The number of chromosomes in each gamete or ploidy surface is also indicated by the letter n. New and modified cultivars of commercially significant species have been developed in recent decades by inducing artificial polyploidies with mutagenic agents. However, polyploidy is not as simple as genome multiplication, and it generates a wide variety of molecular and physiological modifications (Ghasemi et al. 2021). Polyploidy in angiosperms is stable, implying that this genome status has adaptive value and is positively selected (Iannicelli et al. 2020). The majority of polyploidies (natural and artificial) have distinct characteristics from their ancestor (Ghasemi et al. 2021). Polyploidy can disclose novel properties that are not found in either of the diploid progenitors, or it can outperform the progenitor's characteristics. Prior investigations suggest that polyploidy can alter the qualitative or quantitative features of plant metabolites, which has implications for plant metabolism (Park et al. 2021). In most plant species, artificial polyploidy boosted the vitality of determinate plant portions. It has the potential to improve the production of vegetative organs and plant biomass. Polyploidy can be employed to improve the quality and quantity of essential therapeutic substances in plants, as well as to boost secondary metabolites. In flowering plants, chromosome doubling may alter secondary chemistry. DNA methylation, histone changes, and antisense RNA, as well as silence and up- or down regulation of genes involved in secondary metabolite biosynthesis, are all affected by genome doubling (Talebi et al. 2016). Normally, polyploids exhibit an increase in secondary metabolites or enlargement of different plant sections, however, there have been instances of no effect or a negative effect on the number of secondary metabolites. Polyploids make use of their resources through shorter metabolic pathways. They also have a larger cell size and methylation of cytosine (Pradhan et al. 2018). Some of these features, such as bigger organ size (leaf, flower, etc.), a

wider biomass range, dryness tolerance, disease tolerance, and a variety of flowering times, might help polyploids adapt to new environmental changes (Ghasemi et al. 2021). Polyploidy alters physical and physiological alterations, which affect habitat, geographical distribution, reproductive systems, and breeding systems (Talebi et al. 2016). Furthermore, these different polyploidy features increase the likelihood of them being selected for agricultural uses (Ghasemi et al. 2021). It is mostly observed that polyploidy enhances the agronomic traits and attributes such as size of plant leaf and flowers as well as the increased tolerance owing to doubling of genome sets (Iannicelli et al. 2020). Polyploidy promotes growth in secondary metabolites in medicinal plants, as well as more and better adaptation to the environment, and increases the thickness of petals and the size of flower, among other things, in ornamental plants. In reality, artificial polyploidy allows for rapid genetic improvement of plants, making polyploidization one of the most important technologies in plant breeding (Ghasemi et al. 2021). Cell size enlargement is the most well-known and widely recognized result of polyploidization in plants. In reality, the generations of synthetic polyploids allow for rapid genetic improvement in plants, making polyploidization one of the most essential and common technologies employed in plant breeding (Iannicelli et al. 2020). Ploidy induction is an effective strategy for improving the genetics of many plants (Talebi et al. 2016). These distinctive polyploidy cells have almost double the volume of diploid predecessors and a 1.5-fold increase in cell surface area. Polyploids differ from their diploid status in their morpho-physiological parameters, due to the rise in cell size of enlarged flowers, leaf, and fruit sizes. The phenotypic differences are due to two polyploidization-related difficulties that contribute to the most constant impacts of the "polyploid phenotype": an increase in gene dosage. In addition to the reported increases in secondary metabolite production in autopolyploids, the finding in different MAP's suggests that the "polyploid effect" may influence pathways in diverse ways. It has been hypothesized that changes in ploidy cause changes in the control of metabolite production. As a result, while some metabolites are increased, others are reduced in favor of the former (mono and sesquiterpenoids in *A. annua*, morphine/thebaine and codeine in *Papaver somniferum*, and Scopolamine/hyoscyamine in *Centella asiatica)* (Iannicelli et al. 2020). For example, tetraploids exhibited higher levels of sesquiterpenoids in *Artemisia annua* and scopolamine alkaloid accumulation in *H. muticus* plants, presumably through increasing the expression of pathway genes (Talebi et al. 2016).

12.4 Conclusion

Secondary metabolites are of immense importance for the plants and human beings. Their production in plants is under the influence of environmental and genetic constitutions. Ploidy level of plants affects the secondary metabolite accumulation under tight genetic control. In some plants, induction of polyploidy leads to improvement in metabolite accumulation. Various reports are available showing the genetic

variability in different species or cultivars of various medicinal and aromatic plants. These variations also contribute in alteration of secondary metabolites accumulation pattern. Various tools and techniques like genetic markers and QTL mapping can be utilized for identification of genetic diversity in plants and further phytochemical analysis of these plants confer the impact of genetic variance on the chemistry of associated plant up to significant level. Therefore, often plants differing at genetic level also vary in their metabolites either qualitatively or quantitatively. Such studies are very helpful for identification and characterization of improved varieties. Medicinal plants with improved metabolic content will have impact on global trading.

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Part III
Fungi Secondary Metabolites

Chapter 13
Biochemistry of Secondary Metabolism of Fungi

Liege A. Pascoalino, Tânia C. S. P. Pires, Oludemi Taofiq, Isabel C. F. R. Ferreira, Lillian Barros, and Filipa S. Reis

Abstract Fungi, eukaryotic organisms with a kingdom of their own, include microorganisms from moulds and yeasts to the most known and appreciated mushrooms. The incredible biodiversity of these organisms is not limited to their morphology but is reflected in their chemistry, namely in the variety of compounds they produce. Therefore, like other living beings, fungi can be an excellent source of bioactive compounds.

Although they may be primary metabolites, fungal bioactive compounds are mainly produced through secondary metabolism. These compounds have an essential role in the fungal survival and adaptation to almost all *habitats* on earth. Besides, they can also exert beneficial effects on human health, such as antioxidant, antimicrobial, anti-UV radiation, or even anti-inflammatory or antitumor activity. Given the wide bioactivity of the molecules produced, fungi have become, over time, an exciting source of compounds with possible application in various industries, including the food, pharmaceutical, or cosmetics industries.

Fungal secondary metabolites are mainly produced via acetyl-CoA and via the shikimate pathway. Even though it is possible to find in the literature some different classifications regarding secondary metabolites of fungi, in this manuscript, we define polyketides, non-ribosomal peptides, terpenoids, and indole alkaloids as the main structural classes.

The present chapter will present a brief introduction to fungal secondary metabolism, including some examples of the most well-known compounds and their

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principal functions in ecosystems. The biosynthetic pathways of the main classes of fungal secondary metabolites will also be depicted.

13.1 Fungal Secondary Metabolites: An Overview

Fungi are widely known to produce a variety of secondary metabolites that include antibiotics, vitamins, pigments, amino acids, and other organic compounds, which in turn have recognized biological activities (Devi et al. 2020). Penicillins and other β-lactam antibiotics, the cholesterol-lowering lovastatin, or the immunosuppressant cyclosporin are some of the most well-known fungal metabolites used as medicines worldwide.

Albrecht Kossel first introduced the term "secondary" metabolite in 1891 (Devi et al. 2020; Hartmann 2007). Unlike primary metabolites, secondary metabolites are low molecular weight molecules, which are not present in every living cell capable of dividing and are not essential for the producing organism's normal growth, development, reproduction, or energy production (Avalos and Limón 2021; G. F. Bills and Gloer 2016; Thirumurugan et al. 2018). Although the classic definition of secondary metabolism remains in this sense, the truth is that it results from the evolutionary process of the species, and the synthetized compounds have become essential for their existence. In fact, secondary metabolites can confer some adaptative and survival advantages to the producing organisms. Throughout their evolution, fungi conquered a wide range of *habitats*, ubiquitous in terrestrial and freshwater environments, less common in marine territories, and a cosmopolitan distribution (Webster and Weber 2007). This adaptive success to the most diverse ecosystems is also associated with its developed secondary metabolism, which is complex and capable of originating an enormous diversity of compounds with several functions.

Although they seem very different mechanisms, the line separating primary from secondary metabolism becomes very thin, as secondary metabolites are derived from central metabolic pathways and primary metabolite pools, with acetyl-CoA as the initial building block, leading to the synthesis of polyketides and terpenes, and amino acids being used for the synthesis of non-ribosomal peptides (Keller 2019). This cross-linkage between fungal primary and secondary metabolism is represented in Fig. 13.1.

As shown in Fig. 13.1, the production of primary and secondary metabolites is a dynamic process, with common biochemical pathways. Indeed, some products of the primary metabolism are often considered secondary metabolites, depending on their need and function. Some examples are amino acids and other organic acids, such as oxalic acid, alcohols, or sugars. This classification of primary/secondary metabolite is influenced by the organism's growth, cell differentiation and development, combined with the edaphoclimatic conditions in which it develops (G. F. Bills and Gloer 2016; Keller 2019; Thirumurugan et al. 2018).

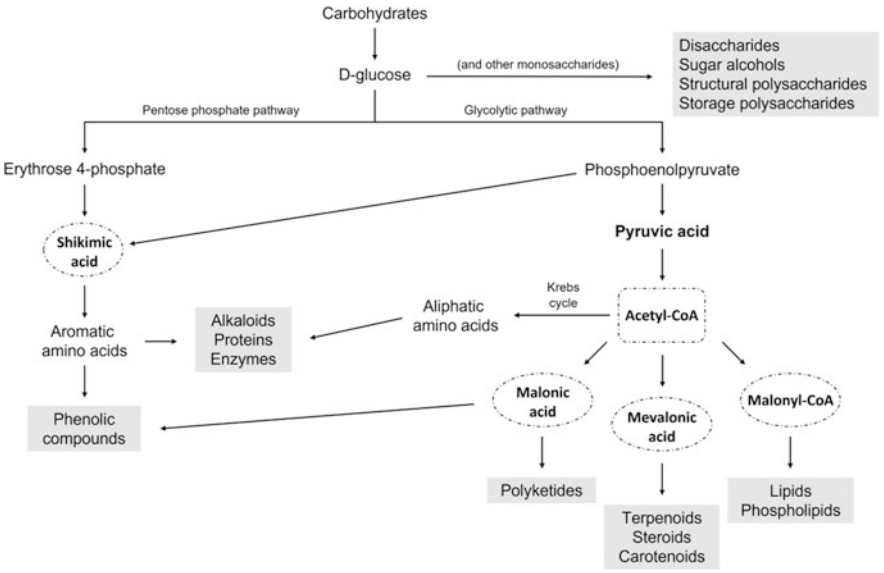


Fig. 13.1 Schematic diagram representing the cross-linkage between primary and secondary metabolism in fungi. There is main evidence on the central pathways (), leading building blocks (), and main originated compounds ()

The main structural classes or chemical families of fungal secondary metabolites are polyketides (PKs), non-ribosomal peptides (NRPs), terpenoids, and indole alkaloids (Daley et al. 2017; Devi et al. 2020). It is important to highlight that there are also hybrid molecules, resulting from the joint action of different classes, namely polyketide–terpene, non-ribosomal peptide–polyketides, and polyketide–fatty acid (Keller 2019). Some authors even consider hybrid non-ribosomal peptide/polyketides the fifth class of fungi secondary metabolites (Avalos and Limón 2021). However, after a critical reading of the information available in the literature, this chapter will consider and address the four chemical classes initially mentioned.

Fungi secondary metabolites are primarily synthesized via the shikimic acid pathway and acetyl-CoA through the malonic and mevalonic pathways (Fig. 13.1). In addition to differences at the function level, primary and secondary metabolism also have their particularities at the genomic level. The genes encoding the synthesis of primary metabolites are dispersed throughout the fungal genome. In contrast, the genes encoding the enzymatic activities to produce secondary metabolites are organized in biosynthetic gene clusters (BGC), ranging from two genes to over twenty genes (Brakhage 2013; Keller 2019). BGCs mainly contain genes that encode one or more enzymes that synthesize the core structure of the compound, the so-called backbone enzymes. Since most secondary metabolites are derived from polyketides (PKs) or non-ribosomal peptides (NRPs), the most common backbone enzymes are polyketide synthases (PKSs) and non-ribosomal peptide synthetases (NRPSs) (Brakhage 2013; Kjærbølling et al. 2019). Accordingly, secondary

metabolites are essentially originated through the polymerization of primary metabolites by backbone enzymes, which will thus determine the chemical class of the generated compounds. For example, polyketide synthases (PKSs) produce polyketides from acyl-CoAs, non-ribosomal peptide synthetases (NRPSs) generate non-ribosomal peptides from amino acids, and terpene synthases and terpene cyclases generate terpenes from activated isoprene units. However, other enzymes can alter the metabolites' bioactivities (Keller 2019).

The secondary metabolism is mainly developed in fungi of the division Ascomycota and Basidiomycota, while underdeveloped in the unicellular forms of the divisions Ascomycota, Basidiomycota, Zygomycota, Blastocladiomycota, and Chytridiomycota (G. F. Bills and Gloer 2016). The diversity of fungal species, particularly in the Ascomycota and Basidiomycota divisions, along with the diversification and clustering of the biosynthetic genes present, contribute highly to the enormous variety of metabolites originated (G. F. Bills and Gloer 2016; Keller 2019; Keller and Hohn 1997).

As previously mentioned, fungi secondary metabolites display a comprehensive range of biological activities, allowing fungi to survive in the most diverse ecosystems. For example, fungi pigments may confer protection against environmental stress, playing a pivotal role against photo-oxidation effects (carotenoids) or function as UV radiation protectors (melanin) (Dufossé et al. 2014; Gmoser et al. 2017; Kalra et al. 2020). Volatile compounds are released by fungi and used for species communication (Farh and Jeon 2020). Phenolic compounds and organic acids may act as signalling molecules in host-parasite/symbiotic relationships (Gaude et al. 2015), and antibiotics are produced to avoid species competition, limiting bacterial and fungal growth (Fan et al. 2017). In response to warmth, humidity, and moisture, the production of toxins by fungi (mycotoxins) is also well-known, mainly by those growing on crops (Brown et al. 2021). Overall, given the bioactive potential of their secondary metabolites, fungi produce these molecules as a response to biotic and abiotic factors, being involved in both communication and competition processes (Netzker et al. 2015; Shalaby and Horwitz 2015).

Therefore, fungi constitute a great source of attractive compounds for different pharmaceutical, cosmetic, and food sectors. In recent years fungi have become a good source of microbial metabolites, accounting for about 45% of the total production. This percentage includes metabolites produced by Basidiomycetes (mushrooms; 11%) and filamentous fungi such as *Penicillium*, *Aspergillus,* or *Trichoderma* (33%). Other types of fungi, such as yeasts and slime moulds, account for nearly 1.5% of the production of all metabolites (Bérdy 2012). Among the incredible variety of secondary metabolites produced by fungi, we can highlight molecules that have provided benefits that have revolutionized society, such as penicillins, cyclosporin, and statins (Cole et al. 2003; Devi et al. 2020; Keller et al. 2005; Rosazza 1984; Turner and Aldridge 1971). On the other hand, some fungal secondary compounds are also associated with severe problems, like mould-contaminated indoor environments or food and livestock contaminants, including aflatoxins and trichothecenes (Bills and Gloer 2016; Bräse et al. 2009, 2013; Hoffmeister and Keller 2007).

Overall, secondary metabolites synthesized by fungi are organized into four main classes and are extremely important to their environment adaptation and survival. Given their chemical diversity and bioactive potential, they have become attractive to humans from a food and pharmaceutical/cosmetic point of view.

13.2 Biosynthetic Pathways of Secondary Metabolites in Fungi

Fungal secondary metabolites may be divided into four major chemical classes: polyketides (PKs), non-ribosomal peptides (NRPs), terpenoids, and indole alkaloids. Beneath, some of the most known fungal secondary metabolites will be displayed, describing their biosynthetic pathway, including the enzymes involved in their origin.

13.2.1 Polyketides

Polyketides are one of the most chemically and functionally diverse compounds, synthesized by fungi (Cox et al. 2018). This chemical class encompasses molecules with an essential ecological function that man uses to develop products with diverse applications in agriculture or pharmacology.

Polyketides are often associated with toxicity, being the precursors of several toxins such as T-toxins (trichothecene mycotoxins), fumonisins, cytochalasin, or the well-known aflatoxins (Fig. 13.2). Although these polyketide mycotoxins are frequently associated with harmful effects on the production of diverse crops, affecting the agricultural sector and animal and human health, in nature, they allow the fungus to perform balanced maintenance of the pathogens that share its environment, competing with the surrounding species (Sakhkhari et al. 2019; Schuemann and Hertweck 2009).

The polyketides class also includes pigments associated with fungal protection, as is the case of melanins (Fig. 13.2e). Melanins play essential ecological and biochemical roles in the fungal lifecycle. Their functions are associated with protection against adverse conditions, including UV light or heavy metals toxicity, and are also involved in charge transport and structural stability phenomena (Cordero and Casadevall 2017; Gómez and Nosanchuk 2003). Figure 13.2 displays the chemical structure of some of the described polyketide-derived compounds with a crucial function in fungal survival in ecosystems.

Given their role in ecosystems, polyketides have inherent bioactivities that are interesting to explore. Therefore, the man tries to synthesize them and use them at an industrial level, as is the case of strobilurins and griseofulvins. Strobilurins are a group of natural products and synthetic analogues used as pesticides in agriculture.

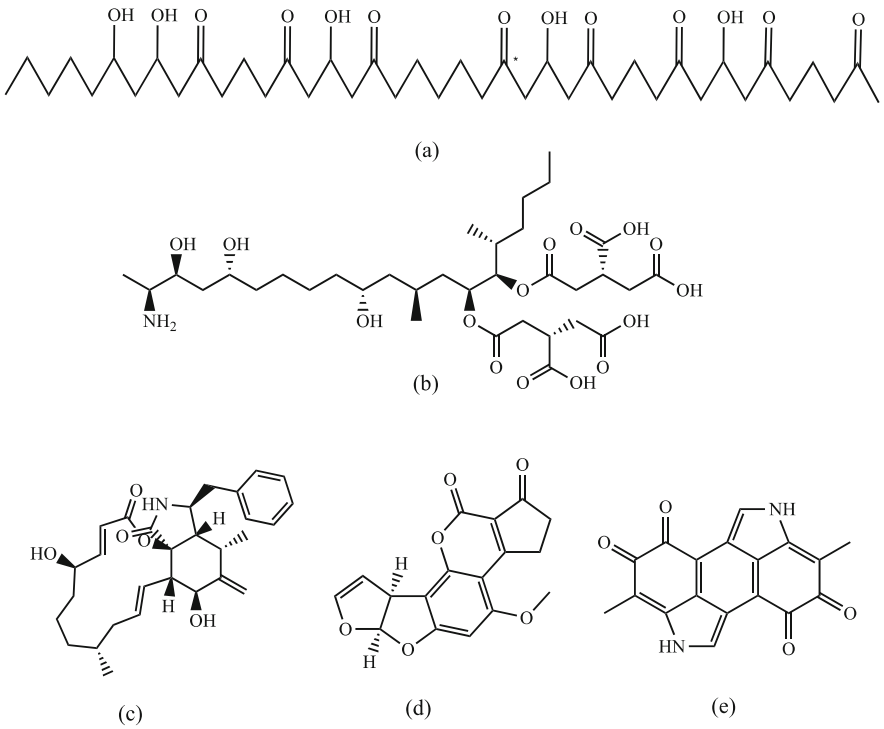


Fig. 13.2 Chemical structure of fungal polyketide-derived compounds with important ecological roles. (**a**) T-toxin (C41); (**b**) Fumonisin B1; (**c**) Cytochalasin B; (**d**) Aflatoxin B1; and (**e**) Melanin

The first natural strobilurin, strobilurin A, was extracted from the fungus *Strobilurus tenacellus*. Nowadays, these polyketides are produced at an industrial level through chemical modifications that promote their activity and photostability, and there are more than ten strobilurins available on the market. Indeed, these broad-spectrum fungicides represent an essential component in the agricultural fungicide trade (Cox et al. 2018; Feng et al. 2020). Griseofulvin (Fig. 13.3a), first obtained from the fungus *Penicillium griseofulvum*, is also an antifungal polyketide but used as medicine for years to treat ringworm in animals and humans (Petersen et al. 2014).

Polyketides also include other valuable and revolutionary drugs used in medicine, such as erythromycin or lovastatin. Erythromycin A (Fig. 13.3b) is an antibiotic (macrolide family of antibiotics) first isolated in 1952 from *Saccharopolyspora erythraea* (McGuire et al. 1952). It is widely used against several respiratory infections, being the main treatment for many pulmonary infections such as Legionnaire's disease. It is also used to treat some sexually transmitted infections, such as chlamydia or syphilis (Farzam et al. 2021). Lovastatin (Fig. 13.3c) is a statin, the class of agents mostly used to treat hypercholesterolemia (Mulder et al. 2015). First isolated from the filamentous fungi *Aspergillus terreus*, this polyketide-derived

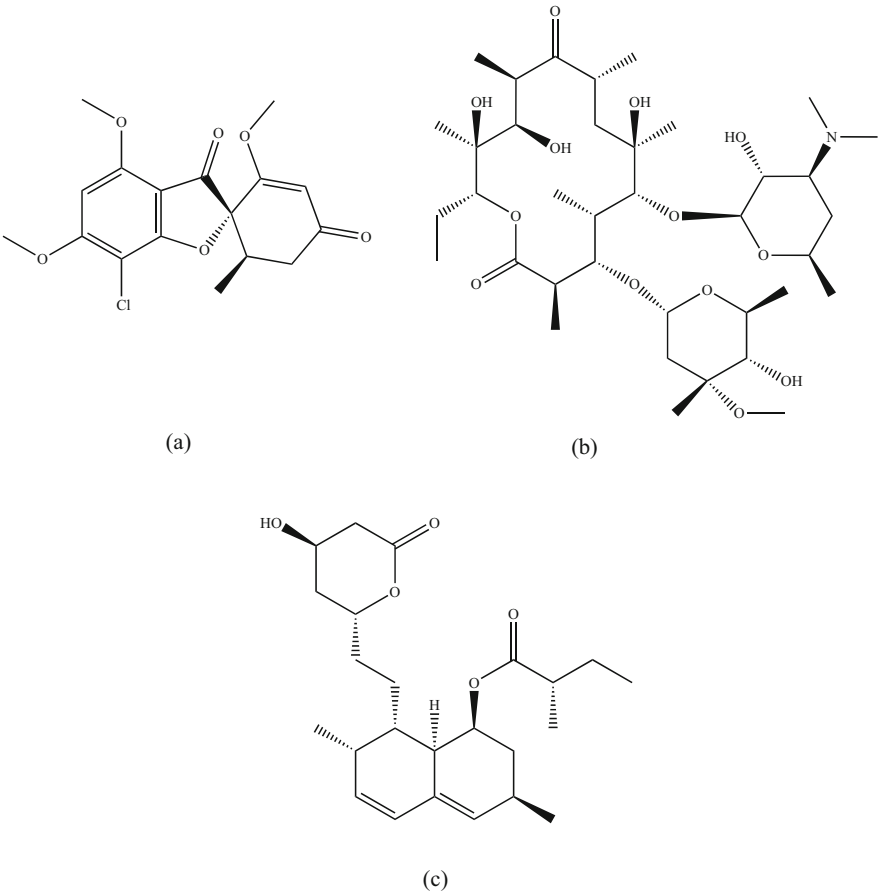


Fig. 13.3 Chemical structure of fungal polyketide-derived compounds with important pharmacological activity. (**a**) Griseofulvin; (**b**) Erythromycin A; and (**c**) Lovastatin

natural product may also be present in higher fungi, such as *Pleurotus ostreatus*, *Cantharellus cibarius*, and *Lentinula edodes* (Kała et al. 2020). The commercial lovastatin is derived from *A. terreus* batch fermentation. It acts on the liver, reducing its ability to produce cholesterol by blocking the enzyme HMG-CoA reductase (Casas López et al. 2004; Mulder et al. 2015). Figure 13.3 displays the chemical structure of some of the described polyketide-derived compounds used worldwide due to their pharmacological potential.

Polyketide's biosynthesis is highly related to fatty acids biosynthesis. Indeed, their carbon backbone is formed by a series of decarboxylative condensation reactions between acetyl-CoA thioesters units and malonate, using enzyme complexes homologous to fatty acid synthases, called polyketide synthases (PKSs). Following the condensation reactions, several chemical reactions occur, justifying the great diversity of this class of compounds (Avalos and Limón 2021; Bhattarai et al. 2021;

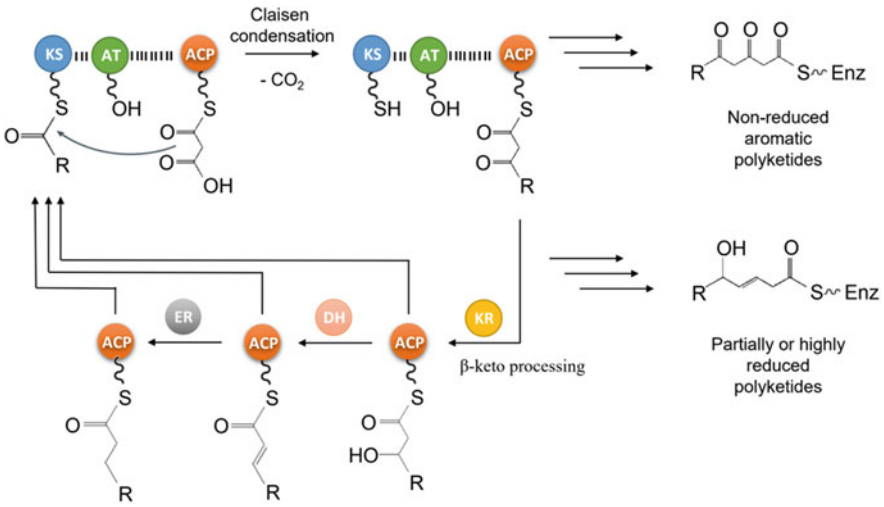


Fig. 13.4 Basic schematic representation of the mechanism of polyketide biosynthesis. *KS* ketoreductase, *AT* acyltransferase, *ACP* acyl carrier protein, *KR* ketoreductase, *DH* dehydrase, *ER* enoyl reductase, *Enz* KS or ACP domain. (Original image based on Crawford and Townsend 2010 and Schuemann and Hertweck 2009)

Cox et al. 2018; Gupta and Rodriguez-Couto 2017; Simpson and Cox 2012). PKS enzymes have been classified into (i) type I, (ii) type II, and (iii) type III PKS. However, fungi polyketides biosynthesis involves mainly single modular iterative type I polyketide synthases (iPKSs), responsible for their carbon backbone construction. Type III PKSs, enzymes with a single keto synthase (KS) domain, may also be present; nevertheless, they are less common. Data on literature reveals that only eleven type III PKSs from fungi have been characterized so far (Avalos and Limón 2021; Bhattarai et al. 2021; Kaneko et al. 2019; Manoharan et al. 2019; Ramakrishnan et al. 2018; Skellam 2022; Yan et al. 2018).

PKSs have a standard set of conserved domains always consisting of ketosynthase (KS), acyltransferase (AT), and an acyl carrier protein (ACP). These three domains are complemented by other catalytic domains such as ketoreductase (KR), dehydratase (DH), enoyl reductase (ER), methyltransferase (MeT) and thioesterase (TE) (Fujii 2010; Fujii et al. 2004; Schuemann and Hertweck 2009; Skellam 2022). Figure 13.4 briefly depicts the biosynthesis of polyketides based on the structure of the PKSs that give rise to them.

In polyketides biosynthesis, acyltransferase (AT) recognizes the monomer that will be used in the synthesis and transfers acyl groups from CoA onto the KS and ACP domains. The Claisen condensation is catalyzed by the KS domain to which acetyl-CoA binds, being condensed with malonyl-CoA units that are carried by the ACP domain (Fig. 13.4). In this stage, a ketide unit is added in each catalytic step. During condensation, the acetyl-CoA continuously bonded to malonyl-CoA loses its acidic group, resulting in the β-polyketide chain (Avalos and Limón 2021; Bhattarai

et al. 2021; Crawford and Townsend 2010; Javidpour et al. 2011). The ACP, transporting the intermediates through the catalytic cycle, serves as a covalent binding site for the intermediate formed (Avalos and Limón 2021). Therefore, ACP domains serve as anchors for both malonyl-extending units and the acyl chain under construction (Avalos and Limón 2021; Schuemann and Hertweck 2009). The molecules that are being formed move between KS and ACP by thioester transfers, and in the end, a β-ketone is obtained (Fig. 13.4). The complexity of the β-ketone depends on the number of cycles (chain length control) and if it is fully reduced at the β-carbon of the extending chain or not. The keto groups formed in the elongating process can be reduced by introducing different possible groups on the β-carbon during the assembly of the polyketide, by a ketoreductase (KR), dehydrase (DH) or enoyl reductase (ER). This process, also called β-ketone processing, allows the obtention of non-reducing, partially reducing, or highly reducing polyketides (Fig. 13.4) (Avalos and Limón 2021; Crawford and Townsend 2010; Schuemann and Hertweck 2009). In addition, other changes carried out by different domains such as methyltransferase (MT) or condensation/heterocyclization (HC) can be performed, increasing the already enormous variety of polyketides existing in nature (Avalos and Limón 2021).

Overall, fungal polyketides assemblage is carried out mainly by iPKSs, being type III PKSs less frequent. According to the degree of reduction of the β-ketone chain, polyketides and PKSs can be classified as non-reducing, partially reducing, or highly reducing PKs or PKSs.

13.2.1.1 Non-reducing Polyketides

The most simple and well-known non-reducing polyketide is the phenolic compound orsellinic acid. The first PKS activity was observed in 1968 and was associated with its production; the orsellinic acid synthase present in a cell-free extract of *Penicillium madriti* (Gaucher and Shepherd 1968; Schuemann and Hertweck 2009).

Non-reducing PKSs (nrPKSs) usually lack domains for β-keto processing. They generally have a conserved set of domains composed of an N-terminal starter unit acyl transferase (SAT) domain, followed by the standard KS, AT, and ACP domains, essential to the chain elongation. Following the general mechanism of polyketides biosynthesis, the SAT domain is responsible for selecting and loading the starter unit, an acetate, a fatty acyl chain or another polyketide. Then, AT loads the malonate extender units, and the KS catalyzes the chain extension of the ACP-bound acyl chain (Cox 2007). What distinguishes these PKSs is a specific structural feature, an additional domain called product template (PT) domain, located between AT and ACP (Cox et al. 2018; Crawford and Townsend 2010; Schuemann and Hertweck 2009; Simpson and Cox 2012). Thus, these enzymes are constituted by an N-terminal loading component (SAT domain), a backbone extension component consisting of KS, AT, PT, and ACP domains, and a C-terminal processing component (Schuemann and Hertweck 2009; Simpson and Cox 2012). The PT domain is responsible for the cyclization of poly-β-ketone intermediates

gathered during polyketide biosynthesis, being involved in the chain length determination, and controlling the final product's structure. This domain can also promote the product release from the enzyme (Barajas et al. 2017; Schuemann and Hertweck 2009; Simpson and Cox 2012; Zheng et al. 2020). Therefore, nr-PKSs are responsible for the synthesis of fungal aromatic polyketides. Some examples are norsolorinic acid and aflatoxin B1 produced by *Aspergillus* sp. or the fungal red pigment bikaverin produced by *Fusarium* sp.

Many nrPKSs do not end after the ACP, having a diverse array of domains, including Claisen cyclase/thioesterases (CLC/TE) (Watanabe et al. 1998), *C*-methyl transferases (*C*-MeT) (Shimizu et al. 2005), and reductases (R) (Cox 2007), as well as additional ACPs (Fujii et al. 2001). TE domains are the most common C-terminal processing components found in nrPKSs (Schuemann and Hertweck 2009). On the other hand, although few nrPKSs with *C*-methylation domains are known, many non-reducing polyketides are *C*-methylated. In this case, the *C*-methylation (*C*-MeT) domain after the ACP domain presumably acts during chain extension. This has been particularly studied in the biosynthesis of the mycotoxin citrinin from *Monascus ruber* or *M. purpureus* (Cox et al. 2018; Schuemann and Hertweck 2009; Simpson and Cox 2012). Reductases (R) are rare in nrPKSs. This domain is usually used as a mechanism to release an aldehyde or primary alcohol (Simpson and Cox 2012). In the nrPKSs with additional ACP domains, although not all are required for polyketides biosynthesis, all of them are functional (Fujii et al. 2001; Watanabe and Ebizuka 2002).

13.2.1.2 Partially Reducing Polyketides

Partially reducing PKSs (prPKSs) are much rarer when compared with the nrPKSs, or the hrPKSs. These PKSs (and highly reduced PKSs) have a core domain, named the ketoreductase (KR) domain. So, prPKSs have an N-terminal KS, followed by AT, DH, KR, and a *C*-terminal ACP. Unlike nrPKSs, they do not possess SAT, PT, or TE domains (Cox et al. 2018; Schuemann and Hertweck 2009).

Although several genes coding for prPKSs are known, only three of these genes are related to the production of secondary metabolites in fungi. One of the best known is associated with the biosynthesis of the fungal metabolite 6-methylsalicylic acid (6-MSA) and has been isolated from *P. griseofulvum*, *A. terreus,* and *Glearea lozoyensis*. During the biosynthetic process of 6-MSA, a single reduction catalyzed by the KR domain occurs (Cox et al. 2018; Schuemann and Hertweck 2009; Simpson and Cox 2012).

13.2.1.3 Highly Reducing Polyketides

Highly reducing PKSs (hrPKSs), a class of enzymes also very common in fungi, are responsible for the synthesis of highly reduced polyketides, such as the previously mentioned lovastatin, T-toxin or fumonisin B1 (Simpson and Cox 2012).

The N-terminal KS from hrPKSs is followed by AT, DH, ER, and KR domains, ending with a C-terminal ACP. Some hrPKSs lose the ER domain, and instead, they possess an equivalent length sequence without known function. These PKSs are therefore grouped into hrPKSs with functional ER domain and hrPKSs with missing or non-functional ER domain. In addition to the β-keto processing domains, many hrPKSs have a *C*-MeT domain following the DH (Cox et al. 2018; Simpson and Cox 2012). For example, for the biosynthesis of lovastatin, two enzymes are required, lovastatin nonaketide (LNKS) and lovastatin diketide synthase (LDKS). The first has an inactive ER domain and a *C*-terminal truncated condensation (C) domain. LDKS encompasses KS, AT, *C*-MeT, ER, KR and ACP domains (Cox et al. 2018).

In general, fungal polyketides are classified as non-reducing, partially reducing, or highly reducing PKs. This classification is mainly based on the enzymes that originate them and the differences in their structure that induce the formation of different compounds. nrPKSs, one of the most common enzymes, consist of an SAT domain, followed by KS, AT, PT and ACP domains, and a C-terminal varying component. prPKSs have an N-terminal KS followed by AT, DH, KR, and a *C*-terminal ACP. Finally, hrPKSs have an N-terminal KS followed by AT, DH, ER, and KR domains, ending with a C-terminal ACP.

13.2.2 Non-ribosomal Peptides

Ribosomes play an essential role in the biosynthesis of proteins that form the building blocks of life. Nonetheless, evidence suggests that some peptides' formation in soil-inhabiting Actinomycetes and Bacilli, eukaryotic filamentous fungi, and marine microorganisms are based on distinct ribosome-independent mechanisms, resulting in the synthesis of non-ribosomal peptides (NRP) (Martínez-Núñez et al. 2016). These structurally diverse NRPs are synthesized by the mega-enzyme complex referred to as non-ribosomal peptide synthetases (NRPSs), playing specific roles in host protection, stress tolerance and interactions with the environment (Oide and Turgeon 2020). Still, the characterization of these fungal metabolites has led to the development of ground-breaking pharmaceutical formulations, including antimicrobial agents, tumour suppressors, enzyme inhibitors, siderophores, and immunosuppressants in past decades (Guzmán-Chávez et al. 2018; Le Govic et al. 2019; Süssmuth et al. 2011).

Biosynthesis of NRPs begins by a series of repeating steps catalyzed by the three core catalytic domains of NRPS: adenylation (A), thiolation (T), and condensation (C). The multidomain NRPSs act as a molecular assembly line in which an amino acid is incorporated from one module to the next, as shown in Fig. 13.5.

The A domain starts the NRPS biosynthesis cycle by carrying out an ATP-dependent activation of the amino acid substrates, then loading onto the T domain's serine residue (Miller et al. 2016). The substrates that the A domain can recognize are proteinogenic and non-proteinogenic amino acids in their D- and L-configurations, fatty acids, α-hydroxy acids, α-keto acids, heterocycles, and other

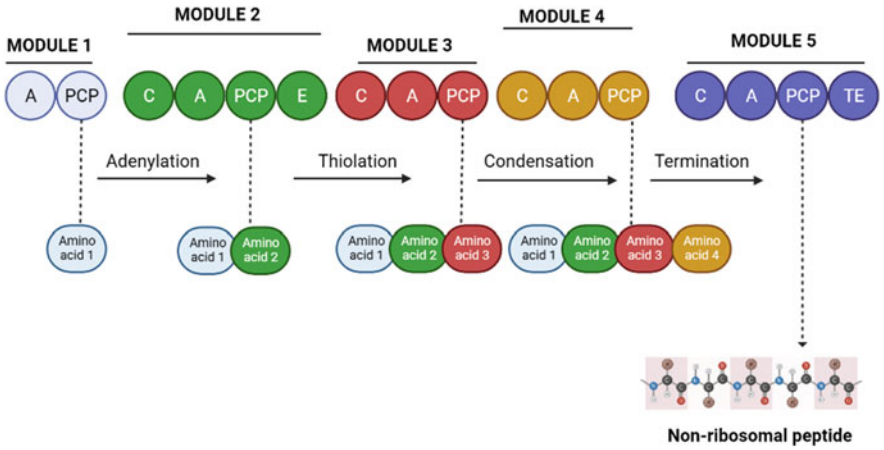


Fig. 13.5 Assembly line of a non-ribosomal peptide synthetase (NRPS)

acyl moieties (Iacovelli et al. 2021). The flexibility of the biosynthetic programming pathway of NRPs based on their ability to utilize an extended range of over 500 substrates compared to ribosomal peptides results in the synthesis of structurally diverse NRPs (Izoré et al. 2021). The T domain, also known as the peptidyl carrier protein (PCP), binds the activated substrate to a 4′-phosphopantetheine cofactor (ppan) attached to a serine residue, forming a covalent linkage between the monomer and the enzyme. After adenylation and thiolation, the C domain, usually located at the beginning of each module, condenses the two substrates loaded onto the T domains, producing the peptide bond. After the generation of the complete peptide, the thioesterase (TE) domain, located on the C-terminal of NRPSs by an intra- or intermolecular cyclization event, catalyze peptide release from NRPSs (Guzmán-Chávez et al. 2018). The peptide then goes through a series of modifications, including methylation, glycosylation, acylation, halogenation, heterocyclization, or hydroxylation, generating structurally diverse peptide scaffolds (Le Govic et al. 2019). According to the collinearity rule, the number of modules in the NRPS is expected to correspond to the number of amino acid building blocks incorporated into the peptide metabolite. This implies that a 3 module NRPS will produce a tripeptide, while a 5 module NRPS will produce a pentapeptide (Challis and Naismith 2004). In addition, each module and the active site of each domain are utilized once in the assembly line, but in sporadic cases, these rules of collinearity and module skipping might be violated due to deletion of a specific domain (Gao et al. 2018). NRPs often have cyclic and/or branched structures that are easily recognizable by a peptide backbone, modified side chain on the amino acids, and can carry either methylated, glycosylated, acylated, halogenated, or hydroxylated modifications on the peptide backbone (Fig. 13.6).

The discovery of penicillin in 1928 by Alexander Fleming represents a historical milestone in searching for effective antimicrobial agents and is recognized as the earliest advancement in therapeutic medicine. Penicillins and cephalosporins are

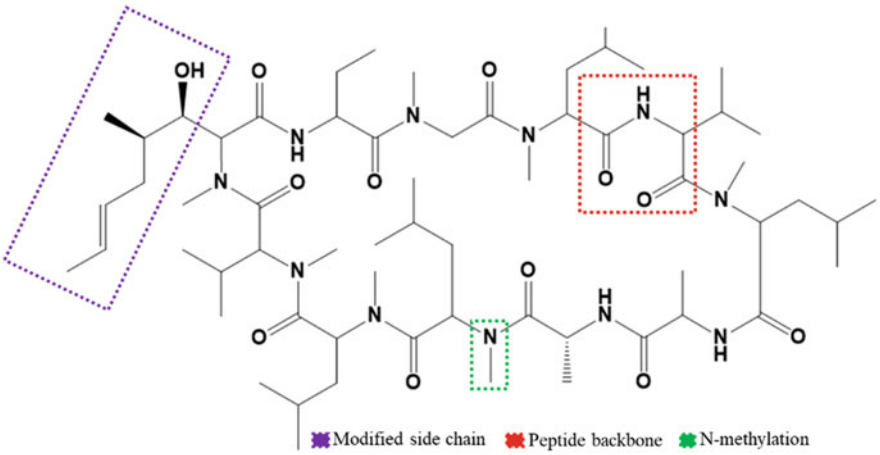


Fig. 13.6 Structural backbone of a typical NRP

NRPs that are mainly produced in *Penicillium chrysogenum*, *Aspergillus nidulans*, and *Acremonium chrysogenum*. Structurally, they contain a β-lactam ring formed by cyclisation of the linear tripeptide δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV), made up of L-α-aminoadipic acid, L-cysteine, and L-valine (von Döhren 2004). ACVS is the most well-characterized NRPS and is responsible for conducting the first reaction in the pathway leading to the biosynthesis of penicillin (Iacovelli et al. 2021). As shown in Fig. 13.7, the biosynthesis of penicillin begins with the activation of the three amino acids, followed by them loading onto the PCP domain. The activated substrates are condensed, forming ACV, a reaction catalyzed by ACV synthetase (ACVS). The next step involves the cyclization of ACV by isopenicillin cyclase, an iron-dependent enzyme, forming isopenicinilin N (Niu et al. 2020). In *Penicillium chrysogenum*, isopenicillin N is converted to penicillin by the enzyme isopenicillin N acyltransferase. In contrast, in *Acremonium chrysogenum*, isopenicillin N is converted to cephalosporin via a series of distinct enzymatic reactions (Guzmán-Chávez et al. 2018).

Cyclic depsipeptides (CDPs) are cyclooligomers containing one or more amino acids being replaced by a hydroxylated carboxylic acid, forming a lactone bond in the core ring. These compounds have been reported in several fungal genera, including *Acremonium*, *Calcarisporium*, *Fusarium*, *Phomopsis*, and *Ramalina* (X. Wang et al. 2018). This class of fungal peptides has received increased attention due to their potential biological properties as antibacterial, insecticidal, herbicidal, anti-viral, cytotoxic, and cholesterol-lowering agents (Süssmuth et al. 2011; X. Wang et al. 2018). Besides their promising potential in pharmaceutical formulation development, they also confer several advantages to the producing fungal strain by enhancing uptake of nutrients, protection against other microbes, and more ecological functions (X. Wang et al. 2018). Biosynthesis of cyclic depsipeptides follows a similar module architecture with identical domain arrangement, as shown

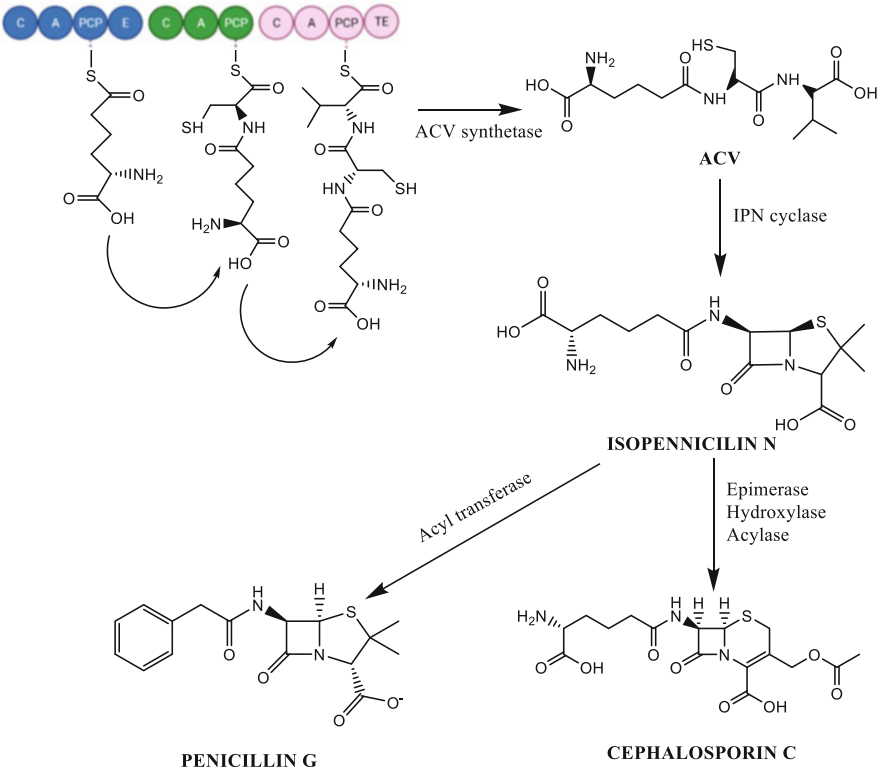


Fig. 13.7 Structural backbone of a typical NRP

in Fig. 13.8. The A domain of the first module activates the D-hydroxycarboxylic acid substrate and is covalently bonded onto the PCP of the same module. The L-amino acid substrate is activated at the A domain of the second module and loaded onto the PCP of the corresponding module, which is linked to a terminal C3 domain, where ester bond formation and cyclization take place (Boecker et al. 2018; Süssmuth et al. 2011).

Hexadepsipeptides are the largest cyclic fungal depsipeptides that have been well characterized and distributed in the genera *Aspergillus*, *Beauveria*, *Cordyceps*, and *Fusarium* (Novak et al. 2021). Beauvericins are mycotoxins formed by D-hydroxyisovaleric acid and N-methyl-L-phenylalanine, produced by the soil-inhabiting entomopathogenic fungus *Beauveria bassiana*, *Fusarium proliferatum*, *Fusarium oxysporum*, *Aspergillus terreus*, *Cordyceps cicadae*, *Peacilomyces tenuipes*, and *Paecilomyces fumosoroseus* (Ulusoy et al. 2022; X. Wang et al. 2018). Increasing scientific research has shown the very promising potential of beauvericin in anti-viral therapy against SARS-CoV-2 and antimicrobial effects in the nematode *Caenorhabditis elegans* (Al Khoury et al. 2022; Büchter et al. 2020). Enniatins are structurally similar to beauvericin, having the phenylalanine moieties

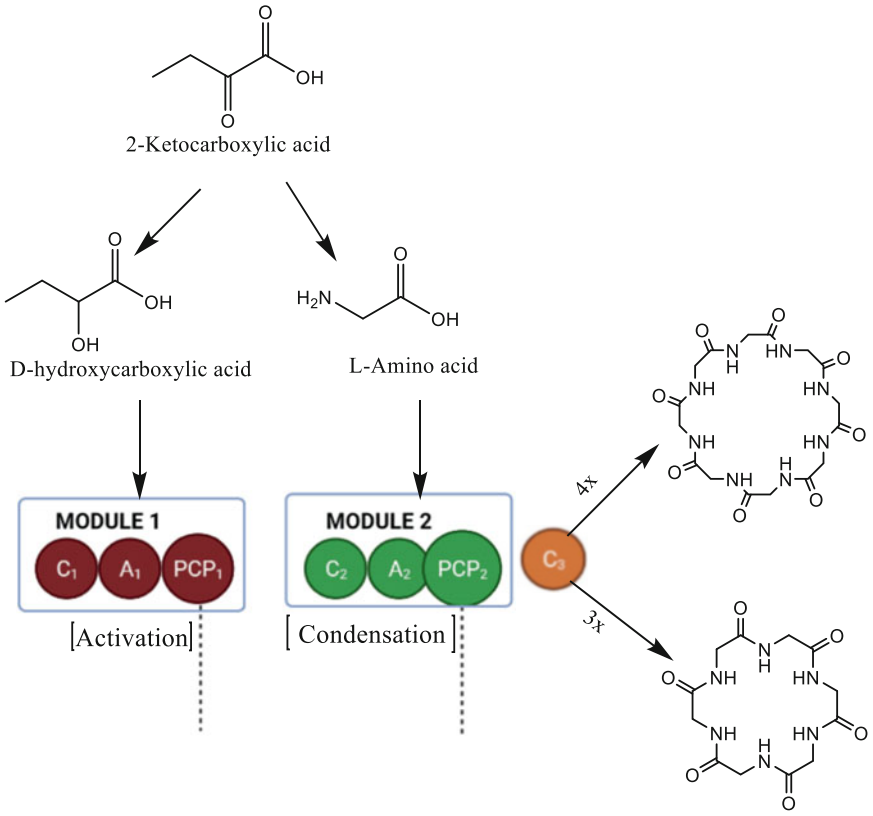


Fig. 13.8 The bimodular architecture in fungal cyclodepsipeptides biosynthesis

replaced by isoleucine, valine or leucine, forming enniatin A (Fig. 13.9), enniatin B or enniatin C, respectively. These compounds are mainly produced by fungal strains belonging to *Alternaria*, *Fusarium*, *Halosarpheia*, and *Verticillium* genus and currently, over twenty enniatin analogues have been isolated from fungal cultures (Süssmuth et al. 2011). These highly ionophoric and lipophilic compounds have shown promising potential in several in vitro models of cytotoxicity, oxidative stress, inflammation, and genotoxicity (Novak et al. 2021; Pallarés et al. 2020).

Other fungal cyclic hexadepsipeptides include allobeauvericin, aspergillicin, bursaphelocide, cardinalisamide, cordycecin, desmethyldestruxin, desmethylisaridin, destruxin, emericellamide, guangomide, hirsutatin, homodestcardin, isaridin, isoisariin, isariin, oryzamide, pullularin, sporidesmolide, and trichomide, among others (X. Wang et al. 2018). Bassianolide is the most well-characterized fungal cyclic octadepsipeptides isolated from *Beauveria bassiana*, *Verticillium lecanii*, and wood-decaying *Xylaria* spp. Structurally (Fig. 13.9), it is a tetramer containing D-hydroxyisovaleric and N-methyl-leucine monomer units similar to enniatin C (Süssmuth et al. 2011).

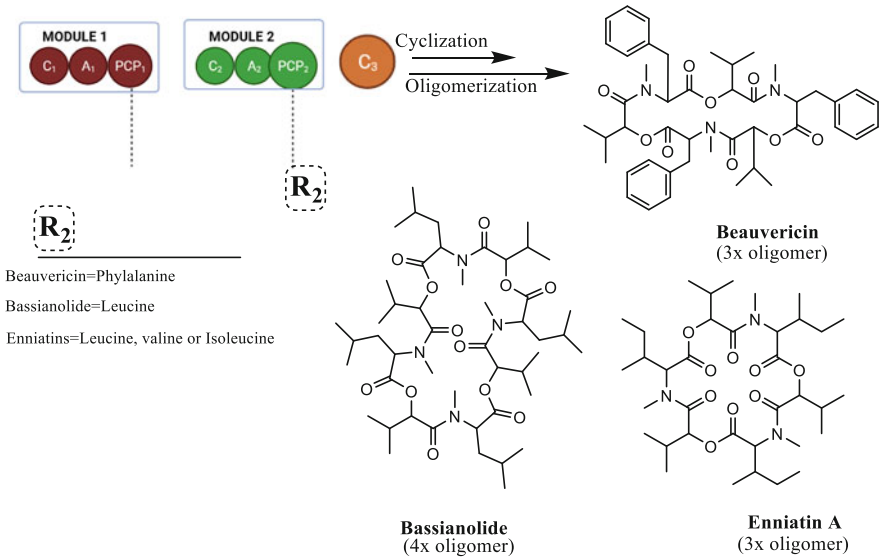


Fig. 13.9 Biosynthetic pathway of beauvericin, bassianolide, and enniatin

Siderophores such as ferrichrome and ferricrocin are also well characterized NRPs (Fig. 13.10). They are cyclic hexapeptides that act as iron chelators and mainly produced in various fungal strains, including *Aspergillus fumigatus*, *Ustilago maydis*, *Aspergillus nidulans*, *Omphalotus olearius*, *Schizosaccharomyces pombe*, *Magnaporthe grisea*, *Cochliobolus heterostrophus*, *Fusarium graminearum*, and *Alternaria brassicicola* (Eisfeld 2009). The siderophore synthetases of ferrichrome combine three N-acylated N-hydroxyornithine residues that form the core heme-binding unit and a ring of glycine, alanine, or serine, forming Ferrichrome A, Ferrichrome C, and ferricrocin, respectively (Bushley et al. 2008).

Overall, non-ribosomal peptides represent structurally diverse metabolites in several fungal strains with significant impact and application in pharmaceutical, food, cosmetic, and agricultural industries. Advances in genomic sequencing have ensured the correct identification of most NRPS biosynthetic genes in several fungal strains. This has encouraged re-engineering these NRP metabolites using several combinatorial biosynthetic methods for industrial-scale production of diverse molecular scaffolds of NRPs with improved biological and pharmacological properties (G. Bills et al. 2014).

13.2.3 Terpenoids/Terpenes

Ascomycota and Basidiomycota are known to produce an array of well-known terpenoid natural products, including mycotoxins, antibiotics, antitumor

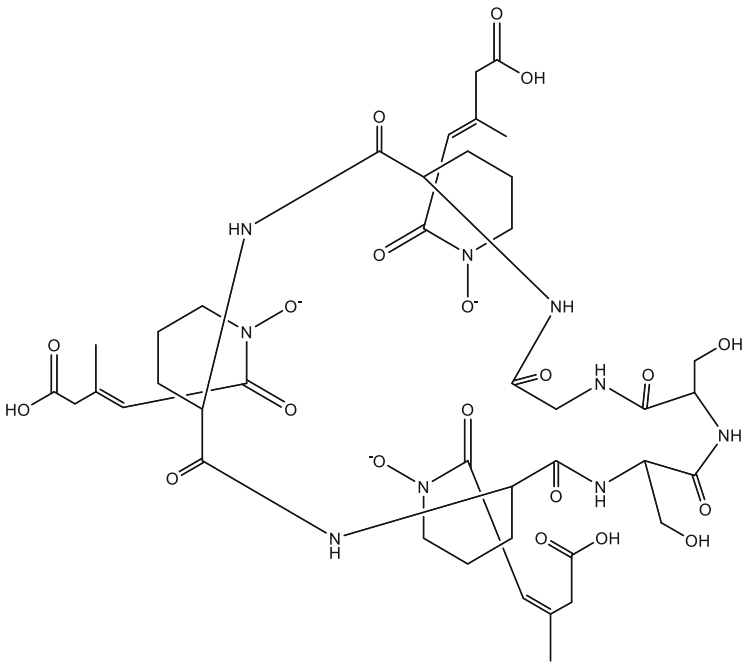


Fig. 13.10 Structure of ferrichrome-type siderophore

compounds, and hormones (G. F. Bills and Gloer 2017). However, the studies that have been developed (secondary metabolic pathways at molecular and biochemical levels) focus mainly on Ascomycota, as the Basidiomycota fungi, in general, are difficult to grow under *in vitro* conditions (G. F. Bills and Gloer 2016).

Fungal terpenoids are derived from five-carbon intermediates of isoprenyl diphosphate intermediates, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), synthesized from one of two pathways, the eukaryotic MVA (via mevalonate) pathway or the prokaryotic MEP (methylerythritol phosphate) pathway (G. F. Bills and Gloer 2017; Z.-J. Li et al. 2021). Condensation of IPP and DMAPP monomers results in linear hydrocarbons of varying length: C10 geranyl pyrophosphate (GPP), C15 (2E,6E)-farnesyl pyrophosphate ((2E,6E)-FPP, or FPP), and C20 geranyl geranyl pyrophosphate (GGPP) (Schmidt-Dannert 2015). These linear hydrocarbons undergo a dephosphorylation and cyclization cascade to produce terpenes. Terpene synthases are the enzymes responsible for these highly complex reactions and two distinct classes of terpene synthase exist, defined according to substrate activation mechanism (G. F. Bills and Gloer 2017; Liao et al. 2016; Schmidt-Dannert 2015). Class I terpene synthases catalyze an ionization-dependent cyclization of the substrate, while class II terpene synthases catalyze a protonation-dependent cascade. Depending on the length of the precursor molecule, fungal terpene synthases are known to produce sesquiterpenes (C15), diterpenes (C20) and triterpenes (C30) (Liao et al. 2016).

Terpenoids are classified into two groups based on whether their scaffolds are derived solely from isoprenyl units or mixed biosynthetic origin. The first group includes the carotenoids, rare sesterterpenoids, and it is divided into mono-, sesqui-, di-, or triterpenoids, which contain two to six C-5 isoprene units. The second group includes the meroterpenoids, the indole diterpenoids, and the structurally and biosynthetically diverse group of prenylated aromatic natural products (G. F. Bills and Gloer 2017).

13.2.3.1 Carotenoids

Carotenoids are terpenoid pigments of yellow, orange, and red colour. Since they are not essential molecules for fungi, they accumulate smaller amounts in these organisms than plants or algae. However, given their antioxidant properties, they can protect fungi from UV radiation, as observed in the parasitic fungi *Microbotryum violaceum* and the mould *Neurospora crassa* (Cacciola and Sandmann 2022).

According to their chemical characteristics, microbial carotenoids are classified as carotenes and xanthophylls. Carotenes, such as α-carotene, β-carotene, γ-carotene, δ-carotene, and torulene, are the most well-known, containing carbon and hydrogen atoms in their chemical structure. Torularhodin, astaxanthin, and canthaxanthin are xanthophylls that, in addition to carbon and hydrogen, also contain oxygen in their chemical structure (Mussagy et al. 2019). The biosynthesis of microbial carotenoids (Fig. 13.11) is derived from acetyl CoA, obtained from fatty acids via the β-oxidation pathway in the microorganism mitochondria (Mussagy et al. 2019). Phytoene is the first carotenoid formed from two geranylgeranyl pyrophosphate (GGPP) molecules a reaction catalyzed by phytoene synthase. Depending on the biocatalytic reactions (cyclization, substitution, elimination, addition, and rearrangement), the phytoene molecule can originate different molecular structures of carotenoids. The desaturation of phytoene by the phytoene desaturase results in the lycopene molecule. β-carotene is formed through lycopene cyclization, where the lycopene β-cyclase introduces two β-ionone end-groups into the chemical structure (Cacciola and Sandmann 2022; Mussagy et al. 2019). Xanthophylls results from hydroxylation reactions in the carotene ring. β-carotene is converted into zeaxanthin through two enzymatic reactions by β-carotene hydroxylase (Mussagy et al. 2019).

The beneficial properties of carotenoids allow their use in various industries, from the food industry to the most recent applications in the pharmaceutical and nutraceutical industries. Carotenoids can be used as colouring foods, food additives and supplements with beneficial properties for human health such as antioxidant, antitumor, among others (Amengual. 2019; Mussagy et al. 2019). In recent years, Biotechnology has made progress regarding the use of fungi to produce carotenoids. β-carotene is produced on a large scale by the mould *Blakeslea trispora*, and significant advances (at the laboratory scale) have been made in astaxanthin production, using the yeast *Xanthophyllomyces dendrorhous* (Gassel et al. 2014). This development in the use of fungi, particularly natural or transgenic yeasts, in the

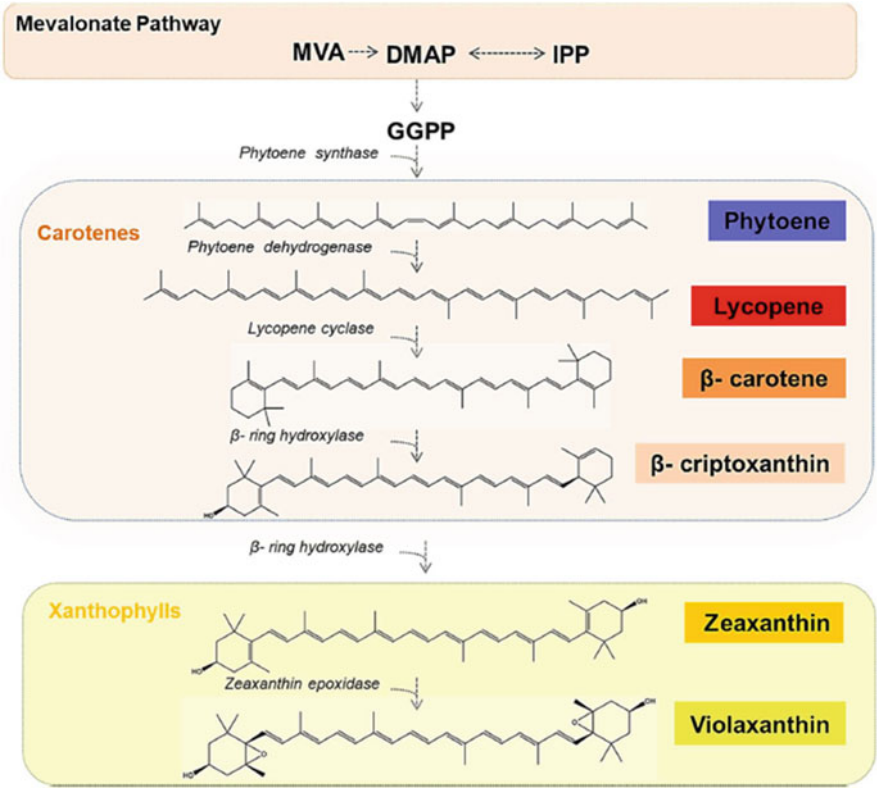


Fig. 13.11 Biosynthesis of carotenoids, derived from acetyl CoA (Source: Mussagy et al. 2019)

production of carotenoids, has been essential in applying concepts that are highly valued today, namely sustainability and circular economy, producing these natural compounds through the cultivation of agro-industrial residues (Cacciola and Sandmann 2022).

13.2.3.2 Sesterterpenoids

Sesterterpenoids that have been isolated from fungi are pentaprenyl terpenoids whose often complex polycyclic structures are derived from the linear precursor geranylfarnesyl diphosphate (GFPP). These compounds are relatively rare among terpenoid natural products (K. Li and Gustafson 2021; Okada et al. 2016).

These molecules are generated from terpenes, and based on the number of C5 isoprene units, they are classified as hemi- (C5), mono- (C10), sesqui- (C15), di- (C20), sester- (C25), tri (C30), and tetraterpenes (C40). Among these, sesterterpenes and their derivatives, known as sesterterpenoids, are ubiquitous secondary metabolites in fungi (Evidente et al. 2015). Their structural diversity encompasses

carbotricyclic ophiobolanes, polycyclic anthracenones, polycyclic furan-2-ones, or polycyclic hydroquinones (Evidente et al. 2015).

Forty-seven sesterterpenoids have been found in *Aspergillus* fungi, including ophyobolins, asperanes, and other type sesterterpenoids (Zhao et al. 2022). This section will focus on the genus *Aspergillus*, where the production of these compounds has been studied, revealing promising biological activities in several areas (Zhao et al. 2022).

13.2.3.2.1 Tricarbocyclic Sesterterpenoids (5/8/5-Membered Ring System)

Ophiobolin A (Fig. 13.12) is a fungal secondary metabolite with cytotoxic properties. It is produced through diverse cyclizations of linear C_{25} precursors, that share the same 5-8-5 carbotricyclic skeleton with fusicoccins and cotylenins, two groups of diterpenoids produced by *Fusicoccum amygdali* and by *Cladiosporum* sp. 501-7 W (Masi et al. 2019; Okada et al. 2016).

Several additional analogues of ophiobolin A were isolated from different fungi strains, and the types of ophiobolins produced vary with the culture conditions (Kinghorn 2020; Okada et al. 2016). Until now, more than 50 naturally occurring ophiobolins have been reported, with the majority coming from *Bipolaris* and *Aspergillus* species (Cai et al. 2019). Several ophiobolin-type sesterterpenoids were isolated from *Aspergillus ustus* and *Aspergillus* spp. These include (5α,6α)-ophiobolin H, (5α,6α)-5-*O*-methylophiobolin H, 5-*O*-methylophiobolin H, (6α)-21,21-*O*-dihydroophiobolin G and (6α)-18,19,21,21-*O*-tetrahydro-18,19-dihydroxyophiobolin, (6α)-21-deoxyophiobolin G, (6α)-16,17-dihydro-21-deoxyophiobolin G, ophiobolins U–W, ophiobolin O, 6-epi-ophiobolin O, ophiobolins X-Z, 21-dehydroophiobolins U and K, 21-epi-ophiobolins Z and O (184) (Zhao et al. 2022).

Ophiobolin B is produced from *Bipolaris oryzae*, ophiobolin C from *B. zizanie*, ophiobolin D from *Cephalosporium caerulens* and ophiobolin F from *B. maydis* (Okada et al. 2016). The formation of the 5/8/5-membered ring system (Fig. 13.13) starts with the cyclization mechanism of geranylfarnesyl diphosphate, where an 11/5-membered ring system is first generated. Subsequently, a 1,5-hydride shift and the formation of another 5-membered ring occur (Kinghorn 2020).

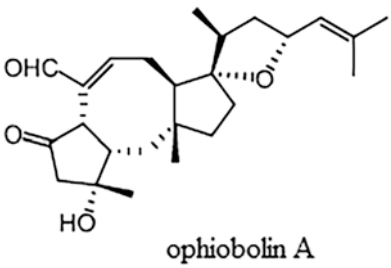


Fig. 13.12 Structure of ophiobolin A (Source: Kinghorn 2020)

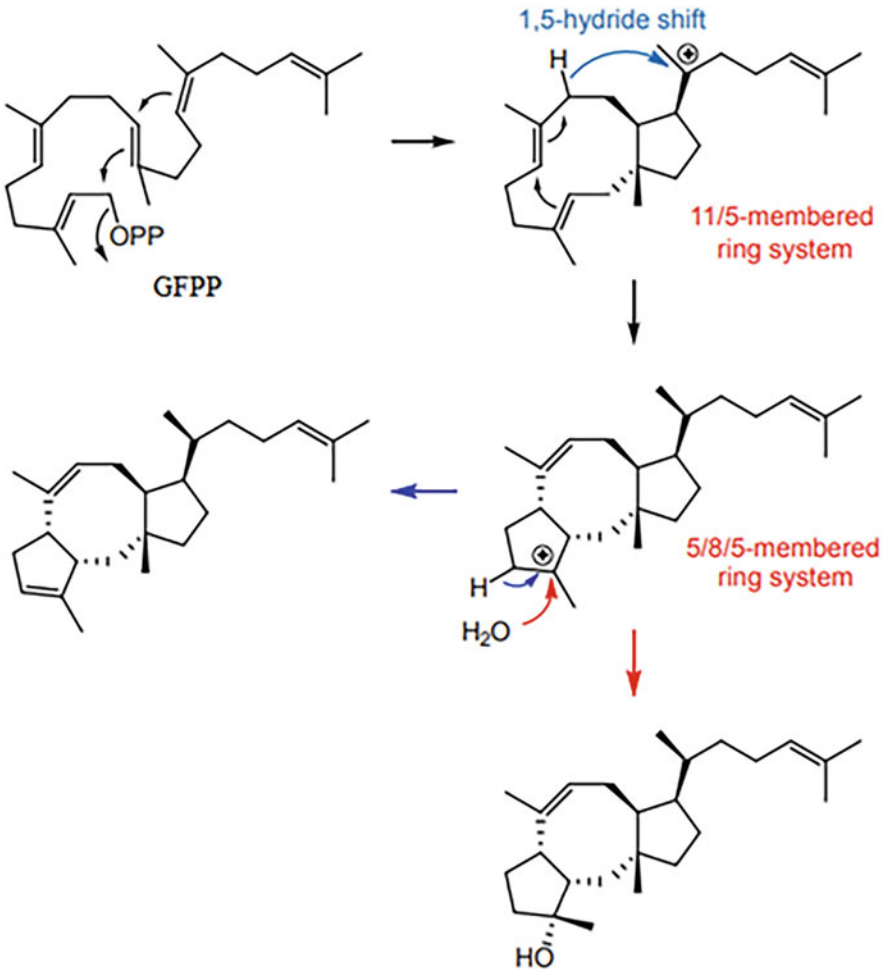


Fig. 13.13 Cyclization mechanism for the formation of the 5/8/5-membered ring system (Source: Kinghorn 2020)

13.2.3.2.2 Tetracarbocyclic Sesterterpenoid (7/6/6/5-Membered Ring System and 5/8/6/6-Membered Ring System)

Aspergilloxide is a tetracarbocyclic sesterterpenoid, isolated from the *Aspergillus* sp. with a 7/6/6/5-membered ring system, and the proposal cyclization mechanism starting from geranylfarnesyl diphosphate is shown in Fig. 13.14 (Kinghorn 2020).

Asperterpenol A is an acetylcholinesterase inhibitor, reported from endophytic fungus *Aspergillus* sp. 085242, and the tetracarbocyclic skeletons are formed as shown in Fig. 13.15 (Kinghorn 2020).

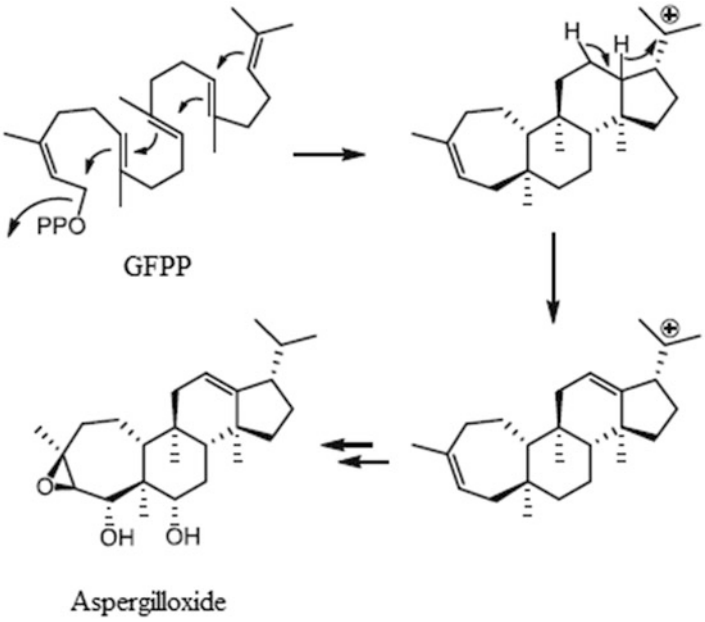


Fig. 13.14 Cyclization mechanism for the formation of the 7/6/6/5-membered ring system of aspergilloxide (Source: Kinghorn 2020)

13.2.3.2.3 Pentacarbocyclic Sesterterpenoids (5/7/3/6/5-Membered Ring System; 5/3/7/6/5 and 5/4/7/6/5-Membered Ring)

Asperterpenoid A is a potent inhibitor of the *Mycobacterium tuberculosis* protein-tyrosine phosphatase B (PtpB), and it is isolated from the endophytic *Aspergillus* sp. 16-5c. The possible cyclization reaction for the formation of the 5/7/3/6/5-membered ring system is shown in Fig. 13.16 (Kinghorn 2020).

Aspterpenacid A, isolated from the fungi *A. terreus* H010, has a 5/3/7/6/5-membered ring system, while astellatol, isolated from *A. variecolor*, possesses a 5/4/7/6/5-membered ring system. The proposed pathway for synthesizing these compounds starting from geranylfarnesyl diphosphate is represented in Fig. 13.17 (Kinghorn 2020).

13.2.3.2.4 Hexacarbocyclic Sesterterpenoids (5/5/5/5/3/5-Membered Ring Systems)

Niduterpenoid A and niduterpenoid B were first isolated from *Aspergillus nidulans* and possess hexacarbocyclic sesterpenoids. The cyclization reaction is quite complicated and starts from geranylfarnesil diphosphate. The possible cyclization

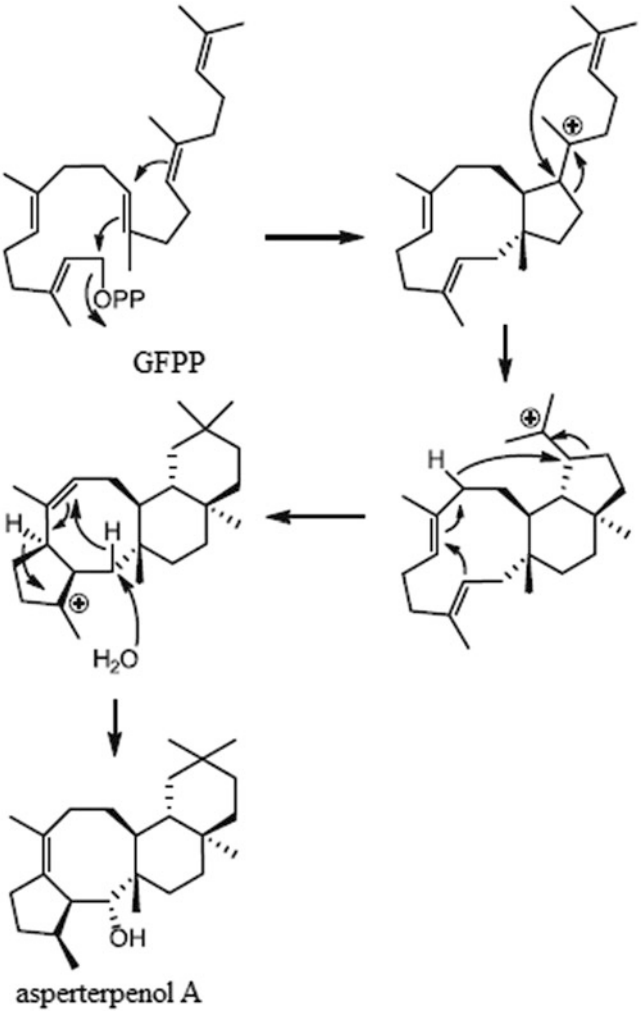


Fig. 13.15 Cyclization mechanism for the formation of the 5/8/6/6-membered ring system of asperterpenol A (Source: Kinghorn 2020)

reactions for forming the hexacarbocyclic skeleton of niduterpenoid A and niduterpenoid B is shown in Fig. 13.18 (Kinghorn 2020).

13.2.3.3 Meroterpenoids and Isoprenoids

Meroterpenoids and isoprenoids, like other natural compounds, may be confused with sestertepenoids; but not all compounds with 25 carbon atoms are sesterterpenoids.

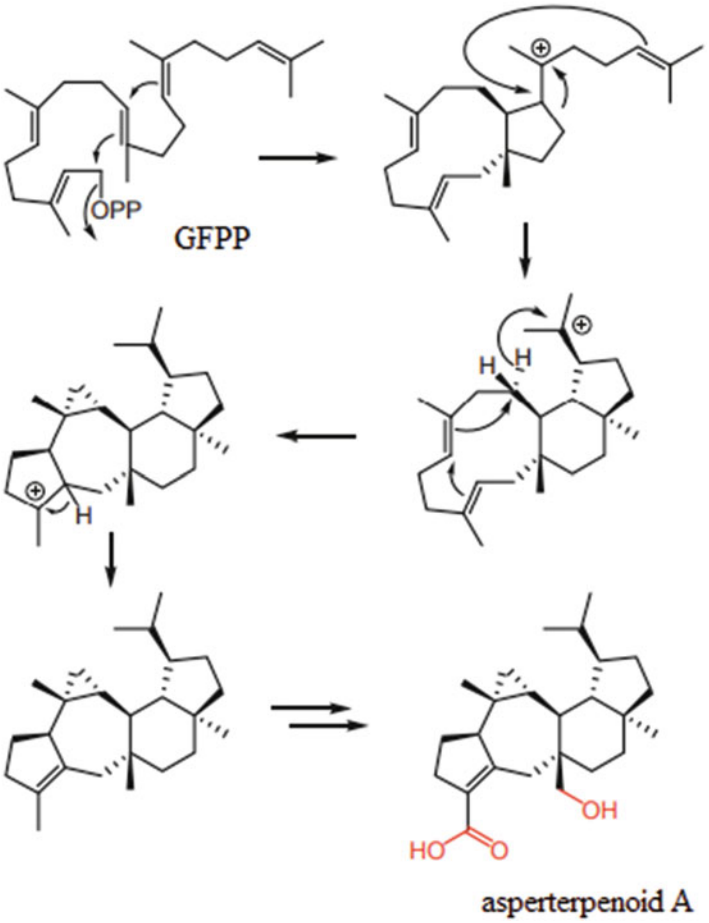


Fig. 13.16 Possible cyclization reaction for the formation of the 5/7/3/6/5-membered ring system of asperterpenoid A (Source: Kinghorn 2020)

Meroterpenoids are natural products with a C10 polyketide moiety (*e.g.*, preterretonin A, protoaustinoid A, and andrastin E), but they are not biosynthesized via geranylfarnesyl diphosphate (GFPP). Instead, they are generated from a C15 terpenoid moiety and a C10 polyketide moiety. These C15 and C10 moieties are combined in their biosynthesis to form the C25 basic carbon skeleton (Kinghorn 2020) as showed in Fig. 13.19.

Highly branched isoprenoids are a member of the terpenoids family, with a 25 carbon atoms skeleton. Isoprenoids are not considered sesterterpenoids, because they do not derive from the C25 polyprenyl diphosphate, but from (C10) geranyl diphosphate (GPP) and (C15) farnesyl diphosphate (FPP) (Kinghorn 2020) as showed in Fig. 13.20.

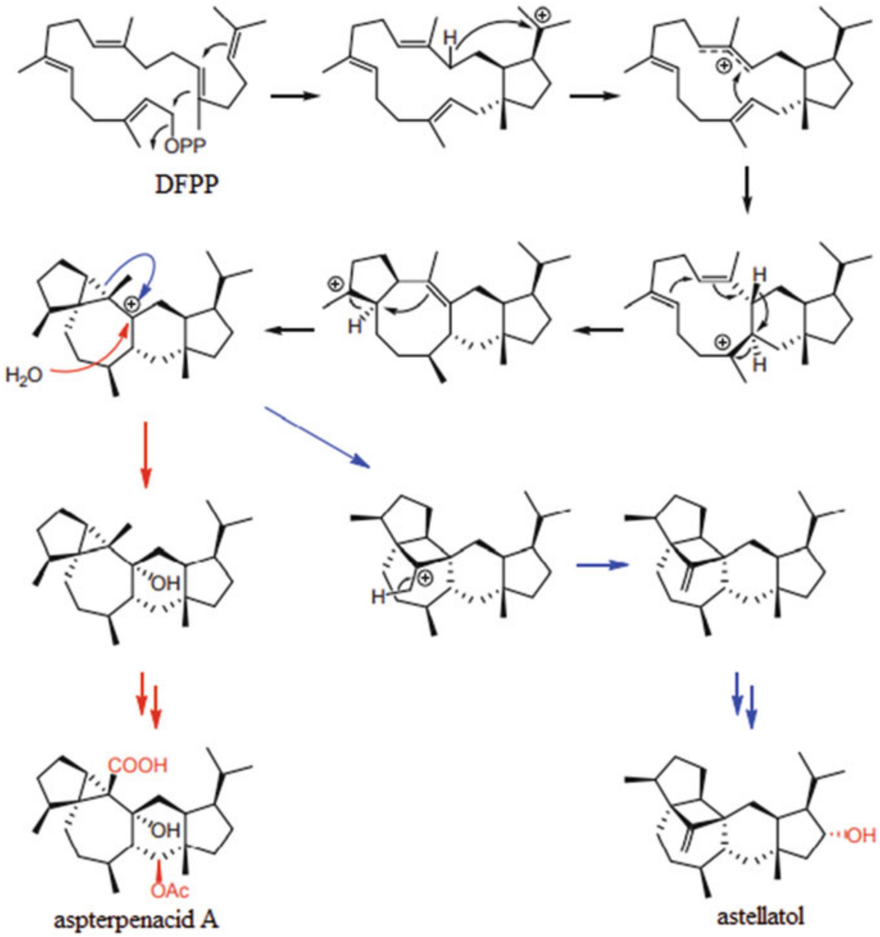


Fig. 13.17 Possible cyclization reactions for the formation of the basic carbon skeletons of aspterpenacid A and astellatol (Source: Kinghorn 2020)

13.2.4 Indole Alkaloids

Indole alkaloids are one of the largest classes of nitrogen-containing secondary metabolites that are widely found in plants, bacteria, fungi, and animals (Fig. 13.21). About 12,000 alkaloids have been discovered, many of which are pharmacologically active and traditionally used as antitussives, purgatives, sedatives, and anticancer drugs (Oudin et al. 2007). Previous phytochemical investigations have led to the characterization of indole alkaloids with cytotoxic, anti-diabetic, and anti-inflammatory activities (Khyade et al. 2014). Therefore, this important class of secondary metabolites has aroused great interest in natural products research due to its structural complexity and significant pharmacological

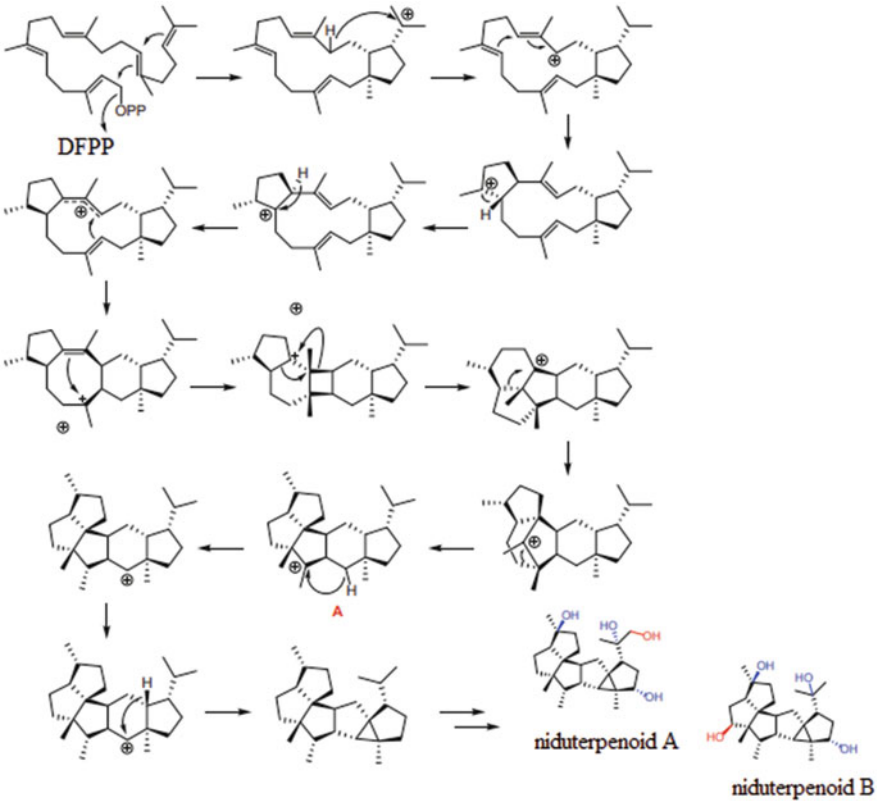


Fig. 13.18 Possible cyclization reactions for the formation of the hexacarbocyclic skeleton of niduterpenoid A and niduterpenoid B. The intermediate A possesses a 5/5/5/6/5-membered ring system (Source: Kinghorn 2020)

activities (Z. W. Wang et al. 2021; Yu et al. 2021). Fungi, especially *Ascomycota*, have been reported as prolific producers of indole alkaloids (Hanson 2008). The availability of fungal genome sequences has, in recent years, significantly accelerated the identification of the biosynthetic genes involved in the biosynthesis of secondary metabolites from fungi (Wiemann and Keller 2014; Yaegashi et al. 2014).

Many fungal metabolites, collectively designated as indole alkaloids, contain in their structures a prenylated indole nucleus (Fig. 13.21) that derives from l-tryptophan and mevalonate. These metabolites include two large groups: (a) the ergot alkaloids produced by the plant parasitic *Claviceps* species (Tudzynski et al. 2001), and (b) the indole alkaloids produced by species of *Aspergillus*, *Penicillium*, and *Neosartorya*, among others (S. M. Li 2009). These alkaloids differ: (i) in the carbon atom of the indole molecule bearing the isopentenyl group, (ii) in modifications of the diketopiperazine ring, and (iii) in modifications of the N1 atom of indole, that are introduced by "late" modification enzymes encoded by additional genes in the clusters. One of the best-known indole alkaloid groups is that of the ergot

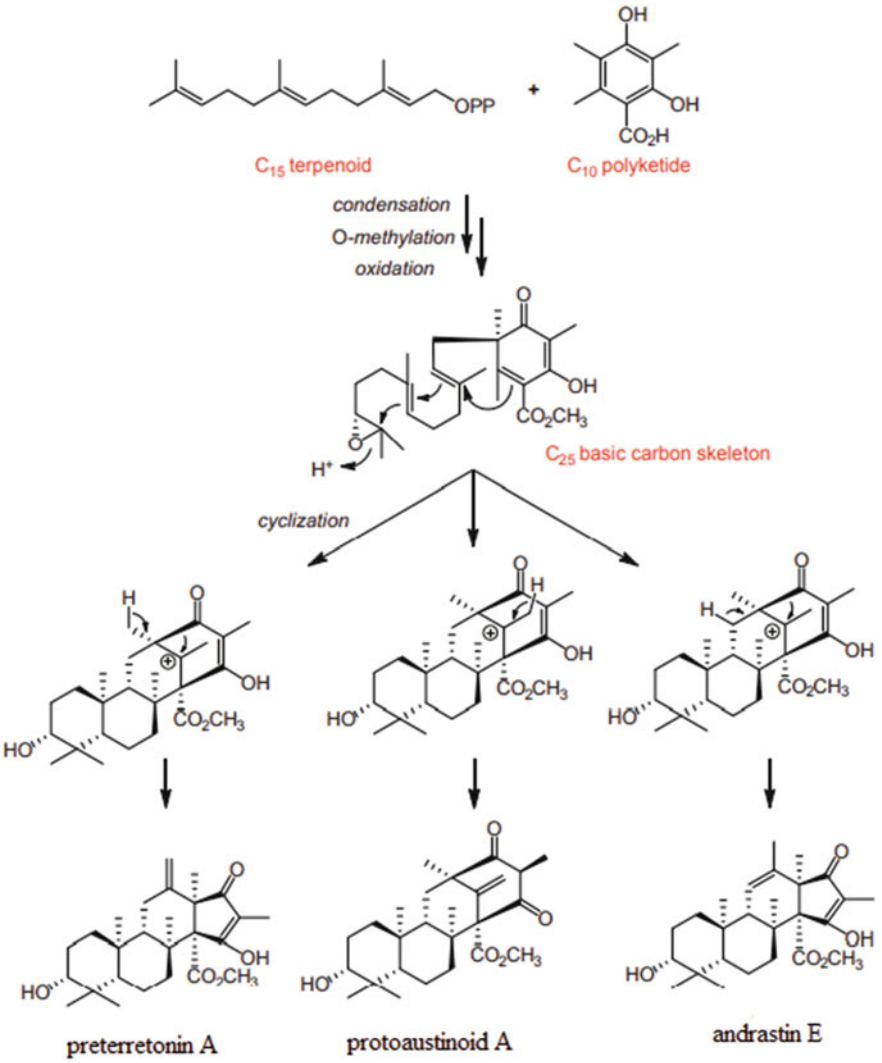


Fig. 13.19 Biosynthesis of preterretonin A, protoaustinoid A, and andrastin E (Source: Kinghorn 2020)

alkaloids (Tudzynski et al. 2001) and another important group is that of roquefortine C (mycotoxin) and related indole alkaloids (glandicoline, meleagrin, neoxaline) (García-Estrada et al. 2011; Sumarah et al. 2005). Several of these compounds are produced by *Penicillium* species of the *Corymbifera* family (Martín et al. 2014).

Indole alkaloids are usually derived from tryptophan and dimethylallyl pyrophosphate, although sometimes amino acids other than tryptophan are used as precursors (Keller et al. 2005). Different strategies to incorporate indole moieties into the final alkaloid structures are found in fungal secondary metabolism. Not surprisingly, most

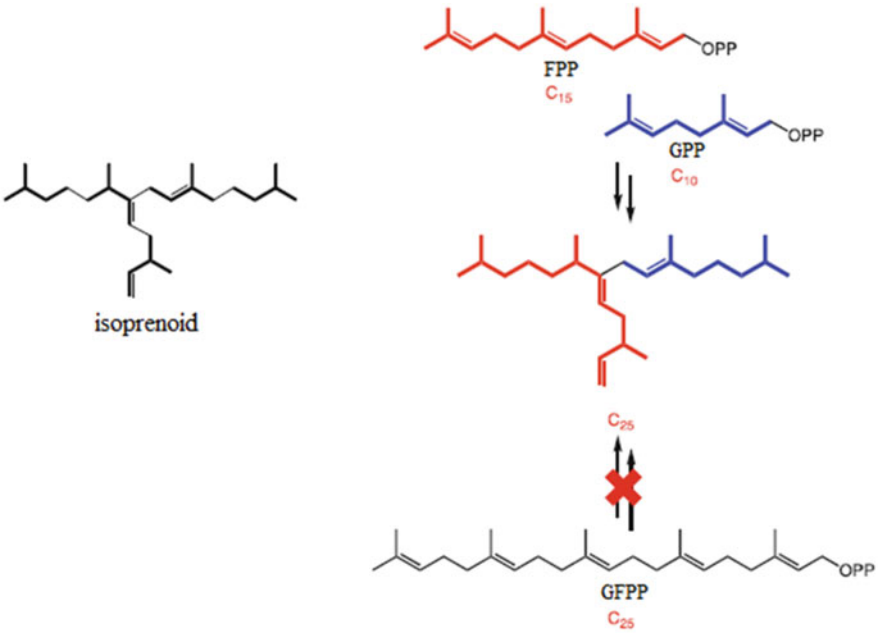
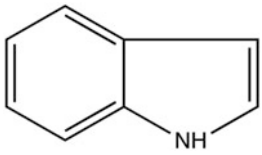


Fig. 13.20 Biosynthesis pathway of Highly branched isoprenoid (Source: Kinghorn 2020)

Fig. 13.21 Basic structure of the indole nucleus



of the indole precursors are related to L-tryptophan (1), the most abundant indole-containing species in the cell. The biosynthesis of (1) itself starts from chorismate in the shikimic acid pathway and involves the intermediates anthranilate and indole-3-glycerol-phosphate. Phosphate intermediate is transformed into indole, which can be coupled with serine to form (1) (Dunn et al. 2008). Tryptophan (1) can be decarboxylated and converted into tryptamine, (Lovenberg et al. 1962) or be prenylated at C4 to yield 4-dimethylallyl tryptophan (4-DMAT) (2), as summarized below in Fig. *13.22* (Lee et al. 1976; Unsöld and Li 2005). Feeding experiments with isotope-labeled precursors have shown that L-tryptophan and indole-3-glycerol-phosphate, tryptamine and 4-DMAT, can each serve as the biosynthetic precursor for the indole/indoline moieties in fungal indole alkaloids (Flieger et al. 1997; Xu et al. 2014).

The best-understood pathway is ergotamine synthesis in *Claviceps purpurea* and related species (Králová et al. 2021; Tudzynski et al. 1999). The biosynthetic pathway is shown in Fig. *13.22*, which starts with the C4-prenylation of L-tryptophan (1) with dimethylallyl diphosphate (DMAPP) as prenyl donor. This reaction is

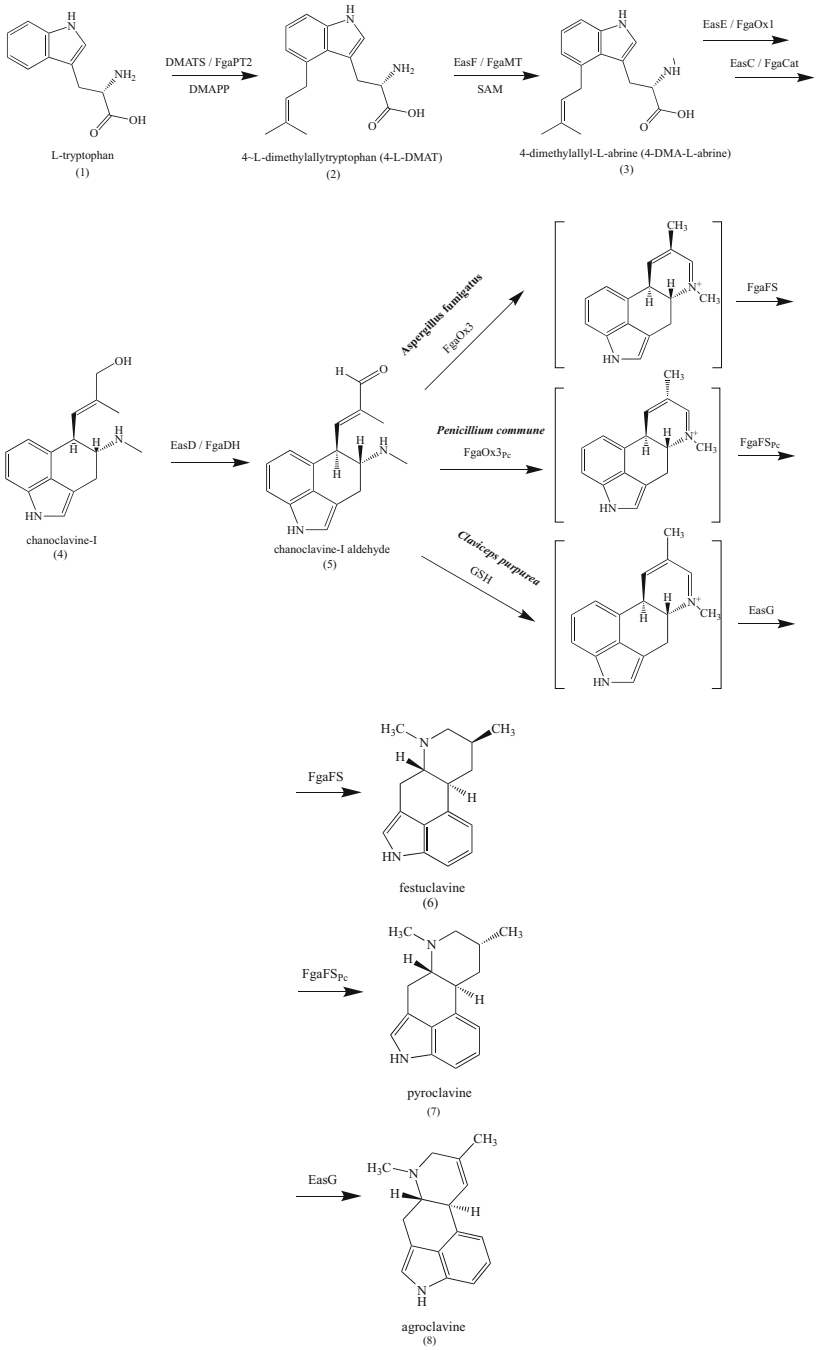


Fig. 13.22 Formation of the ergoline scaffold-biosynthetic pathway (Gerhards et al. 1950)

catalyzed by the prenyltransferase 4-dimethylallyltryptophan synthase (DMATS), also named FgaPT2 in *A. fumigatus* (Coyle and Panaccione 2005; Lee et al. 1976; Unsöld and Li 2005). Biochemical and structural elucidations clearly show the formation of 4-γ,γ-dimethylallyltryptophan (DMAT (2)) as a product (Metzger et al. 2009; Steffan et al. 2007; Steffan and Li 2009). Metzger et al. reported the X-ray structure of FgaPT2 in complex with L-tryptophan, proposing a three-step mechanism: the formation of a dimethylallyl cation, a nucleophilic attack of the indole nucleus to that cation and a deprotonation step, which led to a better understanding of the reaction mechanism (Luk and Tanner 2009; Metzger et al. 2009). Evolutionary investigations have indicated that the gene *fgaPT2* from *A. fumigatus* has the same origin as prenyltransferase genes from another *Ascomycota*, including the ergot-alkaloid-producing *Clavicipitaceae* (Gerhards et al. 1950; Liu et al. 2009).

After the reaction, the pathway reaches a branch point. Several products arise from (5), depending on the fungus. For example, the next intermediate in *A. fumigatus* is festuclavine (6), in *P. commune* pyroclavine (7) and in *C. purpurea* agroclavine (8) (Matuschek et al. 2012). The branch point is mainly controlled by the old yellow enzyme EasA (also termed FgaOx3), and the functional differences in this enzyme result in divergent ergot alkaloid pathways (Coyle et al. 2010). For the formation of festuclavine in *A. fumigatus*, a second enzyme (the festuclavine synthase FgaFS) is required, as shown by Wallwey et al. (Wallwey et al. 2010; Xie et al. 2011). Cheng *et al.*, reported the formation of agroclavine catalyzed by an enzyme from *E. festucae* var. *lolii* (Cheng et al. 2010). However, in *C. purpurea in vitro* investigations on the respective reaction showed that EasG (a homologue of FgaFS from *A. fumigatus*) can catalyze the formation of (8) via a non-enzymatic adduct with reduced glutathione (Gerhards et al. 1950; Matuschek et al. 2011). As shown by Matuschek et al., the formation of pyroclavine in *P. commune* requires both homologues: $FgaOx3_{PC}$ and $FgaFS_{PC}$ (Matuschek et al. 2012).

Other tryptophan-derived alkaloids such as the fumigaclavines and fumitremorgens of *A. fumigatus* undergo one or more prenylation steps. The details of these pathways are yet to be elucidated, but it is likely that the fumigaclavine biosynthetic pathway proceeds through agroclavine and might therefore have some early steps in common with the ergotamine pathway (Keller et al. 2005; von Nussbaum 2003).

The biosynthetic pathway for indole alkaloids has been investigated extensively in *Claviceps* species and *A. fumigatus* and the elucidation of the pathway is of interest especially because of broad range of pharmaceutical uses, being able to increase knowledge concerning the genes and enzymes. Therefore, molecular genetic manipulations may be used to improve industrial production of medically important indole alkaloids, and novel forms that could act as drugs with new or improved pharmacological activities and minimal side effects might be created by synthetic microbiology or other related techniques.

13.3 Conclusions

In this chapter, the most abundant secondary metabolites from fungi, namely their biosynthesis, were discussed.

Fungal secondary metabolites exhibit impressive chemical structures and biological activities, but their biosynthetic pathways share some key points with primary metabolites or even with each other. Four main classes of fungal secondary metabolites can be considered, originating through acetyl-CoA and via the shikimate pathway, *i.e.* polyketides, non-ribosomal peptides, terpenoids, and indole alkaloids.

Although some of these compounds are associated with adverse effects, such as mycotoxins, the truth is that others have brought benefits that have revolutionized the world of pharmacy/medicine and agriculture, namely antibiotics or pesticides. This dichotomy regarding fungal secondary metabolites is thus indicative of the enormous diversity of natural products that fungi can produce.

Conflicts of Interest

The authors declare no conflict of interest.

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Chapter 14
Phenolic Acids from Fungi

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Abstract For centuries, mushrooms have been worldwide appreciated not only for their culinary attributes, but also for their nutritional and therapeutic properties. Different species of mushrooms have been identified as sources of bioactive compounds, such as polysaccharides, proteoglycans, terpenes, phenolic compounds, and others. Among these, phenolic acids are the most commonly occurring phenolics in mushrooms, to which several bioactive properties are attributed, namely antitumor, antioxidant, immunomodulatory, radical scavenging, cholesterol reducing, antimicrobial, anti-inflammatory, hepatoprotective, detoxifying activities, anti-diabetic, analgesic, and several others. Some of these phenolic acids include *p*-coumaric, cinnamic, *p*-hydroxybenzoic, benzoic, ferulic, and gallic acids. In this context, there has been a growing increase in the use of different mushroom species as potential nutraceuticals, liable to be used directly in the diet or in individual fractions (extract or isolated phenolic compounds) and incorporated into functional foods. Thus, future investigations should focus on complementary studies that allow improving the action of these compounds, while maintaining their bioactive characteristics and bioavailability throughout the different processing stages. Furthermore, new encapsulation techniques, which may allow the incorporation of compounds of interest into products with different characteristics should be strongly promoted.

14.1 Introduction

Worldwide, fauna, flora, and mycobiota are the main sources of secondary metabolites, taken as preeminent natural resources in the upkeep of human health and the manufacture of many medicines (David et al. 2015). Natural products, by adapting to different biotic and abiotic stresses, arise as a result of years of uninterrupted

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evolution of living organisms. These evolving bioactive compounds have been used for a long time in traditional medicine, enduring today as pools of potential drugs (David et al. 2015).

Mushrooms are a group of macrofungi belonging to ascomycetes and basidiomycetes that obtain their nutrition through being saprotrophs, parasites, or symbiotic as mycorrhiza, and that holds a distinctive fruiting body (hypogeous or epigeous; reproductive phase) and mycelia (vegetative phase) (Sánchez 2017). In several countries, numerous varieties of fresh and preserved mushrooms are consumed as delicacies due to their unique flavor and texture (Carrasco et al. 2018). As well, cultures all over the world use mushrooms not only in their diet, given their nutritional profile rich in proteins, carbohydrates, elements (phosphorous, potassium, calcium, copper, magnesium, iron, zinc), vitamins, and lower fat amounts, but also for medicinal purposes and creative inspiration. Different mushroom species have been identified as a source of valuable bioactive compounds, which are produced during their cultivation, and associated with several beneficial biological properties (Aprotosoaie et al. 2017). As natural remedies, mushrooms and their extracts have been used in the treatment and prevention of various acute and chronic diseases, including asthma, cancer, heart disease, infections, insomnia, ulcers, among others (Muszyńska et al. 2018).

Nowadays, after several years of research in this field, medicinal mushrooms are considered as functional foods and occurs as over-the-counter health supplements used in complementary and alternative medicines, being globally known as important biological resources of secondary metabolites which are chemically quite diverse and with a wide range of biological activities pursued in folk medicine (Ayeka 2018; Hrudayanath and Sameer 2014). These metabolites, despite not having nutritional value, are widely differentiated by their biological properties, playing a major role in several biological systems (Bal et al. 2017). For that, chemically pure bioactive metabolites originating from fungi have assumed a key role in modern pharmacological research including, in addition to antibiotics, anticancer agents, immunosuppressants, enzyme inhibitors, hormone antagonists and agonists, and a wide variety of psychotropic substances, among others (Pennerman et al. 2015).

Specifically, several of the inherent bioactive properties of mushrooms were ascribed to the incidence of phenolic compounds, mainly phenolic acids, in their composition, namely antioxidant, antitumor, antimicrobial, and others (Kostić et al. 2020a; Sadowska et al. 2020a; Selli et al. 2021b; Y. Zhang et al. 2020a). Given their bioactive characteristics, these constituents have been added to various products that promote human health and well-being. In this context, the growing interest in the nutraceutical effects of mushroom extracts and isolated phenolic compounds has been the motto for the development of functional food products with bioactive potential (Elkhateeb et al. 2019; Stoffel et al. 2019).

14.2 Mushrooms As a Source of Bioactive Compounds

In the past few decades, mushrooms have had particular notice owing to their rich constitution in phenolic acids, labeled as the main influencers of its associated medicinal properties. In addition to the fruiting bodies, the mycelium and the culture medium used in mushroom cultivation were also investigated as prospective sources of bioactive compounds (Ma et al. 2016). Particularly, cultivated mycelia have been acknowledged as promising alternative sources of fungal bioactive compounds, mainly due to the shorter incubation time and easier cultivation settings, counting with the smaller space required for their cultivation, the low prospect of contamination, and greater biomass production when likened with fruiting bodies (Zhang et al. 2016).

Among different mushroom species, the concentration of the phenolic acids varies between its different parts and with growth conditions, concentrating mostly in fruiting bodies, as in *Ganoderma lucidum* (Curtis) P. Karst., or in mycelia, as in the case of *Pleurotus ostreatus* (Jacq. ex Fr.) P. Kumm., in which cinnamic acid is present in much greater quantity than in whole mushroom fruiting bodies (Reis et al. 2012). Also, the concentration and individuality of specific compounds may oscillate due to the dissimilar origins of the fungus and the substrate on which it grows, which has been proven by some investigations in which wild mushrooms from Serbia (Karaman et al. 2010) had much higher concentrations of phenolic acids than other purchased mushrooms in Portuguese supermarkets (Reis et al. 2012).

14.2.1 Phenolic Acids

Phenolic acids are the main phenolic compounds present in mushrooms (Ferreira et al. 2009). These are a class of organic compounds consisting of an aromatic benzene ring directly linked to a hydroxyl group and a carboxylic acid substituent (Velderrain-Rodríguez et al. 2014). Phenolic acids can be divided into two main groups: hydroxybenzoic and hydroxycinnamic acids, which are derived from the non-phenolic molecules of benzoic and cinnamic acids, respectively (Choi et al. 2012; Ferreira et al. 2009; Manach et al. 2004). The former normally occur in the bound form and constitute complex structures, such as hydrolyzable lignins and tannins, and can also be found bound to sugars and organic acids. On the other hand, hydroxycinnamic acid derivatives are mainly linked to structural components of the cell wall, such as cellulose, lignin, and proteins, as well as organic acids, such as tartaric or quinic acid (chlorogenic acids), through ester bonds (Ferreira et al. 2009; Manach et al. 2004).

In mushrooms, hydroxycinnamic acids are more commonly found than the hydroxybenzoic ones, the former being mainly constituted by *p*-coumaric, caffeic, ferulic, synapic, and caffeoylquinic acids. These are rarely found in their free form, except in processed foods subjected to freezing, sterilization, and fermentation

processes. The linked forms are glycosylated derivatives or esters of quinic, shikimic, and tartaric acid. Furthermore, the presence of ellagic and tannic acids may be observed. On the other side, the most prevalent phenolic acids derived from benzoic acid found in mushrooms are *p*-hydroxybenzoic, protocatechuate, gallic, gentisic, homogentisic, vanillic, 5-sulfosalicylic, syringic, veratric, and vanillin acids (Bahadori et al. 2019; Ferreira et al. 2009; Mutukwa et al. 2019; Yahia et al. 2017). The highly predominant phenolic acids found in different mushroom species are listed in Table 14.1.

14.2.1.1 Bioactive Properties Related with Mushrooms Phenolic Acids

As mentioned above, over the years mushrooms have been used not only as a food but also as a medicinal resource. Several studies have established the numerous biological activities associated with the phenolic profile of mushrooms, which is mainly composed of phenolic acids (Nowacka et al. 2014; Taofiq et al. 2016). Some of the health benefits related with the presence of phenolic acids in mushrooms are presented in Table 14.2.

Oxidative stress is assumed to be the main cause of the production of reactive oxygen species (ROS) in living organisms. The overproduction of these ROS promotes oxidative damage in essential macromolecules, such as DNA, RNA, lipids, proteins, among others, culminating in tissue injury or death (Vaz et al. 2011). Additionally, ROS are involved in the pathogenesis of various chronic diseases such as diabetes, cardiovascular diseases, cancer, aging, rheumatoid arthritis, among others (Jeong et al. 2012). In this sense, the investigation of antioxidant compounds present in mushroom extracts has taken important proportions in the pharmaceutical and food industries, replacing synthetic homologous substances and providing bioactive components from natural sources (Zielinski et al. 2016). The profile and concentration of phenolic compounds present in mushrooms are identified as responsible for variations, not only in the antioxidant activity of the extracts from different species of mushrooms but also for other bioactivities (Abd Razak et al. 2019). Furthermore, the concentration of polyphenols and the antioxidant activity of mushrooms are shown to be strongly correlated with the ability to scavenge their hydroxyl groups (Abd Razak et al. 2019; Contato et al. 2020b). The high concentration in polyphenols, as well as a strong antioxidant activity, was observed in methanolic and acetic ester extracts of diverse mushroom species, including *Meripilus giganteus* (Pers.) Karst., *Agaricus silvaticus* Schaeff., and *Hydnum rufescens* Pers., reinforcing the idea that these extracts should be used as food or pharmaceutical product in the upkeep of human health (Garrab et al. 2019). Also, different extracts of other mushroom species are able to protect DNA by scavenging free radicals that promote H_2O_2-induced destruction, thus proving that the antioxidant characteristics of these polyphenols act as reducing agents or donors of hydrogen atoms (Ahmad et al. 2014; Aprotosoaie et al. 2017). Among the mushrooms extracts obtained with solvents such as ethanol, acetone, ethyl acetate, chloroform, *n*-hexane, and pure aqueous extract, acetone and ethanolic extracts seem

Table 14.1 Main phenolic acids in mushrooms

Phenolic acids	Chemical formula	Species	References
Gallic acid	$C_7H_6O_5$	*A. auricula-judae, A. bisporus, B. edulis, G. lucidum, M. esculenta, L. edodes, H. erinaceus, P. eryngii, R. delica*, and *R. senecis*	(Eliuz 2021; Fernandes et al. 2014; Khatua et al. 2015; Magdziak et al. 2019; Selli et al. 2021a; Singh et al. 2020; Yahia et al. 2017; Yildiz et al. 2015)
p-hydroxybenzoic acid	$C_7H_6O_3$	*A. arvensis, A. blazei, A. flavoconia, A. pantherina, B. s edulis, C. militaris, C. rutilus, G. lucidum, L. edodes, L. índigo, M. cognata, M. stridula, M. elata, M. esculenta, P. ostreatus, P. eryngii,* and *S. floccopus*	(Babak et al. 2019; Çayan et al. 2020; Dogan et al. 2018; Gąsecka et al. 2016, 2018; Pintathong et al. 2021; Selli et al. 2021a; Yahia et al. 2017; Yildiz et al. 2015; Y. Zhang et al. 2020b)
Protocatechuic acid	$C_7H_6O_4$	*A. bisporus, A. flavoconia, A. mellea, A. pantherina, A. arvensis, Boletus frostii, B. edulis, G. lucidum, H. erinaceus, H. lactiflorum, L. edodes, M. cognata, M. stridula, M. esculenta, P. ostreatus, P. eryngii, R. flava, R. emética, S. imbricatus,* and *S. floccopus*	(Babak et al. 2019; Dogan et al. 2018; Eliuz 2021; Erbiai et al. 2021; Gąsecka et al. 2016; Selli et al. 2021a; Yahia et al. 2017; Yildiz et al. 2015)
Vanillic acid	$C_8H_8O_4$	*A. bisporus, A. auricula-judae, A. mellea, G. lucidum, H. erinaceus, L. edodes, M. procera, P. ostreatus, P. eryngii, R. flava,* and *R. emética,*	(Erbiai et al. 2021; Gąsecka et al. 2016; Kaewnarin et al. 2016; Kakoti et al. 2021; Selli et al. 2021a; Yahia et al. 2017; Yildiz et al. 2015)
Caffeic acid	$C_9H_8O_4$	*A. auricula-judae, A. flavoconia, A. pantherina, A. arvensis, B. frostii, B. edulis, B. luridus, G. lucidum, I. obliquus, P. ostreatus, P. eryngii, P. pulmonarius, R. flava, R. aurea,* and *S. floccopus*	(Alkan et al. 2020; Contato et al. 2020a; Gąsecka et al. 2016; Hwang et al. 2019; Kakoti et al. 2021; Yahia et al. 2017)
Cinnamic acid	$C_9H_8O_2$	*A. auricula-judae, A. bisporus, A. bisporus Portobello, A. mellea, B. edulis, L. edodes, M. procera, P. ostreatus, P. eryngii, P. tuber-regium, R. senecis,* and *S. floccopus*	(Eliuz 2021; Erbiai et al. 2021; Gąsecka et al. 2016, 2018; Junior et al. 2019; Kakoti et al. 2021; Khatua et al. 2015; Lin et al. 2015; López-Vázquez et al. 2017; Selli et al. 2021a; Yahia et al. 2017)
Chlorogenic acid	$C_{16}H_{18}O_9$	*A. bisporus, A. caesarea, A. pantherina, B. frostii, B. edulis, B. luridus,*	(Eliuz 2021; Gąsecka et al. 2016, 2018; López-Vázquez et al. 2017; Pintathong et al.

(continued)

Table 14.1 (continued)

Phenolic acids	Chemical formula	Species	References
		C. cibarius, C. militaris, G. lucidum, L. índigo, L. edodes, M. esculenta, P. eryngii, P. ostreatus, and *S. imbricatus*	2021; Yahia et al. 2017; Yildiz et al. 2015)
p-Coumaric acid	$C_9H_8O_3$	*A. brasiliensis, A. mellea, A. bisporus, B. aereus, B. impolitus, C. militaris, C. cornucopioides, G. lucidum, H. erinaceus, L. edodes, M. procera, M. cognata, M. stridula, M. esculenta, P. eryngii,* and *P. ostreatus*	(Babak et al. 2019; Erbiai et al. 2021; Ferrari et al. 2021; Gąsecka et al. 2016, 2018; Kosani et al. 2019; Pintathong et al. 2021; Selli et al. 2021a; Taofiq et al. 2016; Yildiz et al. 2015)
Ferulic acid	$C_{10}H_{10}O_4$	*A. bisporus, A. arvensis, A. brasiliensis, A. campestris, A. aegerita, A. flavoconia, B. frostii, H. erinaceus, M. esculenta, P. ostreatus, P. eryngii, P. pulmonarius,* and *R. emética*	(Çayan et al. 2020; Contato et al. 2020a; Gąsecka et al. 2016, 2018; Lin et al. 2017b; Selli et al. 2021a; Yahia et al. 2017; Yildiz et al. 2015)
Sinapic acid	$C_{11}H_{12}O_5$	*A. arvensis, A. aegerita, A. virosa, B. edulis, B. frostii, G. lucidum, L. deliciosus, L. indigo, L. perlatum, R. flava, R. extremiorientalis, R. aurea,* and *S. floccopus*	(Alkan et al. 2020; Kaewnarin et al. 2016; Lin et al. 2017b; Yahia et al. 2017)
Syringic acid	$C_9H_{10}O_5$	*A. bisporus, A. mellea, A. brasiliensis, A. silvaticus, C. cornucopioides, G. lucidum, H. erinaceus, I. obliquus, L. edodes, M. procera, M. cognata, M. stridula, P. tuber-regium, P. ostreatus,* and *P. eryngii*	(Babak et al. 2019; Erbiai et al. 2021; Gąsecka et al. 2018; Hwang et al. 2019; Kosani et al. 2019; Lin et al. 2015; Yildiz et al. 2015)

to be the most efficient in terms of extraction quality and antioxidant activity, being also quantified a higher phenolic concentration in the same extracts. In this field, phenolic acids, including chlorogenic, gallic, and protocatechuic acids, are responsible for the high antioxidant performance in extracts of different mushroom species (Sezgin et al. 2020).

More recently, the significance of some mushroom species as antitumor agents has been also investigated, pointing out their use in the control of numerous cancerous conditions. Mushrooms appear to exert their antitumor activity by inhibiting the growth and proliferation of cancer cells *in vivo* and *in vitro* (Hu and Luo 2016; K. Liu et al. 2017; Nowacka-Jechalke et al. 2018), and by inducing apoptosis through the hydroxyl groups interface covered in the phytochemical

Table 14.2 Health benefits related with the presence of phenolic acids in mushrooms

Species	Phenolic acids	Health benefits	References
A. bisporus, A. auricula-judae, L. arcularius, Lentinus sajor-caju, L. squarrosulus, L. velutinus, L. scabrum, P. lecomtei, P. giganteus, P. ostreatus, P. pulmonarius, and *P. arcularius*	Gallic, caffeic, 3,4-dihydroxybenzoic, vanillic, *p*-coumaric, and *t*-cinnamic	Antioxidant and antihemolytic	(Kakoti et al. 2021)
A. bisporus and *P. ostreatus*	Chlorogenic, cinnamic, ferulic, gallic, gentisic, *p*-hydroxybenzoic, *p*-coumaric, protocatechuic, and vanillic	Antioxidant	(Selli et al. 2021a)
A. mellea and *M. procera*	Cinnamic, ferulic, gallic, *p*-coumaric, *p*-hydroxybenzoic, protocatechuic, syringic, and vanillic	Antioxidant	(Erbiai et al. 2021)
P. ostreatus	Cinnamic, *p*-coumaric, and protocatechuic	Antioxidant	(Cardoso et al. 2021)
R. integra, R. rosea, and *R. nigricans*	Cinnamic and *p*-hydroxybenzoic	Antioxidant, antimicrobial, antibiofilm, and cytotoxic	(Kostić et al. 2020b)
P. pulmonarius	Caffeic, ferulic, gallic, vanillic, and *p*-coumaric	Antioxidant	(Contato et al. 2020a)
C. rutilus	Protocatechuic and *p*-hydroxybenzoic	Antioxidant and anti-inflammatory	(Zhang et al. 2020b)
C. comatus	Caffeic, cinnamic, coumaric, *p*-hydroxybenzoic, and protocatechuic	Antioxidant	(Stilinović et al. 2020)
H. annosum	Gentisic and protocatechuic	Anticancer	(Sadowska et al. 2020b)
M. cognata and *M. stridula*	Benzoic, *p*-coumaric, *p*-hydroxybenzoic, protocatechuic, syringic, and *t*-cinnamic	Antioxidant	(Babak et al. 2019)
M. lobayensis	Cinnamic and ferulic	Antioxidant	(Khatua et al. 2019)
T. indicum	Erucic, gallic, and *p*-coumaric	Antioxidant	(Li et al. 2019)
G. lucidum, H. erinaceus, and *L. edodes*	4-hydroxybenzoic, gallic, and *t*-cinnamic	Antioxidant	(Magdziak et al. 2019)
L. edodes	Gallic	Chemopreventive	(Finimundy et al. 2018)
P. squamosus	Cinnamic, *p*-hydroxybenzoic, and *p*-coumaric	Antioxidant and antimicrobial	(Mocan et al. 2018)

(continued)

Table 14.2 (continued)

Species	Phenolic acids	Health benefits	References
A. arvensis, A. bisporus (brown and white), *A. bitorquis, A. brasiliensis, A. campestris,* and *A. silvaticus*	Gallic, caffeic, *p*-hydroxybenzoic, *p*-coumaric, ferulic, chlorogenic, syringic, *t*-cinnamic, and protocatechuic	Antioxidant	(Gąsecka et al. 2018)
A. arvensis	Protocatechuic and *p*-hydroxybenzoic	Antioxidant	(Dogan et al. 2018)
A. aegerita	Chlorogenic, ferulic, gallic, protocatechuic, and sinapic	Anti-angiogenic and antitumor	(Lin et al. 2017b)
A. arvensis, A. flavoconia, A. pantherina, A. virosa, B. edulis, B. frostii, B. luridus, C. alboviolaceus, G. lucidum, H. lactifluorum, H. sordidus, L. perlatum, L. indigus, R. emética, R. flava, S. floccopus, and *S. imbricatus*	Caffeic, chlorogenic, cinnamic, gallic, ferulic, fumaric, *p*-coumaric, *p*-hydroxybenzoic, protocatechuic, vanillic, and sinapic	Antioxidant	(Yahia et al. 2017)
P. ostreatus and *A. bisporus*	Cinnamic, *p*-hydroxybenzoic, *p*-coumaric, and protocatechuic	Antioxidant and cytotoxic	(Cardoso et al. 2017)
A. caesarea, B. edulis, C. cibarius, L. indigo, and *Ramaria sp.*	Chlorogenic and cinnamic	Antioxidant	(López-Vázquez et al. 2017)
P. eryngii and *S. bellinii*	*p*-hydroxybenzoic and cinnamic	Antioxidant, anti-inflammatory, and cytotoxic	(Souilem et al. 2017)
A. molesta, B. plúmbea, L. deliciosus, R. flava, T. terreum, and *V. gloiocephalus*	Gallic, rosmarinic, *p*-coumaric, and syringic	Antioxidant, cytotoxic, and antibacterial	(Sadi et al. 2016b)
P. squamosus	Cinnamic and p-hydroxybenzoic	Antioxidant and antimicrobial	(Fernandes et al. 2016)
P. ostreatus and *P. eryngii*	Chlorogenic, syringic, ferulic, *p*-coumaric, caffeic, *t*-cinnamic, and vanillic	Antioxidant	(Gąsecka et al. 2016)
P. portentosus, R. emetica, R. extremiorientalis, and *Russula sp.*	Ferulic, gallic, *m*-coumaric, protocatechuic, rosmarinic, sinapic, syringic, and vanillic	Antioxidant, anti-tyrosinase, and hyperglycemic inhibition	(Kaewnarin et al. 2016)
G. lucidum, M. esculenta, L. edodes, and *H. erinaceus*	Gallic, protocatechuic, *p*-hydroxybenzoic, chlorogenic, vanillic, syringic, *p*-coumaric, ferulic, and *t*-cinnamic	Antioxidant	(Yildiz et al. 2015)
A. bisporus, A. bisporus Portobello, A. caesarea, B. aereus, B. edulis,	*p*-hydroxybenzoic, *p*-coumaric, and cinnamic	Anti-inflammatory	(Taofiq et al. 2016)

(continued)

Table 14.2 (continued)

Species	Phenolic acids	Health benefits	References
B. fragrans, B. impolitus, B. reticulatus, C. cibarius, L. deliciosus, M. esculenta, M. procera, P. eryngii, and *P. ostreatus*			
P. tuber-regium	Cinnamic, chlorogenic, coumaric, ferulic, gallic, protocatechuic, and syringic	Antioxidant and anti-angiogenic	(Lin et al. 2015)

molecules, such as polyphenols, with the polar receptor site of the mitochondrial cytochrome P450 enzyme (Abdelshafy et al. 2021; Ukaegbu et al. 2020). Among other mushroom species, the aqueous extract rich in phenolic compounds of *Agrocybe aegerita* (Brig.) Sing. stands out for its antitumor potential and low cytotoxicity, showing potential in the control of cancerous diseases. Here, phenolic acids, namely ferulic, chlorogenic, protocatechuic, gallic, and sinapic acids, have been identified as the main responsibles for the observed antitumor activity, through the inhibition of the vascular endothelial growth factor (VEGF)-induced proliferation in human umbilical vein endothelial cells (HUVECs), and its secretion in Caco-2 cells exposed to the same extract (Lin et al. 2017a). Also, gallic and *p*-coumaric acids from *Volvopluteus gloiocephalus* (DC.) Vizzini, Contu & Justo mushroom extract were investigated for their possible antitumor activity against liver cancer (HepG2) cell line, the results showing that the antitumor activity for tried phenolic acids may be due to their antioxidant activity or cell growth inhibitory potential (Sadi et al. 2016a).

Some mushroom species also exhibit anti-inflammatory activity by inhibiting the production of inflammatory mediators (Dennis and Norris 2015). Inflammation is the normal response of the human organism immune system to the harmful effects related with pathogenic microorganisms and other physical and chemical agents (Elsayed et al. 2014). This bioactivity performed by some edible mushrooms such as *P. ostreatus*, *Boletus edulis* Bull., *Cantharellus cibarius* Fr., *Agaricus bisporus* (J.E. Lange) Imbach, *Calocybe gambosa* (Fr.) Donk, *Hygrophorus marzuolus* (Fr.) Bres., *Craterellus cornucopioides* (L.) Pers., and *Lactarius deliciosus* (L. ex Fr.) S.F.Gray has been related to their composition in phenolic acids such as homogentisic, ferulic, caffeic, gentisic, *p*-hydroxybenzoic, protocatechuic, gallic, and catechin acids, detected in *in vitro* studies with LPS-activated RAW 264.7 macrophages. In these investigations, it was possible to verify the anti-inflammatory effects of different mushroom extracts by the expression of inflammatory markers, including the production of nitric oxide (NO), IL-6, and IL-1β (Palacios et al. 2011). Likewise, Taofiq et al. (2016) showed that the phenolic extracts of *A. bisporus*, *Boletus impolitus* (Fr.) Šutara, *Macrolepiota procera* (Scop.) Singer, and *P. ostreatus* were responsible for the greatest anti-inflammatory potential through the inhibition of NO production, and the authors attributed this significant bioactivity to the presence of high concentrations of cinnamic acid.

In the last decade, the bioactive potential of mushrooms against other diseases such as diabetes *mellitus* has also been investigated, given its rich composition in phenolic compounds with potential anti-hyperglycemic activity. This chronic disease is characterized by an irregular increase in blood glucose levels as a consequence of an imbalance in insulin production/insensitivity (Shobana et al. 2009). The control of this type of diabetes can be achieved through the inhibition of the pancreatic α-amylase enzyme or the intestinal α-glucosidase enzyme, which directly influence the hydrolysis and absorption of polysaccharides (Koike et al. 1995). In addition to its ability to control diabetes disease, different mushroom extracts have also been shown to be able to prevent and control complications associated with this condition (hypertension and cardiovascular diseases) (Garduño-Diaz and Khokhar 2012), given the presence of antioxidants, such as phenolic acids, in their composition which will act in the inhibition of enzymes like aldose reductase (Kato et al. 2009) and the aforementioned α-amylase and α-glucosidase (Liu et al. 2012). The *in vitro* anti-diabetic activity of *Coprinus comatus* (O.F.Müll.) Pers., *Phellinus linteus* (Berkeley & Curtis) Teng, *Inonotus obliquus* (Ach. ex Pers.) Pilát, *Cordyceps militaris* (L.) Fr., *Agaricus blazei* Peck, and *Morchella conic*a Pers. was investigated by Stojkovic et al. (2019), who detected the presence of high amounts of phenolic acids, such as cinnamic and *p*-coumaric acids, as well as the inhibition of the α-glucosidase enzyme in all the analyzed mushroom species. Given that the presence of these phenolic acids is also associated with the antioxidant activity exerted by different mushroom extracts, it can be said that there is a close relationship between this bioactivity and the resulting anti-diabetic effects (Wu and Xu 2015). It has also been claimed that the antioxidant activity performed by several phenolic acids from mushrooms are positively correlated with anti-tyrosinase effects, namely *p*-hydroxybenzoic, *p*-coumaric, and gallic acids, which have a significant effect as tyrosinase inhibitors (Alam et al. 2019; Alkan et al. 2020; Taofiq et al. 2016).

Other mushrooms have also been shown to perform antimicrobial activity against numerous pathogenic organisms (Erjavec et al. 2016). Specifically, *Tricholosporum goniospermum* (Bres.) Guzmán ex T.J.Baroni mushroom methanolic, ethyl acetate, and *n*-hexane extracts were tested against some Gram-positive and Gram-negative bacteria and yeasts, with all extracts presenting an antimicrobial potential against the selected microbial strains. Here, authors attribute the occurrence of this bioactivity to the presence of phenolic acids such as gallic acid and catechin (Angelini et al. 2020). However, Gram-negative bacteria seem to be more resistant to mushroom extracts, probably due to the complex permeability barrier, since it has a supplemental outer lipopolysaccharide barrier that limits the breakthrough of many compounds while being permeable to nutrients. The antimicrobial properties wielded by mushroom extracts seem, therefore, to be related to their composition in phenolic acids, which act at the level of cell wall and nucleic acid inhibition mechanisms and in the synthesis of cellular proteins (Bach et al. 2019; Oliveira et al. 2016).

14.3 Biodynamic Food Products Designed Through Mushrooms Incorporation

The population is increasingly interested in nutritious, healthy, and safe food products with beneficial effects on human health, improving well-being and quality of life, and potentially reducing disease risk. Therefore, the scientific community, food, and pharmaceutical industries are constantly searching for natural bioactive ingredients that, in addition to their nutritional value, offer health benefits without altering the quality and safety of the final products (Guiné et al. 2019; Patinho et al. 2019).

Mushrooms have been seen as treasures and highlighted as the new generation of healthy food ingredients given their low-fat, high protein, dietary fiber, vitamins, minerals amount, and many bioactive molecules, making them much appreciated ingredients to develop functional foods, dietary supplements, and medicines (Das et al. 2021; Lu et al. 2020). Mushrooms have numerous bioactive components, responsible for their well-reported bioactive effects. Thus, phenolic acids, representing a class of these structurally diverse molecules, are frequently being investigated for their bioactive properties, such as antioxidant, antitumor, antimicrobial, anti-inflammatory, among others (Fig. 14.1) (Heleno et al. 2015; Kumar and Goel 2019; Reis et al. 2017; Yadav and Negi 2021). Phenolic acids are present in extracts obtained from different parts of mushrooms, including their fruiting bodies and mycelium, and are constantly incorporated into the human diet (Das et al. 2021) (Table 14.3).

Mushrooms or extracts thereof have been marketed as functional food and dietary supplements for improving health in general (Erbiai et al. 2021; Yadav and Negi 2021). They possess a distinctly pleasant appetizing flavor enhanced by

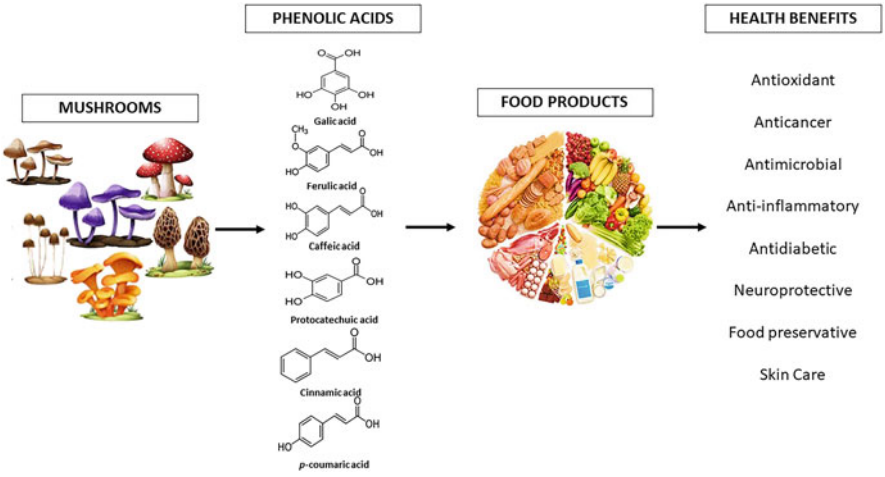

Fig. 14.1 Possible health benefits of functional foods fortified with mushrooms

Table 14.3 Food products fortified with mushrooms and their acquired beneficial properties

Fortified products	Species	Beneficial properties	References
Cookies	*P. ostreatus*	Higher content of health promoting components and antioxidant potential	(Uriarte-frías et al. 2021)
Bread	*L. edodes*, *B. edulis*, and *A. bisporus*	Improved nutritional and sensory quality and antioxidant activity	(Lu et al. 2021)
Beef salami	*P. ostreatus*	Antioxidant properties	(Özünlü and Ergezer 2021)
Extruded products (snacks)	*L. edodes*, *B. edulis*, and *A. bisporus*	Antioxidant properties	(Lu et al. 2020)
Frankfurter	*B. edulis*	Microbial inhibition, antioxidant properties, and sensory improvement	(Novakovic et al. 2020)
Chicken patties	*P. sapidus*	Increase the level of antioxidant activity and improve sensory quality	(Abd et al. 2020)
Goat meat nuggets	*F. velutipes*	Antioxidant properties and physico-chemical qualities	(Banerjee et al. 2020)
Pork meat sausage	*L. edodes*	Improved nutritional quality, shelf life, and antioxidant activity	(L. Wang et al. 2019a)
Cantonese sausages	*F. velutipes*	Antioxidant properties	(X. Wang et al. 2019b)
Wheat bread	*B. edulis*	Improve nutritional content and antioxidant activity	(Vlaic et al. 2019)
Rice muffin	*L. edodes*	Antioxidant properties	(Olawuyi and Lee 2019)
Noodles	*P. ostreatus*	Improve nutritional profile and add antioxidant potential to noodles	(Arora et al. 2018)
Pasta	*L. edodes* and *B. edulis*	Lowering the potential glycemic response and improving antioxidant capacity	(Lu et al. 2018)
Ground beef	*A. bisporus*	Antioxidant properties	(Alnoumani et al. 2017)
Brown rice extrudates	*A. auricula*	Antioxidant effect and lower glycemic load after ingestion	(Vallée et al. 2017)
Sponge cake	*A. bisporus*	Improve the nutritional and antioxidant quality	(Arora et al. 2017)
Milk	*A. blazei*	Antioxidant properties	(Vital et al. 2017)
Fermented sausages	*L. edodes*	Antioxidant and antimicrobial properties	(Van Ba et al., 2016, 2017)
Frankfurter	*L. edodes*	Better sensory scores and antioxidant quality	(Pil-nam et al. 2015)
Sutchi catfish patties	*A. bisporus*	Increases the nutritional quality and improves the shelf life of patties	(Nayak et al. 2015)

monosodium glutamate, making them preferable and adaptable in most food formulations. Mushrooms are used directly as food but also as ingredients in the production and formulation of new functional foods and dietary supplements for those concerned with health. The food industry also uses them as additives due to their smell, taste, and bioactive properties that also favorably influence flavor, appearance, acceptability, and shelf life when incorporated into other food products (Das et al. 2021; Ma et al. 2018).

Today, food products are being developed incorporating mushrooms as bioactive ingredients that enhance health benefits (Fig. 14.1) (Table 14.3). Patinho et al. (2019) used *A. bisporus* mushroom in beef burgers to provide antioxidant properties. Given that phenolics are one of the main bioactive compounds responsible for antioxidant activity, *A. bisporus* has been reported to possess a variety of phenolic acids with this biological effect (Eliuz 2021). A recent study also created a version of cookies by adding *Pleurotus eryngii* (DC.) Quél. mushroom flour to wheat flour to improve their nutritional profiles and potential health benefits (Biao et al. 2020). *P. eryngii* presented good antioxidant, anti-inflammatory, and anticancer properties, and indeed possesses some phenolic acids responsible for these characteristics (Gąsecka et al. 2016; Yuan et al. 2017). Francisco et al. (2018) added *A. bisporus* extract to yogurt to make it a functional food. As mentioned above, this mushroom has phenolic acids related to different bioactive properties.

Other formulations have been developed exploring a variety of research centered on the use of mushrooms as a functional ingredient and additive replacer due to their nutritional and bioactive assets related to their phenolic acids content, which bring health benefits to consumers. (Lu 2018; Pintathong et al. 2021; Rangel-vargas et al. 2021; Reis et al. 2017; Süfer et al. 2016) (Table 14.3).

Several findings have reported the beneficial effect of phenolic acid-rich mushroom extracts as functional ingredients in several food formulations (Rangel-vargas et al. 2021). However, after incorporation, studies on the technical aspects, functionality, and sensory properties of the final food formulation must be conducted to ensure consumers' safety and health benefits of the final product.

14.4 Conclusion

Phenolic compounds are one of the most important classes of secondary metabolites present in mushrooms, presenting different bioactive properties already studied and verified. Phenolic acids, such as *p*-coumaric, cinnamic, *p*-hydroxybenzoic, benzoic, ferulic, gallic acids, and others, are the most frequently reported in different extracts of various species of mushrooms, like *A. bisporus* and *P. ostreatus,* among others. Phenolic acids extracted from mushroom and/or its extract seem to perform several biological activities, such as antitumor, antioxidant, anti-hyperglycemic, antimicrobial, anti-inflammatory, anti-tyrosinase, among others, also functioning as chemotherapeutic and cytotoxic agents. Given their functional properties with beneficial effects on human health, mushrooms rich in this type of metabolites have been

extensively studied in the last few years and used as nutraceutical agents in several food products already available, such as cookies, bread, pasta, and other formulations.

Despite the proven benefits of using mushrooms in a balanced diet and against different health conditions, complementary studies should tend to focus on improving the effectiveness of these compounds, while maintaining their bioactive properties, bioavailability, and stability through storage, preparation, and consumption. Additionally, the development of novel drugs delivery techniques through the development of nano formulations seems to be an important step in the immediate future to further take advantage of the benefits of these compounds.

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Chapter 15
Terpenes from Fungi

Ana F. Vinha, Marlene Machado, and M. Beatriz P. P. Oliveira

Abstract Terpenes are naturally occurring compounds produced by an extensive variety of plants, animals, and microorganisms. A wide range of the biological properties of terpenoids is recognized, including cancer chemopreventive effects, antimicrobial, antifungal, antiviral, antihyperglycemic, anti-inflammatory, and antiparasitic activities. Fungi are known to produce several well-known terpenoids, including mycotoxins, antibiotics, antitumor compounds, and phytohormones. This chapter provides a comprehensive overview of the current knowledge on fungal terpenoid biosynthesis from biochemical viewpoint, taking into account the main differences between the terpenome of the major fungal phyla. We also propose opportunities and challenges for the introduction of these secondary metabolites as biologically active compounds for the development of drugs, agrochemicals, as well as nutraceuticals in food industry.

15.1 Introduction

For millennia that fungi have been recognized by several communities as a valuable source for nutrition and medicine; however, the traditional view of the mycobiota has changed across history (De Obeso Fernandez Del Valle and Scheckhuber 2021; Kotowski 2019). Moreover, all plants in natural ecosystems appear to be symbiotic with fungal endophytes. Over the years, many researchers suggested the importance of fungi in the creation of early religious practices, by using fungal fruiting bodies

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containing psychoactive compounds in order to achieve metaphysical experiences (Winkelman 2019; Failla 2015, Guzmán 2009, 2008). Nowadays, fungi present an outstanding importance due to the production and development of beneficial compounds (enzymes, pharmaceuticals, food additives), participating in important ecological processes (biocontrol of pathogenic organisms, waste fermentation), and possessing ludic uses (e.g., fungi producing hallucinogenic substances) (De Obeso Fernandez Del Valle and Scheckhuber 2021). Thus, features of taxonomic significance are integrated with natural functions, including their relevance in human health. The main classes of secondary metabolites in fungi include polyketides, non-ribosomal peptides, terpenoids, and siderophores (Koczyk et al. 2021). Terpenes, commonly known as terpenoids are the largest and most divergent group of secondary metabolites mostly found in plants and fungi. Nowadays they have been largely studied for their potential due to the high biological properties. This review provides an overview of terpene types synthesized from different classes of fungi, projecting them into future applications in different human health areas.

15.2 Fungi

Fungi are an antique group, which fossil evidence suggest to be over than 3.5 billion years old (Redecker et al. 2000). According to fossil evidence, the earliest vascular land plants did not appear until about 425 million years ago, and some researchers believe that fungi may have played a key role in the colonization of land by these early plants (Carris et al. 2012). Over the last few decades, the fungi were described by the significant role both in nature and in the human economy. According to several authors, approximately 80,000 to 120,000 species of fungi have already been described, despite the estimated value being around 1.5 million (Hawksworth and Lücking 2017; Hawksworth 2001; Kirk et al. 2001). Among them, it is estimated that 700 species of fungi possess therapeutic activities (Niazi and Ghafoor 2021; Slusarczyk et al. 2021) and therefore they may be a good source of biologically active compounds for use in the pharmaceutical and food industries. Several research on the health-promoting properties of fungi is being carried out intensively in many research centers around the world. Furthermore, many living organisms use terpenes for ecological interactions, including predation, competition, commensalism, amensalism, mutualism, symbiosis, and parasitism (Jiang et al. 2020). Also, terpene research as an active ingredient in pesticide development has been considered a global resurgence due to its low risk to the human health and environment.

All fungi are heterotrophic, that is, unlike plants, they are not capable of producing their own food, nourishing themselves by absorption of them from other sources. In addition, as heterotrophic, fungi are eukaryotic beings and can be unicellular, as in the case of yeasts, or multicellular, such as mushrooms. The kingdom Fungi contains five major phyla that were established according to their mode of sexual reproduction or using molecular data. The five true phyla of fungi are: *Chytridiomycota* (Chytrids) (Medina and Buchler 2020), *Zygomycota* (conjugated fungi) (Chang et al.

2015), *Ascomycota* (sac fungi) (Nagy et al. 2017), *Basidiomycota* (club fungi) (X. L. Li et al. 2019a), and the recently described *Glomeromycota* (Martins et al. 2017). There are also many fungus-like organisms, including slime molds, and oomycetes (water molds) that do not belong to the kingdom Fungi but are generally called fungi. Many of these fungus-like organisms are included in the kingdom Chromista (Lourenço et al. 2020).

15.2.1 Chytridiomycota *(Chytrids)*

The only class in the *Phylum Chytridiomycota* is the *Chytridiomycetes*. The chytrids are the most primitive *Eumycota* and, therefore the simplest or true fungi. Like all fungi, chytrids possess chitin in their cell walls, but one group of chytrids contains both cellulose and chitin in the cell wall. This phylum is an early-diverging, mostly unicellular, lineage of fungi that consists of significant aquatic saprotrophs, parasites, and pathogens, and is of evolutionary interest because its members retain biological traits considered ancestral in the fungal kingdom (Laundon and Cunliffe 2021).

15.2.2 Zygomycota *(Conjugated Fungi)*

The zygomycetes, a relatively small group of fungi, are classified as a single phylum, *Zygomycota*, based on sexual reproduction by zygospores, frequent asexual reproduction by sporangia, absence of multicellular sporocarps, and production of coenocytic hyphae, all with some exceptions (Spatafora et al. 2016). The zygomycetes include: (i) *Phycomyces blakesleeanus* and other important model organisms; (ii) species such as *Rhizopus stolonifer* that cause economically significant pre- and postharvest diseases of fruits; (iii) members of Glomeromycota that colonize roots and form endomycorrhizal symbioses with more than 80% of land plants; and (iv) diverse and important pathogens or commensals of insects, nematodes, and other soil invertebrates (Redecker and Schüßler 2014). Moreover, several zygomycetes can synthesize bioactive compounds such as lycopene, fatty acids, even though they can also cause rare and deadly human diseases such as zygomycosis (Doggett and Wong 2014). Mucoromycotina includes *Mucor*, *Rhizopus*, *Actinomucor* and the majority of the most common and best known zygomycetes that play a considerable commercial role, with several species used in industry for fermentation and organic acid production (Spatafora et al. 2016). The metabolic products of other species of *Rhizopus* are intermediates in the synthesis of semi-synthetic steroid hormones. This form of sexual reproduction in fungi is called conjugation (although it differs markedly from conjugation in bacteria and protists), giving rise to the name "conjugated fungi."

15.2.3 Ascomycota *(Sac Fungi)*

The majority of known fungi belong to the phylum *Ascomycota*, which is characterized by the formation of an ascus (plural, asci), a sac-like structure that contains haploid ascospores (Berman 2012). Many ascomycetes possess high commercial importance, providing beneficial roles, such as the yeasts used in baking, brewing, and wine fermentation, plus truffles and morels, which are held as gourmet delicacies (Maicas 2020). Also, *Aspergillus oryzae* used in the fermentation of rice to produce sake. Other ascomycetes parasitize plants and animals, including humans. Lichens (i.e., green algae or cyanobacteria) are classified as fungi and the fungal partners belong to the *Ascomycota* and *Basidiomycota*. They are acknowledged for producing bioactive substances with great medical potential (Grimm et al. 2021; Slusarczyk et al. 2021), among them, amino acid derivatives, sugar alcohols, aliphatic acids, macrolytic lactones, monocyclic aromatic compounds, quinines, chromones, xanthones, dibenzofurans, depsides, depsidones, depsones, terpenoids, steroids, carotenoids, and diphenyl ethers (Zhao et al. 2021c).

15.2.4 Basidiomycota *(Club Fungi)*

The fungi in the phylum *Basidiomycota* are easily recognizable under a light microscope by their club-shaped fruiting bodies called basidia (singular, basidium), which are the swollen terminal cell of a hypha. The basidia, which are the reproductive organs of these fungi, are often contained within the familiar mushroom, commonly seen in fields after rain, on the supermarket shelves, and growing on lawn. They are part of the phylum *Basidiomycota*, which is a phylogenetic sister group of *Ascomycota*. These two phyla are part of a wide category called "higher fungi" (Sandargo et al. 2019). These mushroom-producing basidiomycetes are sometimes referred to as "gill fungi" because of the presence of gill-like structures on the underside of the cap. The accumulated secondary metabolites produced by mushrooms have been widely accepted, as sources of nutraceuticals, food additives, cosmeceuticals, and pharmaceuticals (Bakratsas et al. 2021). Until now, about 80–85% of bioactive compounds described in mushrooms comprise polysaccharides, polyketides, phenolic compounds, triterpenoids, steroids, proteins, nucleotides, fatty acids, and lactones (Fernandes et al. 2021; Kardideh et al. 2019; Keller 2019). More recent research has shown that plenty of basidiomycetes can grow in submerged cultures and generate advantageous bioactive compounds for nutrition, medicine, and pharmaceuticals industries (Dudekula et al. 2020).

15.2.5 Glomeromycota

The *Glomeromycota* is a newly established phylum which comprises about 230 species that all live in close association with the roots of trees. It appears that all members of this family form arbuscular mycorrhizae: the hyphae interact with the root cells forming a mutually beneficial association where the plants supply the carbon source and energy in the form of carbohydrates to the fungus, and the fungus supplies essential minerals from the soil to the plant (Schubler et al. 2001). According to Sharma et al. (2017) the colonization of roots by arbuscular mycorrhiza fungi is recognized to influence secondary metabolism in plants, including changes in the concentration and composition of terpenoids, which can boost both direct and indirect plant defense against herbivorous insects.

15.3 Terpenes

Terpenes constitute a highly diverse class of chemical compounds, playing an important role in many plant physiological processes, including, in plant growth and development, and in the ability to protect against environmental adversities (W. Yang et al. 2020a). As raw materials, presenting various structures and a wide variety, terpenoids are also widely used in pharmaceuticals, food, and cosmetics industries (Carsanba et al. 2021). To date, more than 50,000 terpenoids have been identified in nature (Fei et al. 2021; Sun et al. 2017). A vast number of different terpenoids have been widely used in medicine and medical sciences to prevent and treat several diseases due to their pharmaceutically bioactive properties. Although the majority of terpenoids are predominantly present in plants, they can be also found in other organisms such as bacteria, fungi, insects, and animals (Moser and Pichler 2019). Some terpenoids are currently used in medicine, food, cosmetics, fine chemistry, pharmacy, agriculture, and biofuel (Zhang et al. 2017), since their chemical diversity provides different activities, and, therefore, these compounds are divided into sub-classes. Chemically, terpenes are grouped together because of their distinctive carbon skeleton. It consists of a basic five-carbon isoprene unit. Displaying a wide range of biological properties, terpenoids are the most attractive metabolites among the other natural compounds from higher fungi.

As the focus, this chapter will discuss the most recent approaches on the relationship between terpenes and fungi, emphasizing the importance of fungi in the synthesis of terpenoids with therapeutic action. Table 15.1 summarizes the main types of terpenoids found in fungi.

Table 15.1 Main sub-classes of terpenoids described in fungi

Terpene	Type	Reference
Monoterpenoids	Osmane	Niu et al. (2020a)
	Carene	Liu et al. (2017)
	Menthene	Liu et al. (2017)
Sesquiterpenoids	Eremophilane	Li-Bin et al. (2021), Wang et al. (2020a)
	Bisabolane/phenolic bisabolane	Chen et al. (2021c), Li et al. (2021), Niu et al. (2020b), Qu et al. (2020), Sun et al. (2020)
	Guaiane	Tan et al. (2021)
	Drimane	Fang et al. (2021), Gou et al. (2021), Ma et al. (2020)
	Tremulanes	Shi et al. (2021), Chen et al. (2020a)
	Cyclonerane	Liu, X. H. et al. (2020a), Ma et al. (2021)
	Sterpurane	Zhai et al. (2021)
	Illudane	Perera et al. (2020), Zhai et al. (2021)
	Trichothecenes	Shi et al. (2020)
	Caryophyllene	Zhang, Y. et al. (2020a)
	Muurolane	Yang et al. (2020b)
	Triquinane	Liu, H. X. et al. (2020b)
	Botryanes	Su et al. (2020)
	Irlactane	Chen et al. (2020c)
	Cadinane	Cui et al. (2020), Shi et al. (2019)
	Tropolonic	Chen et al. (2020b)
Meroterpenoids	DMOA-derived	Huang et al. (2021), Tang et al. (2021)
	Cochlioquinones	Liu et al. (2021a)
	Picoline-derived	Jiang et al. (2021b)
	Andrastin	Cheng et al. (2020a), Ren et al. (2021)
	Austalides	Antipova et al. (2019), Cheng et al. (2020b)
	Tricycloalternarenes	Hwang et al. (2020), Wang et al. (2020b)
	Phenolic Sesquiterpene	Chen et al. (2020d)
Diterpenoids	Isopimarane	Yang et al. (2020b)
	Pimarane	Hou et al. (2020), Xu et al. (2019)
	Nor-isopimarane	Chen et al. (2020c)
	Fusicoccane	Zhang et al. (2020b)
	Cyathane	Yin et al. (2021)
	Indole	Chen et al. (2021b), Zhou et al. (2021), Zhou et al. (2019)
Sesterterpenoids	GFPP-derived	Wang et al. (2021)
	Asperane	Y. L. Li et al. (2019b)
	Ophiobolins	Cai et al. (2019), Chi et al. (2020), Fang et al. (2021), Liu et al. (2019b), Wei et al. (2004)
Triterpenoids	Hopane	Isaka et al. (2010)
	Tetracyclic	Ibrahim et al. (2016), Liu et al. (2021a)
	Lanostane/ lanostanoid	Isaka et al. (2017), Gao et al. (2021), Rincon et al. (2021), Zou et al. (2020)

15.3.1 Terpenes in Fungi

Fungal secondary metabolites can be classified in distinct chemical classes, including polyketides, terpenoids, shikimic acid derived compounds, and non-ribosomal

peptides (Pusztahelyi et al. 2015). Also, hybrid metabolites, such as meroterpenoids, formed via the hybridization of the terpene moiety and the nonterpene moiety, are common (K. Chen et al. 2021a; Pusztahelyi et al. 2015). According to the nonterpene part involved in their biosynthetic pathways, meroterpenoids can be divided into four categories: polyketide-terpenoids, indole-terpenoids, shikimate-terpenoids, and miscellaneous terpenoids (K. Chen et al. 2021a). Polyketide-terpenoids (46.1%) and shikimate-terpenoids (44.2%) comprise the majority of fungal meroterpenoids, followed by indole-terpenoids (7.2%) and miscellaneous meroterpenoids (2.6%) (Jiang et al. 2021a).

On the basis of the number of acyl units employed in the polyketide part biosynthesis, polyketide-terpenoids can be further categorized into triketide-terpenoid (4-hydroxybenzoic acid (4-HB), 4-hydroxy-6-methylpyrone (HMP), 4-hydroxy-6-phenyl-2H-pyran-2-one (HPPO), 3-dehydroquinic acid (DHQ), 4-hydroxy-6-(3-pyridinyl)-2H-pyran-2-one (HP-3-PO), gentisyl alcohol (GA)), tetraketide-terpenoid (orsellinic acid (OA), 3,5-dimethylorsellinic acid (DMOA), 6-methylsalicylic acid (6-MSA), 6-hydroxymellein (6-HM), 5,7-dihydroxy-4-methylphthalide (DHMP), 3-methylorsellinic acid (3-MOA)), pentaketide–terpenoid, and hexaketide–terpenoid meroterpenoids (Jiang et al. 2021b; M. Liu et al. 2021a; M. Zhao et al. 2021a). Among them, meroterpenoids derived from 4-HB and DMOA demonstrate the most biological activity, including anti-fibrosis, anti-inflammation, anti-Alzheimer, neuroprotection, and anticancer properties (M. Zhao et al. 2021a). There are still andrastin-type meroterpenoids, which are derived from DMOA and farnesyl diphosphate (FPP) via a mixed polyketide-terpenoid pathway and are renowned for their potent cytotoxic, antifeedant, and insecticidal effects (Ren et al. 2021). Fungi like *Ganoderma, Penicillium*, and *Aspergillus* are the most common producers of meroterpenoids, accounting for more than half of the total number of meroterpenoids (Jiang et al. 2021a). Thus, the use of fungal metabolites as an alternative to conventional antibiotics/chemotherapeutics increases the probability of a successful eradication therapy (Cen et al. 2021).

Also, some secondary metabolites produced by fungi are phytotoxic and can lead to major illnesses in agrarian and forestry plants resulting considerable economic losses. At least 545 fungal phytotoxic secondary metabolites have been documented, including 207 polyketides, 46 phenols and phenolic acids, 135 terpenoids, 146 nitrogen-containing secondary metabolites, and 11 others. According to Xu et al. (2021), the main phytotoxic compounds are aromatic polyketides and sesquiterpenoids. Also, algae have been described to be the second most important source of marine fungus (Fang et al. 2021).

15.3.1.1 Monoterpenoids (C10)

Monoterpenes are the first and the simplest sub-class of terpenes, containing ten carbons in their chemical structure. As previously mentioned, several terpenoids possess medicinal properties, such as anticancer (taxanes), antimicrobial, or antimalarial activity (artemisinin) (Škubník et al. 2021). Also, these compounds, including

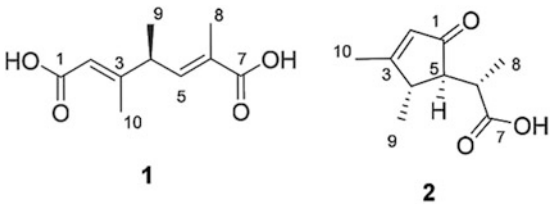

Fig. 15.1 Aspermonoterpenoid A (1) and B (2): osmane-type monoterpenoids isolated from *Aspergillus sydowii*. (Niu et al. 2020a)

the monoterpenes, are often used as fragrance or flavoring compounds. Monoterpenes have also been studied as biofuel targets. For instance, pinene can be chemically dimerized for use as a potential jet fuel, whereas limonene can be used as a biofuel and a precursor for commodity chemicals. Because of the interest in terpenes for an assortment of uses, the pathways that produce these molecules have been engineered for increased production in *Saccharomyces cerevisiae* and *Escherichia coli*. Endophytic fungi within the genera *Hypoxylon*, *Annulohypoxylon*, and *Daldinia* can produce 1,8-cineole, a commercially important monoterpene. Also, Niu et al. (2020a) described two new monoterpenes, Aspermonoterpenoid A and B (Fig. 15.1), synthesized by *Aspergillus sydowii* that have anti-inflammatory activity. According to Shaw et al. (2015) microbial derived 1,8-cineole could be cost-competitive with plant extract sources, and future efforts should be made to correlate the huge variety of fungal terpenes to produce new terpene-derived drugs, commodity chemicals, or biofuels.

15.3.1.2 Sesquiterpenoids (C15)

Sesquiterpenoids (including drimane-, phenolic bisabolane-, eremophilane-, cyclonerodiol-, cadinane-, humulane-, protoilludane-, and other types) are produced by some marine-derived fungal species mainly from the genera *Aspergillus*, *Septoria*, *Trichothecium*, *Graphostroma*, and *Trichoderma* (Y. Chen et al. 2021c; Li-Bin et al. 2021; Liu et al. 2019a, 2019b, Shi et al. 2020). These compounds exhibit several biological activities, including cytotoxic, antibacterial, nitric oxide inhibitory, nematicidal, anti-inflammatory, and growth inhibition of marine phytoplankton activities (Y. Chen et al. 2021c; Hu et al. 2020; Liu et al. 2019a, 2019b). Bracing efforts to discover new sesquiterpenes from nature continue (Gou et al. 2021).

15.3.1.2.1 Drimane-Type Sesquiterpenoids

Drimanes, a type of sesquiterpenoid with a bicyclic scaffold, exhibit several biological activities, such as antimicrobial, anti-inflammatory, cytotoxic,

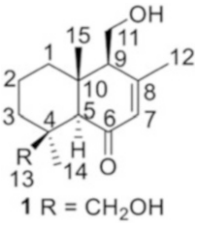

Fig. 15.2 (4S,5R,9S,10R)-11,13-dihydroxy-drim-7-en-6-one: driname-type sesquiterpenoid isolated from *Penicillium citrinoviride* (Gou et al. 2021).

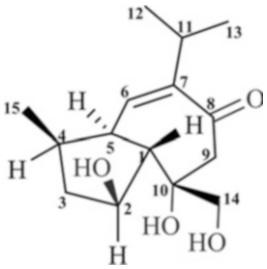

Fig. 15.3 Oxytropiol A: guaiane-type sesquiterpenoid isolated from *Alternaria oxytropis* (Tan et al. 2019)

neurotransmission, anti-diabetic, and antihyperlipidemic activity (W. Y. Zhao et al. 2021b). Because of their interesting structural features and biological activities, fungi-derived drimanes have attracted increasing attention. These compounds (Fig. 15.2) can be synthesized by fungi (primarily *Aspergillus* and *Penicillium* species). Moreover, the well-known drimane dialdehydes act as antifeedants against insects and have potential to be used as alternative insecticides (Inocente et al. 2018).

15.3.1.2.2 Guaiane-Type Sesquiterpenoids

Guaiane-type sesquiterpenoids (Fig. 15.3) are a large group of natural products, and the core skeleton is a five-membered ring fused to a seven-membered ring with two methyl groups and an isopropyl group anchored at C4/C10 and C7, respectively. According to different ring systems, guaiane sesquiterpenoids can be generally divided into five categories: bicyclic guaiane, guaiane-lactone, tricyclic guaiane, dimeric or trimeric guaiane, and other guaiane-type sesquiterpenoids (Tan et al. 2021). Eleven bicyclic guaiane sesquiterpenoids (oxytropiols A-K) were isolated from the notorious locoweed endophytic fungus *Alternaria oxytropis* (Tan et al. 2019, 2021). Englerin A is a guaiane sesquiterpene with potent and selective growth inhibition activity against six human renal cancer cell line (Abu-Izneid et al. 2020). Thus, suberosenone, which was a relatively potent cytotoxic sesquiterpene from marine-derived fungus comprises especially sensitive cytotoxicity toward ovarian, renal, and melanoma lines (Abu-Izneid et al. 2020).

15.3.1.2.3 Tremulanes-Type Sesquiterpenoids

Tremulanes, a class of 5/7-ring-fused sesquiterpenoid, were initially isolated from the wood-decaying fungus *Phellinus tremulae*, and later from the medicinal fungus *Phellinus igniarius*, and *Conocybe siliginea* mushroom (H. P. Chen et al. 2020b). Between 2016 and 2020, over 60 tremulane derivatives were found, more than double the previous number. Most of them were isolated from basidiomycetes *Irpex lacteus* cultures, as shown in Fig. 15.4. All the tremulanes isolated from 2016 to 2020 were derived from fungi, except one derivative, which was obtained from a traditional Chinese medicine tabasheer (Shi et al. 2021). Phellinignins A, B, and C (tremulane sesquiterpenoids) were isolated from *Phellinus igniarius*, which displayed cytotoxicity activity in three human cancer cell lines (Wu et al. 2020).

15.3.1.2.4 Bisabolane-Type Sesquiterpenoids

Bisabolenes are produced from farnesyl pyrophosphate (FPP) and they are produced by various fungi such as *Fusarium verticillioides*, *Synechococcus* sp. PCC 7002, and *A. sydowii* (W. Y. Zhao et al. 2021b). Phenolic bisabolane sesquiterpenoids (Fig. 15.5) are a rarely found family of natural molecules produced by different organisms including sponges, coral, fungi, and plants (Y. Chen et al. 2021c). Structurally, phenolic bisabolanes featured a para-alkylated benzene ring skeleton incorporating an eight carbons side chain (from C-7 to C-14) (Y. Chen et al. 2021c). The structural variability of the phenolic bisabolanes is usually caused by oxidation, reduction, esterification, or cyclization occurring at various carbon positions along the alkyl side chain to yield alcohol, carboxylic acid, double bond, lactone, furan, and pyran functionalities (Y. Chen et al. 2021c; Niu et al. 2020b). Fungal-derived phenolic bisabolanes have been demonstrated a diversity of biological activities,

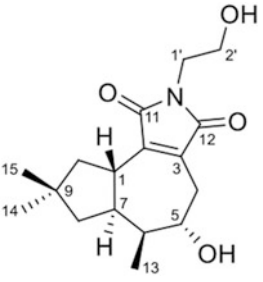

Fig. 15.4 Irpexolactins A: tremulane-type sesquiterpenoid isolated from *Irpex lacteus* (H. P. Chen et al. 2020b)

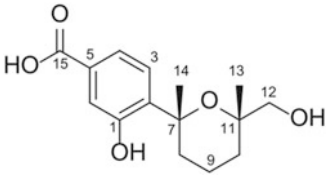

Fig. 15.5 12-Hydroxysydowic acid: bisabolane-type sesquiterpenoid isolated from *Aspergillus versicolor* (Li et al. 2021)

including antimicrobial, cytotoxicity, and anti-inflammatory (Y. Chen et al. 2021c; Niu et al. 2020b).

15.3.1.2.5 Trichothecenes-Type Sesquiterpenoids

Since the first natural trichothecene, trichothecin, was isolated from the fungus *Trichothecium roseum* in 1948, a large number of trichothecene derivatives have been obtained from various fungal species (Fig. 15.6) (Shi et al. 2020). Trichothecenes are, also, sesquiterpenoids mycotoxins, possessing a tetracyclic framework (two carbon rings and two ether rings) in which the 12,13-epoxy ring seems to be responsible for diverse activities, such as antiviral, antifungal, and antimalarial properties (Shi et al. 2020).

15.3.1.2.6 Triquinane-Type Sesquiterpenoids

Linear triquinanes are a prominent compound class of more than 70 reported examples discovered since 1947. Hirsutanes and capnellenes are the most common scaffolds of the linear triquinane sesquiterpenoids isolated from fungi. Linear triquinanes are categorized according to their ring scaffold with the hirsutanes and capnellenes being the most common ones. Some of them have been reported to have significant biological activities, such as cytotoxic activity (H. X. Liu et al. 2020b). For instance, Cerrenins D (Fig. 15.7) showed cytotoxic activity against SF-268 (human glioma cell line), MCF-7 (human breast adenocarcinoma cell line), NCI-H460 (human non-small cell lung cancer cell line), as well as HepG-2

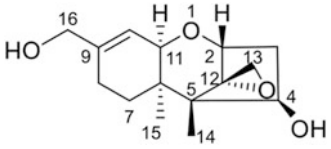

Fig. 15.6 Trichodermarins G: trichothecenes-type sesquiterpenoid isolated from *Trichoderma brevicompactum* (Shi et al. 2020)

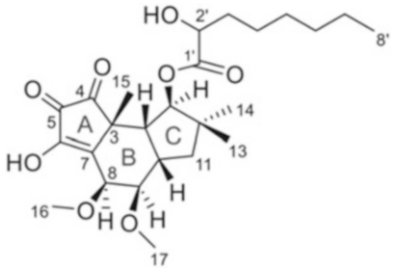

Fig. 15.7 Cerrenin D: triquinane-type sesquiterpenoid isolated from *Cerrena sp.* (H. X. Liu et al. 2020b)

(human liver cell line) cell lines (H. X. Liu et al. 2020b). Thus, many of these compounds displayed other biological activities, including antimicrobial and anti-inflammatory activities (Qiu et al. 2018). Given their structural diversity and complexity, and significant biological activities, linear triquinane sesquiterpenoids have attracted great interest from synthetic chemists.

15.3.1.2.7 Botryane-Type Sesquiterpenoids

Botryanes are a class of fungus-derived sesquiterpene metabolites featuring characteristic bicyclic non-isoprenoid system, with a broad spectrum of biological activities, such as cytotoxicity, phytotoxicity, and antimicrobial properties (Su et al. 2020). For example, Arthrinins E–G (Fig. 15.8), three new sesquiterpenoids possessing non-isoprenoid botryane skeleton, were isolated from the fermentation of *Arthrinium* sp., an endophytic fungus. Presently, fungal endophytes have become an important source for novel and biologically active secondary metabolites, and for these reasons, more studies should be carried out in the future, appreciating fungi as natural sources of synthesis of these bioactive compounds (Tan et al. 2019).

15.3.1.3 Diterpenoids (C20)

Normally, diterpenoids are derived from geranylgeranyl diphosphate (GGPP) with the catalysis of terpene cyclases to form several skeletons with one to four carbon rings (M. Zhang et al. 2020a). Indole diterpenoids are biogenetically derived from tryptophan and GGPP and are one of main compounds described in fungi (Zhou et al. 2021). Dai et al. (2021) described four new indole diterpenoids, named penerpenes K-N (1–4), obtained from the fermentation of *Penicillium* sp. KFD28. Among several other resources, fungal species have an extensive contribution owing to their potential to carry out the bio-transformations and drug synthesis under environmentally acceptable conditions. For instance, hydroxymethylacylfulvene (HMAF) is a semi-synthetic antitumor agent based on the naturally occurring illudin S occurring in the mushroom *Omphalotus olearius.*

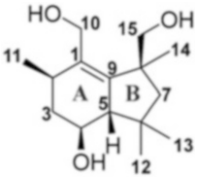

Fig. 15.8 Arthrinin E: botryane-type sesquiterpenoid isolated from *Arthrinium sp.* (Su et al. 2020)

15.3.1.3.1 Fusicoccane Diterpenoids

Fusicoccane diterpenoids are a promising but insufficiently explored family of diterpenoids with the classic dicyclopenta [a, d]cyclooctane (5-8-5-fused ring system) skeleton, which have attracted a great deal of attention due to their diverse structures and biological properties (M. Zhang et al. 2020a). Fusicoccanes are potent phytotoxins known to be synthesized by a few numbers of fungal species (De Boer and Vries-van Leeuwen 2012). In recent years, significant efforts have been devoted to the chemical analysis of these compounds. The isolation of new fusicoccane diterpenoids has resulted in the identification of alternative ring systems (5-9-5, 5-9-4 and 5-8-6). Talaronoids A–D (Fig. 15.9) isolated from the fungus *Talaromyces stipitatus* represent a new class of fusicoccane diterpenoids, being the first examples of natural products identified with an unprecedented benzo[a] cyclopenta[d] cyclooctane (5-8-6-fused) tricyclic carbon skeleton. These compounds have moderate *in vitro* butyrylcholinesterase inhibitory activity (M. Zhang et al. 2020a).

15.3.1.4 Sesterterpenoids (C25)

Sesterterpenes are terpene molecules containing a C25 skeleton, which are rare among terpene compounds. The sesterterpenoid framework derived from geranylfarnesyl diphosphate (GFPP) is among the rarest framework, with little more than 1000 known compounds. Many of these compounds are described in marine fungi, especially those from mangroves, which include neomangicols A–C and mangicols A–G from the mangrove fungus *Fusarium* sp., a phytopathogenic fungi described as a promising source for new drug discovery (Lu et al. 2021). Also, asperterpenoid A, a novel sesterpenoid isolated form *Arpergillus* sp. is an inhibitor of *Mycobacterium tuberculosis* protein tyrosine phosphatase. Nowadays, an increasing number of sesterterpenoids have shown several biological functions, including anticancer, anti-inflammatory, antimicrobial, and antitubercular activities (Wang et al. 2021). For instance, *Fusarium solani* is capable of causing disease in many agriculturally important crops, however it has the ability to produce cyclooxygenase-2 (COX-2) inhibitor fusopoltide A (Chen et al. 2018) and antitumor naphthoquinone compound solaninaphthoquione (Tadpetch et al. 2015). Renner et al. (2000) focused on marine *Fusarium* species to mine novel bioactive secondary metabolites. Endophytic fungi, such as *Aplosporella javeedii*, have been proven to be important sources for bioprospecting for new pharmaceutical lead compounds, including

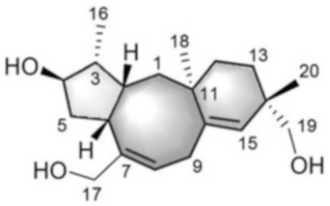

Fig. 15.9 Talaronoid A: fusicaccane-type diterpenoid isolated from *Talaromyces stipitatus* (M. Zhang et al. 2020a)

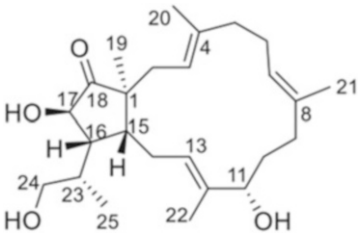

Fig. 15.10 Terpestacin B: sestertepenoid isolated from *Aplosporella javeedii* (Gao et al. 2020)

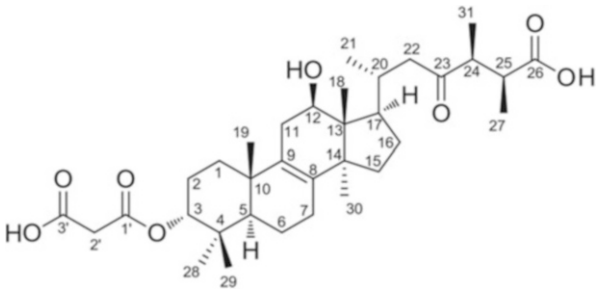

Fig. 15.11 Fomitopsin D: lanostane-type triterpenoid isolated from *Fomitopsis feei* (Isaka et al. 2017)

terpestacins sestertenoids (Fig. 15.10) (Gao et al. 2020). Neomangicols, halogenated sesterterpenoids with cytotoxic activity, and mangicols A−G, sesterterpene polyols with remarkable anti-inflammatory activity, were identified from *Fusarium heterosporum*.

15.3.1.5 Triterpenoids (C30)

Naturally occurring triterpenoids are represented by more than 100 various types of skeletons (Kumar and Dubey 2019). Natural triterpenoids are of interest for researchers due to their availability and multiple biological activities, including antimicrobial, anti-inflammatory, antitumor, cytotoxic, hepatoprotective, among other activities (Feng et al. 2018). Today, pentacyclic triterpenoids and their natural derivatives are mainly obtained by extraction from plant sources. However, the extraction and separation of these compounds (using organic solvents) are extremely labor-intensive, and energy- and time-consuming. Besides, most of pentacyclic triterpenoids are found in very low concentrations in plants, entailing the use of huge amounts of plant raw materials and the formation of waste biomass in large volumes (Luchnikova et al. 2020). An alternative source of pentacyclic triterpenoids seems to be highly efficient to obtain from mycelial fungi, commonly known as mushrooms. Three 24-methyl-lanostane triterpenoids, fomitopsins D–F (Fig. 15.11), were isolated from fruiting bodies of the basidiomycete *Fomitopsis feei* (Isaka et al. 2017).

15.3.1.5.1 Meroterpenoids

The term meroterpenoid was first described in 1968, to characterize natural products of mixed biosynthetic origin which are partially derived from terpenoids. In fact, the prefix mero- (from Greek *merus*) means "part, partial or fragment" and, therefore, a number of secondary metabolites can be described as meroterpenoids. *Hericium erinaceum* (Bull.: Fr.) Pers. (Hericiaceae) is known as an edible mushroom. Its chemical composition has been previously investigated and shown to include meroterpenoids some of which, hericenone A, were found to have significant cytotoxicity against tumor cells and to stimulate activity of the synthesis of the nerve growth factor (Kim et al. 2012; Yaoita et al. 2005). Nine shikimate-terpenoids were isolated from solid culture of the fungus *Guignardia mangiferae*, an endophyte obtained from the leaves of *Dendrobium nobile*. These shikimate-terpenoids exhibited inhibition of nitric oxide production (K. Chen et al. 2021a).

Fang et al. (2021) isolated the meroterpenoid Terretonin F from *Aspergillus* sp., a fungus derived from the red alga *Rhodomela confervoides*, and demonstrated that it inhibited the growth of three harmful microalgae (bloom-forming): *Prorocentrum donghaiense*, *Heterosigma akashiwo*, and *Chattonella marina*. Recently, Jiang et al. (2021b) isolated, for the first time, picoline-derived meroterpenoids possessing a pyrano[3,2-*c*]pyridinyl-γ-pyranone skeleton, possibly deriving from lysine-terpenoid hybrid biosynthetic pathway. These meroterpenoids, amphichoterpenoids A-C, produced from the ascidian-derived fungus *Amphichorda felina* exhibited moderate inhibitory activity against acetylcholinesterase, a promising therapeutic target in Alzheimer's disease.

15.3.1.5.2 Andrastin-Type Meroterpenoids

Andrastin-type meroterpenoids are derived from 3,5-dimethylorsellinic acid (DMOA) and farnesyl diphosphate (FPP), characterized by a five-methyl substituted ent-5α,14β-androstane skeleton (6,6,6,5-tetracarbocyclic skeleton) (X. Cheng et al. 2020a). Ren et al. (2021) described three new andrastin-type meroterpenoids penimeroterpenoids A–C (Fig. 15.12) from marine-derived *Penicillium* species

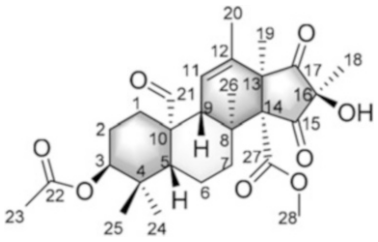

Fig. 15.12 Penimeroterpenoid A: andrastin-type meroterpenoid isolated from *Penicillium sp.* (Ren et al. 2021)

(sp.). Thus, marine-derived fungi, living under extreme environmental conditions such as high salinity, intensely high pressure, absence of sunlight, and deficiency of nutrients, are a new reservoir of structurally diverse and biologically active metabolites for drug discovery. According to Ren et al. (2021), meroterpenoids penimeroterpenoids showed moderate cytotoxicity against A549, HCT116, and SW480 cell lines. Their results suggested that the fungal genus *Penicillium* is a rich source of bioactive secondary metabolites, and thus worthy of in-depth investigations.

15.4 Collection, Fermentation, and Extraction

Currently, the extraction of secondary metabolites is emphasized in ecological research, i.e. quantitative methods are often used to measure the total content of metabolite groups. Throughout this chapter, different groups of terpenes have been described. In fact, it is pertinent to emphasize the importance of the synthesis sources of these compounds, as well as to relate the differences in their chemical structures, to understanding the extraction methods and, consequently, the real identification of each compound. Briefly, on this sub-chapter, we describe the methods used for the characterization of terpenes in fungi. Marine sediments are the main source of terpene-producing fungi. Fungi isolated from marine sponges, corals, marine algae, rhizosphere, plant leaves are also common (Fang et al. 2021; Gao et al. 2021; Pan et al. 2021; Zhou et al. 2021). The fungi collection is a critical step. It is occasionally essential to sample a little bit of support material (e.g., a piece of sponge, coral, sediment, water, or elsewhere) in order to bring viable fungus to the laboratory (Duarte et al. 2012). The identification of the fungus can be performed by morphological and molecular phylogenetic (rDNA, 16S rDNA sequences) techniques (Cao et al. 2020). Following collection, the fungi are cultured on a growth medium, most commonly potato dextrose agar, to create seed cultures (M. Liu et al. 2021a, 2021b). In several research, fungi are cultured for 5 to 8 days at a temperature of 25 to 28 °C, with or without agitation. Afterwards, the fungus cultures are put on a suitable fermentation medium to produce bioactive secondary metabolites (terpenes, polyketides, alkaloids). Solid rice medium is the most used fermentation medium followed by potato dextrose broth, potato dextrose agar, and other formulations. Liquid media containing glucose, yeast extract, corn flour, maltose, mannitol, peptone, and seawater (for marine fungi) are also employed in certain research (Pan et al. 2021; Zhou et al. 2019). Fermentation can last between 21 and 45 days and can occur in either agitated or static conditions. Regarding the fermentation temperature, this can occur at room temperature or range between 25 and 30 °C. After fermentation, cultures of fungi (mycelium and broth) are subjected to process extraction using solvents of different polarities: butanol, chloroform, ethyl acetate, acetone, methanol, ethanol, water, or a proper combination of these solvents (Perera et al. 2020; Antipova et al. 2019; Duarte et al. 2012). Ethyl acetate is the universal organic solvent with medium polarity and minimum toxicity on test strains

(Mohamed et al. 2021). Ultrasonication can occasionally be used to help with solvent extraction (Wang et al. 2021). The organic layer is then dried and concentrated using anhydrous sodium sulfate, vacuum, reduced pressure, and/or lyophilization (Fang et al. 2021; Perera et al. 2020). As bioactive fungi extracts are a complex mixture of different classes of compounds with unknown chemical properties, it is hard to use a standard approach to separate the components in the extract. Fractionation using organic solvent can provide a wide separation of the mixture, producing fractions with different polarities: water-soluble fraction (alkaloids, shikimates, polyketides, amino acids); medium polarity fraction (peptides); and low polarity fraction (hydrocarbons, fatty acids, terpenes). Depending on the polarity of the bioactive fraction, these fractions are further fractionated and purified by column chromatography using a variety of solvent systems and stationary phases. These methods are time-consuming and involve the use of huge volumes of organic solvents. Biological assays are used to determine the bioactivity of extracts, fractions, and purified compounds during the screening process for bioactive compounds. Following the isolation of a pure compound, the elucidation of its chemical structure can be approached, which is a challenging task (Duarte et al. 2012). NMR, MS, and FTIR are some of the techniques used to characterize bioactive compounds (Cui et al. 2020; Liang et al. 2021; Tang et al. 2021). In summary, there is no specific extraction technique for terpenes or other bioactive compounds, owing to the existence of several bioactive chemicals in the fungi extract and their tight association (Duarte et al. 2012).

15.5 Biological Properties of Terpenes Present in Fungus

Extensive biological investigations have been carried out within the group and these studies have revealed a broad spectrum of pharmacological and physiological properties, some of which have led to several terpenoids gaining medicinal application. Nevertheless, biological and ecochemical functions of terpenes have not yet been fully investigated. In an attempt to relate the biological properties of the terpenes present in fungi, Table 15.2 summarizes the major terpene compounds described in fungi, and their biological properties.

15.6 Conclusion

The need to discover new drugs implies resorting to new strategies for the recognition of new natural products. Recently, poorly tapped biological resources, including rare actinobacteria, endophytic fungi, and even phytopathogenic fungi, have been explored as innovative resources for discovering novel natural products with promising bioactivity.

Table 15.2 Main terpenes compounds described in fungi, and their biological properties

	Terpene	Fungi	Phylum	Source	Bioactivity	Reference
Monoterpenoids	Aspermonoterpenoid A and B	*Aspergillus sydowii*	Ascomycota	Deep-sea sediment (South Atlantic Ocean)	NO inhibitory activity	Niu et al. (2020a)
	Aspergerthinol A and B	*Aspergillus* sp.	Ascomycota	*Cynanchum bungei* (rhizosphere soil)	NO inhibitory activity	Pan et al. (2021)
Sesquiterpenoids	Flavilane A and B	*Aspergillus flavipes*	Ascomycota	Fresh seawater (coastal zone of Yantai, China)	Antimicrobial activity; cytotoxicity against MKN-45 and HepG2 cell lines	Y. Chen et al. (2021c)
	Septeremophilane D-F	*Septoria rudbeckiae*	Ascomycota	–	Antibacterial activity; potent inhibition of NO generation in LPS-activated BV-2 microglial cells	Li-Bin et al. (2021)
	(4S,5R,9S,10R)-11,13-dihydroxy-drim-7-en-6-one	*Penicillium citrioviride*	Ascomycota	Hydrothermal vent sediment (Taiwan)	Anti-inflammatory activity; strong α-glucosidase inhibitory effect	Gou et al. (2021)
	5-hydroxyepicyclonerodiol oxide and 4-hydroxyepicyclonerodiol oxide	*Trichoderma hamatum*	*Trichoderma*	*Grateloupia sp.*	Antibacterial activity against *V. anguillarum, V. harveyi, V. parahaemolyticus,* and *V. splendidus;* inhibitory activity against *Chattonella marina.*	Ma et al. (2021)
	Sterpurol D and E	*Cryptomarasmius aucubae*	Basidiomycota	Lichen	Anti-neuroinflammatory activity	Zhai et al. (2021)
	Asperienes A-D	*Aspergillus flavus*	Ascomycota	Marine sediment (Bohai Sea)	Cytotoxicity against HeLa, MCF-7, MGC-803, and A549 cell lines	Y. F. Liu et al. (2019b)
	Septeremophilane D and E	*Septoria rudbeckiae*	Ascomycota	*Karelinia caspia*	Anti-neuroinflammatory activity	Lin et al. (2021)

	Trichodermarin G-I, L and M	*Trichoderma brevicompactum*	Ascomycota	*Chondria tenuissima*	Antifungal activity against phytopathogenic fungi	Shi et al. (2020)
	Asperbisabolane L	*Aspergillus sydowii*	Ascomycota	Deep-sea sediment (South Atlantic Ocean)	Anti-inflammatory activity by inhibiting the NF-κB-activated pathway	Niu et al. (2020b)
	Pestalotiopsin C	*Pestalotiopsis hainanensis*	Ascomycota	*Taxus chinensis* (leaves)	Inhibitory activity against HL-60 and THP-1 cell lines	Y. Zhang et al. (2020a)
	Rhizoperemophilanes E, F, L, M, K, and N	*Rhizopycnis vagum*	Ascomycota	–	Antibacterial activity; cytotoxicity against NCI-H1650 and BGC823 tumor cells; strong phytotoxic activity against the radicle elongation of rice seedlings	A. Wang et al. (2020a)
	Phomoterpenes A and B	*Phomopsis prunorum*	Ascomycota	*Hypericum ascyron* (leaves)	Moderate antibacterial activity against plant pathogenic bacteria	Qu et al. (2020)
	Zopfiellins C	*Zopfiella* sp.	Ascomycota	*Actinidia chinensis*	Cytotoxicity against the A549, MCF-7, and Hela cell lines	Sun et al. (2020)
	Sterostrein H, P, Y	*Stereum complicatum*	Basidiomycota	*Brassica rapa* (leaves)	Phytotoxic activity against *Agrostis stolonifera* and *Lemna paucicostata*; antifungal activity against *Botrytis cinerea, Colletotrichum fragariae,* and *C. acutatum*	Perera et al. (2020)
	Chermesiterpenoid B and C	*Penicillium chermesinum*	Ascomycota	*Pterocladiella tenuis*	Potent activity against human and aquatic pathogenic bacteria and plant pathogenic fungi	Hu et al. (2020)
	Trichodermaloids A and B	*Trichoderma sp.*	Ascomycota	*Dysidea sp.*	Cytotoxicity against human NCIH-460, NCIC-H929, and SW620 cell lines	Cui et al. (2020)

(continued)

Table 15.2 (continued)

	Terpene	Fungi	Phylum	Source	Bioactivity	Reference
	(10E)-isocyclonerotriol	*Trichoderma citrinoviride*	*Ascomycota*	*Laurencia okamurai*	Inhibitory activity against marine phytoplankton species that can cause red tides	Liu et al. (2020a, 2020b)
	Purpurides E-G	*Penicillium minioluteum*	Ascomycota	–	Antimicrobial activity against *Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans*; antiproliferative activities against U251 and U87MG cell lines	Ma et al. (2020)
	12-Hydroxysydowic acid	*Aspergillus versicolor*	Ascomycota	Deep-sea sediment	Antibacterial activity against *Aeromonas hydrophila, E. coli, Edwardsiella tarda,* and *Vibrio harveyi*	Li et al. (2021)
Diterpenoids	Conidiogenone C	*Penicillium* sp., strain	Ascomycota	Deep ocean sediment	Cytotoxicity against the HL-60, A-549, BEL-7402, and MOLT-4 cell lines	Du et al. (2009)
	Hypoxyterpoids A	*Hypoxylon* sp.	Ascomycota	*Bruguiera gymnorrhiza*	Moderate α-glucosidase inhibitory activity	Hou et al. (2021)
	Ascandinine C and D	*Aspergillus candidus*	Ascomycota	Sponge (Pulitzer Bay)	Anti-influenza virus activity; cytotoxicity against HL-60 cell line	Zhou et al. (2021)
	9-epipaxilline and penerpene J	*Penicillium sp.*	Ascomycota	*Meretrix lusoria*	Inhibitory activities against protein tyrosine phosphatase	M. Y. Chen et al. (2021b)
	Talaronoids A-D	*Talaromyces stipitatus*	Ascomycota	Soil (Beijing)	Moderate butyrylcholinesterase inhibitory activity	M. Zhang et al. (2020b)
	Botrysphins G and H	*Botryosphaeria laricina*	Ascomycota	*Rhodobryum umgiganteum*	NO inhibitory activity; quinone reductase inducing activity	H. Yang et al. (2020b)

	Penicichrysogene A	*Penicillium chrysogenum*	Ascomycota	*Huperzia serrata*	Inhibitory activity on ATP release of thrombin-activated platelets	Qi et al. (2020)
	Sarcosenone A	*Sarcosomataceae sp.*	Ascomycota	*Everniastrum sp.*	Moderate cytotoxicity against the MCF-7, HeLa, HepG2, and 786-O cell lines	Hou et al. (2020)
	Xylarilongipins A	*Xylaria longipes*	Ascomycota	*Fomitopsis betulinus*	Moderate inhibitory activity against the cell proliferation of ConA-induced T LYM and LPS-induced B LYM	H. P. Chen et al. (2020a)
	Xylarinorditerpenes B-E, I, and N	*Xylaria longipes*	Ascomycota	*Fomitopsis betulinu*	Cytotoxicity against the cell proliferation by ConA-induced T LYM and LPS-induced B LYM	H. P. Chen et al. (2020c)
	Emindole SB	*Penicillium javanicum*	Ascomycota	Mangrove rhizosphere soil (Hainan Island)	Antifungal activity against Rhizoctonia *solani*, *R. cerealis, Gaeumannomyces graminis,* and *Alternaria alternata*	Liang et al. (2020)
	Stercorin A	*Cyathus stercoreus*	Basidiomycota	–	Neurotrophic activity in PC-12 cells; anti-neuroinflammatory activity	Yin et al. (2021)
	Libertellenone T	*Phomopsis sp.*	Ascomycota	–	Anti-inflammatory activity against the production of IL-1β and IL-6 induced by LPS in macrophages	Xu et al. (2019)
Sesterterpenoids	12-deoxy-16S-hydroxypeniroquesine A	*Penicillium roqueforti*	Ascomycota	*Aconitum vilmorinianum* (root)	Cytotoxicity against the MCF-7 cell line; anti-inflammatory activity	Wang et al. (2021)
	Asperophiobolin H	*Aspergillus sp.*	Ascomycota	*Kandelia candel* (leaves)	Inhibitory activity against LPS-induced NO production	Cai et al. (2019)

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Table 15.2 (continued)

	Terpene	Fungi	Phylum	Source	Bioactivity	Reference
	(5S,6S)-16,17-dihydroophiobolin H	*Aspergillus insuetus*	Ascomycota	Cold seep sediment (South China Sea)	Antibacterial activity	Chi et al. (2020)
	Fusaproliferin	*Aplosporella javeedii*	Ascomycota	*Orychophragmus violaceus* (stem tissue)	Moderate cytotoxicity against the mouse lymphoma cell line L5178Y	Gao et al. (2020)
	Asperunguisin C	*Aspergillus unguis*	Ascomycota	*Xanthoria* sp.	Cytotoxicity against the human cancer cell line A549	Y. L. Li et al. (2019b)
	Bipolaricins B and C	*Bipolaris sp.*	Ascomycota	Wheat (leaves)	Significant inhibitory potency against LPS-induced NO production	M. Liu et al. (2019a)
Triterpenoids	Pericinone A and B	*Periconia sp.*	Ascomycota	*Rosa chinensis* (leaves)	Moderate anti-inflammatory activity against the NO	Gao et al. (2021)
	Asperfumins A and B	*Aspergillus fumigatus*	Ascomycota	*Cleidion brevipetiolatum* (root)	Cytotoxicity against the A549, HGC-27, and H1975 cell lines	L. Liu et al. (2021a)
	Ganodapplanoic acid I	*Ganoderma applanatum*	Basidiomycota	–	Repressed adipogenesis through downregulating the protein expression (PPARγ, CEBPβ, and FAS)	Su et al. (2021)
	Inonotsutriol A	*Inonotus obliquus*	Basidiomycota	–	Neuroprotective activity	Zou et al. (2020)
	Fomitopsins D–F	*Fomitopsis feei*	Basidiomycota	–	Antibacterial activity against *Bacillus cereus*; antiviral activity against herpes simplex virus type 1	Isaka et al. (2017)

	Integracides F and G	*Fusarium sp.*	Ascomycota	*Mentha longifolia* (roots)	Potent cytotoxicity toward BT-549 and SKOV-3 cell lines; anti-leishmanial activity	Ibrahim et al. (2016)
	17(21)-hopene-6α,12β-diol	*Aschersonia paraphysata*	Ascomycota	Scale insect (Hemiptera)	Antimalarial activity	Isaka et al. (2010)
Meroterpenoids	Talaromynoid G–I	*Talaromyces purpureogenus*	Ascomycota	Soft coral (Nanshan Islands)	Activity of reducing triglycerides in 3 T3-L1 adipocytes	Huang et al. (2021)
	Bipolarinoids B and D-F	*Bipolaris zeicola*	Ascomycota	Soil (Mo Mountain of East Lake)	Cytotoxic and immunosuppressive	M. Liu et al. (2021a)
	Mangnardones B, F, I	*Guignardia mangiferae*	Ascomycota	*Dendrobium nobile* (leaves)	Strong anti-inflammatory activity against the NO	K. Chen et al. (2021a)
	(−)-cochlearols B	*Ganoderma cochlear*	Basidiomycota	Commercially acquired	Strong inhibitor of p-Smads, exhibiting renoprotective activity; antifibrotic	Dou et al. (2014)
	Δ^{12}–19-dehydroxy-arthripenoid A and 12,19-didehydroxy-arthripenoid A	*Bipolaris zeicola*	Ascomycota	Soil (Mo Mountain of East Lake)	Immunosuppressive effect against ConA-induced T LYM proliferation; moderate cytotoxicity	M. Liu et al. (2021b)
	Aspergillactone	*Aspergillus sp.*	Ascomycota	Marine sediment (East China Sea)	Antimicrobial activity against *Helicobacter pylori* and *S. aureus*	Cen et al. (2021)
	Terretonin F	*Aspergillus* sp.	Ascomycota	*Rhodomela confervoids*	Inhibitory activity against *Prorocentrum donghaiense*, *Heterosigma akashiwo*, and *Chattonella marina*	Fang et al. (2021)
	Amphichoterpenoids A–C	*Amphichorda felina*	Ascomycota	*Styela plicata*	Moderate inhibitory activity against acetylcholinesterase	Jiang et al. (2021b)

(continued)

Table 15.2 (continued)

	Terpene	Fungi	Phylum	Source	Bioactivity	Reference
	Penimeroterpenoids A	*Penicillium sp.*	Ascomycota	Deep-water sediment (East Pacific)	Moderate cytotoxicity against A549, HCT116, and SW480 cell lines	Ren et al. (2021)
	Talaromyolide K	*Talaromyces purpureogenus*	Ascomycota	*Grateloupia filicina*	Antiviral activity against pseudorabies virus	Cao et al. (2020)
	Tricycloalternarenes X	*Alternaria* sp.	Ascomycota	*Callyspongia* sp.	Cytotoxicity against the HL-60 and HO8910 cells	L. Wang et al. (2020b)
	Furanasperterpenes A	*Aspergillus terreus*	Ascomycota	*Onchidium struma*	Hypolipidemic activity	Tang et al. (2020)
	Austalide Y	*Penicillium thomii*	Ascomycota	Deep-sea water (Yap Trench)	Inhibitory activity against α-glucosidase	Z. Cheng et al. (2020b)
	Phomeroids A and B	*Phomopsis tersa*	Ascomycota	Deep-sea sediment	Cytotoxicity against human SF-268, MCF-7, HepG-2, and A549 cell lines	S. Chen et al. (2020a)
	Photeroids A and B	*Phomopsis tersa*	Ascomycota	Deep-sea sediment	Moderate cytotoxicity against the SF-268, MCF-7, HepG-2, and A549 cell lines	S. Chen et al. (2020b)
	Austalides V and W	*Aspergillus ustus*	Ascomycota	Stone (Moscow Kremlin)	Inhibit proliferation and migration of cancer cells; suppresses activity of key signaling pathways regulating cancer cell growth	Antipova et al. (2019)

NO, nitric oxide; ConA, concanavalin A; LPS, lipopolysaccharide; LYM, lymphocytes; A549, NCIH-460, NCI-H1650, NCI-H1975: lung cancer cell lines; MCF-7, BT-549: breast cancer cell lines; HeLa: cervical carcinoma cancer cell lines; NCIC-H929: myeloma cell line; SW620: colorectal cancer cell line; U251, U87MG, SF-268: glioblastoma cell lines; HepG2, BEL-7402: hepatocellular carcinoma cell lines; 786-O: renal cell adenocarcinoma cell line; L5178Y: mouse lymphoma cell line; SKOV-3, HO8910, HO-8910: ovarian cancer cell lines; HCT116, SW480: colon cancer cell lines; HL-60, THP-1, MOLT-4: leukemia cell lines; MKN45, BGC823, HGC-27: gastric cancer cell lines

In summary, over 100 novel terpenes from 27 genera of fungi were delineated, along with their diverse biological properties. The most investigated genera were *Aspergillus* and *Penicillium*, both of which belong to the phylum Ascomycota. Terpene-producing fungi were found in a variety of habitats, including sponges, mushrooms, and molluscs, although the predominant habitats were marine plants and sediments. In terms of terpene classes, sesquiterpenes and meroterpenes were the most isolated in fungi, with monoterpenes being the least isolated. Terpenoids, regardless of class, have demonstrated potential cytotoxic action against a wide range of cell lines, including colon, ovarian, lung, and breast cancer cell lines. Terpenes also frequently displayed anti-inflammatory, antibacterial, and antifungal action, as well as enzyme inhibitory activity. Thus, these secondary fungi metabolites with a high structural diversity may be useful in both medicine and agriculture. Terpenes isolated from endophyte fungi can protect their host plants from phytopathogenic fungi, and consequently avoid huge food losses in agricultural fields. However, as fungi produce a limited quantity of terpenes, it is critical to investigate techniques for increasing these beneficial compounds. Fungi terpene research provides bioactive candidates for the development of drugs or agrochemicals, but it can also be valuable for the nutraceutical and functional food industry. Incorporating mycelium or fruiting bodies of terpene-producing fungi can help to update existing products while also benefiting health. The research of fungi and their secondary metabolites is a never-ending topic that has the potential to be investigated in a wide range of industrial applications.

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Chapter 16
Alkaloids from Fungi

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Abstract The prospection of unusual sources and undiscovered habitats is valuable in natural product research. Indeed, the fungi kingdom has received special attention since its ability to produce novel and intriguing secondary metabolites with various

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biological uses. Among secondary metabolites, alkaloid-derived structures present a wide range of bioactivities, including antineurodegenerative, antidepressive, anxiolytic, anti-inflammatory, cytotoxic, and insecticidal properties. Furthermore, various studies showed particular properties of those alkaloids in reducing nicotine addiction and alcohol dependence. Alkaloids are categorized into several groups based on their heterocyclic ring system and biosynthetic precursor, such as indole, isoxazoles, and muscarine. Therefore, this chapter focuses on those fungi's bioactive alkaloids with emphasis on pharmacokinetics as well as the current analytical approaches for extraction and compound identification. Furthermore, the main biological activities and action mechanisms of these fungus alkaloids will also be discussed.

Abbreviations

AD	Alzheimer's disease
EGR 1	Early Growth Response Protein 1
EGR 2	Early Growth Response Protein 2
GABA	Gamma-Aminobutyric Acid
GC-MS	Gas Chromatography with Mass Spectrometry
HPLC-ECD	High-Performance Liquid Chromatography with Electrochemical Detection
HTR	Head Twitch Response
LC	Liquid chromatography
LC-MS	Liquid Chromatography and Mass Spectrometry
LC-MS/MS	Liquid Chromatography and Tandem Mass Spectrometry
LOD	Limit of Detection
MDD	Major Depressive Disorder

16.1 Introduction

A characteristic feature of fungi, plants, and other organisms is their capacity to synthesize an enormous variety of low molecular weight compounds, the so-called secondary metabolites (Heitefuss 2011). Alkaloids' chemistry is one of the most fascinating and important topics in bioorganic chemistry. Alkaloids, unlike other natural compounds, have virtually infinite structural frameworks and contain an *N* atom in their molecules, making them exceedingly diverse (Wink 2010). Based on their heterocyclic ring system and biosynthetic precursor, alkaloids have been classified into diverse categories including indole, isoxazoles, purine, quinoline, isoquinoline, tropane, piperidine, imidazole, among others (Kaur and Arora 2015; Roy 2017). These compounds have been described to play a critical role in biomedical research. As so, depending on the amine functionality, alkaloids can act as either a hydrogen-receptor or a hydrogen-donor for hydrogen bonding. This function is crucial for the interaction between alkaloid scaffold-containing drugs (ligands) and their targets (e.g., enzymes, proteins, and receptors) (Derosa and Maffioli 2014; Kittakoop et al. 2014).

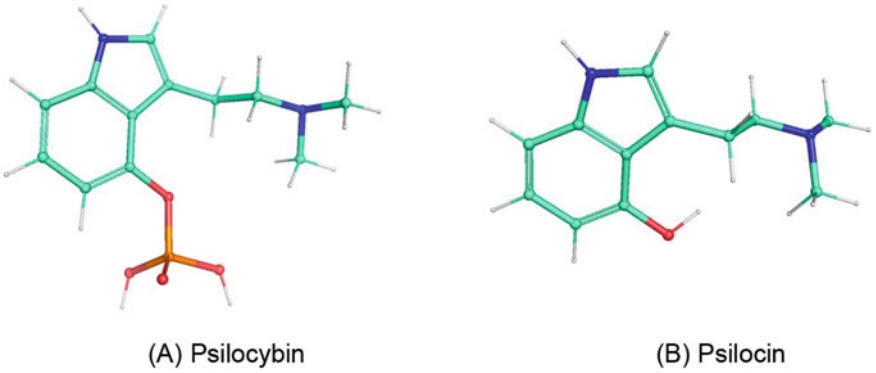

Fig. 16.1 3D structure of (**a**) psilocybin and (**b**) psilocin

Alkaloids derived from macrofungi are mainly divided into two groups: indoles and isoxazoles, which have medicinal and industrial applications (Kaushik et al. 2013). The chemical group of indoles includes the most common heterocyclic compounds, the psilocybin (*O*-phosphoryl-4-hydroxy-*N,N*-dimethyltryptamine) (Fig. 16.1a) and the psilocin (4-Hydroxy-*N,N*-dimethyltryptamine) (Fig. 16.1b), which are found in a variety of psychoactive or hallucinogenic mushrooms (Guzm 2008; Michelot and Melendez-Howell 2003; Nichols 2020). These compounds have been described for their potential to treat several mental and physical conditions, including addiction, anxiety, depression, and post-traumatic stress disorder (Chi and Gold 2020; Davis et al. 2021; Ling et al. 2022). Furthermore, psilocybin analogs, such as baeocystin ([3-[2-(methylamino)ethyl]-1H-indol-4-yl] dihydrogen phosphate), norbaeocystin ([3-(2-aminoethyl)-1H-indol-4-yl] dihydrogen phosphate), bufotenine (N,N-Dimethyl-5-hydroxytryptamine), and aeruginascin ([3-[2-(trimethylazaniumyl)ethyl]-1H-indol-4-yl] hydrogen phosphate), have also been discovered in hallucinogenic mushrooms (Sherwood et al. 2020). *Agrocybe*, *Conocybe*, *Galerina*, *Gymnopilus*, *Hypholoma*, *Inocybe*, *Panaeolus*, *Psilocybe*, *Pholiotina*, *Pluteus*, and *Weraroa* are examples of mushrooms genera whose hallucinogenic compounds have been chemically identified (Wieczorek et al. 2015).

Ibotenic acid (α-amino-3-hydroxy-5-isoxazoloacetic acid) (Fig. 16.2a), muscimol (5-(aminomethyl)-3-hydroxyisoxazole) (Fig. 16.2b), and muscazone (α-amino-2,3-dihydro-2-oxo-5-oxazoloacetic acid) are active components found in hallucinogenic mushroom species belonging to the *Amanita* genus (Michelot and Melendez-Howell 2003). These compounds are from the isoxazole chemical group and have been demonstrated to interact with neurotransmitter receptors in the central nervous system, thus, exhibiting extensive and pharmacologically important biological activities (Michelot and Melendez-Howell 2003; Satora et al. 2005).

Muscarine (2,5-anhydro-1,4,6-trideoxy-6-(trimethylammonio)-D-ribo-hexitol) (Fig. 16.2c), the main alkaloid in the genera *Inocybe* and *Clitocybe*, was initially isolated from the bright orange-cap fungus *Amanita muscaria* (Michelot and Melendez-Howell 2003). Based on genetic and pharmacological characterizations,

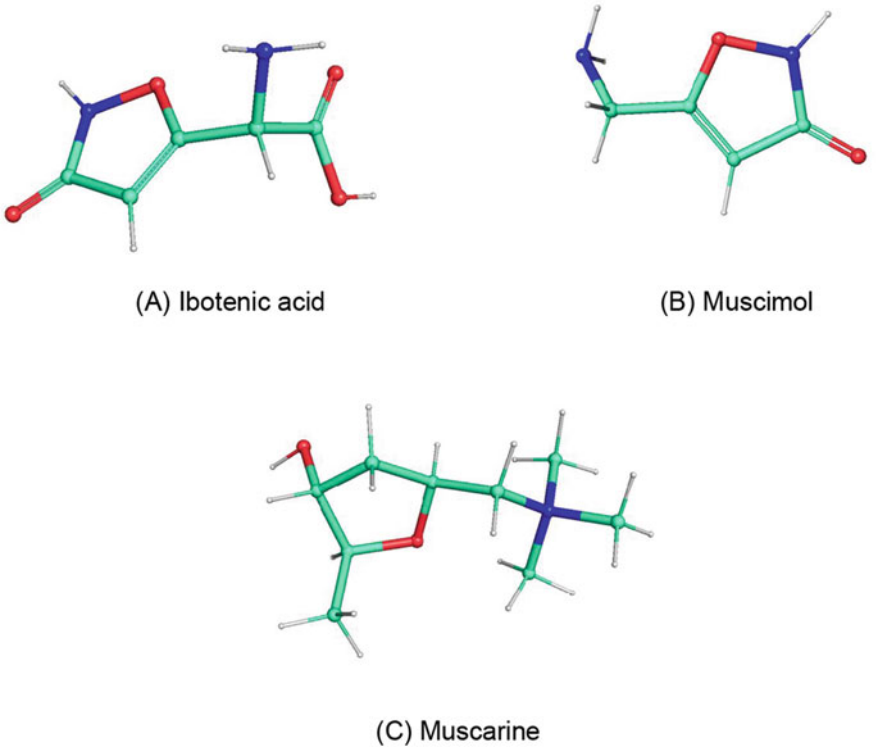

Fig. 16.2 3D structure of (**a**) ibotenic acid, (**b**) muscimol, and (**c**) muscarine

this compound functions as a competitive agonist of the acetylcholine neurotransmitter, being also responsible for psychotropic effects on humans (Jin 2009).

Therefore, the present chapter aimed to review the pharmacokinetics, and analytical methods of alkaloids, and clarify the biomedical relevance of these compounds found in Fungi, particularly in hallucinogenic mushrooms, with a focus on their possible therapeutic activity.

16.2 Indoles

This is the most diverse and intriguing alkaloid family obtained from tryptophan. Simple tryptamine derivatives, carbazoles (alkaloids lacking the ethanamine chain), a variety of alkaloids containing one or more prenyl residues, and others containing normal monoterpenoid or diterpenoid units are all examples of significant alkaloids from this category (Dey et al. 2020). Psilocybin will receive special attention because it has been the subject of extensive research over the previous few decades.

16.2.1 Psilocybin

Hallucinogenic mushrooms that commonly contain psilocybin and psilocin include *Psilocybe* spp., *Panaeolus* spp., and *Stropharia aeruginosa*. The most often ingested *Psilocybe* species are *Psilocybe semilanceata*, *Psilocybe cubensis*, *Psilocybe mexicana*, *Psilocybe bohemica*, and *Psilocybe baeocystis* (Graeme 2014). Psilocybin, the psychoactive compound, was effectively isolated and identified for the first time in 1958 from *P. mexicana* (Heim and Hofmann 1958a,b). Parallel to psilocybin, a second chemical structurally similar but present in traces was isolated and named psilocin. Psilocybin is a white and soft crystal compound that melts at 220–228 °C and is soluble in 20 parts of boiling water or 120 parts of methanol, while in chloroform and benzene is completely insoluble. In methanol, the psilocin forms white crystals that melt around 173–176 °C and is insoluble in water, however, it dissolves in most organic solvents (Andersson and Kristinsson 2009). Ingestion of mushrooms containing psilocybin and psilocin causes psychedelic effects and has become a common type of drug addiction among teenagers and young adults.

16.2.1.1 Pharmacokinetics and Pharmacodynamics

The pharmacokinetics and tissue distribution of psilocybin/psilocin in rats and humans have been extensively studied (Kalberer et al. 1962). Several studies showed evidence that psilocybin is a prodrug that is dephosphorylated to psilocin, a reaction catalyzed by alkaline phosphatase and unspecific esterases of the intestine mucosa (Eivindvik et al. 1989; Horita and Weber 1962; Kalberer et al. 1962). Once absorbed, psilocin is further metabolized in plasma to 4-hydroxytryptophol and 4-hydroxyindole-3-acetic acid (Hasler et al. 1997). Clinical data from healthy volunteers is becoming an increasingly important milestone for psilocybin pharmacokinetic studies. In order to study psilocybin pharmacokinetics, oral doses of 0.2 mg psilocybin per kg body weight (maximum 15 mg per person) were administered to seven healthy volunteers (Lindenblatt et al. 1998). A series of blood samples were taken at specified times and analyzed using a validated high-performance liquid chromatography with electrochemical detection (HPLC-ECD). The psilocybin was rapidly absorbed and dephosphorylated to psilocin since it could be determined in the volunteers' plasma at 15–50 min after administration. Psilocin was still present in the plasma of all volunteers after 360 min. The time of maximum plasma concentration was between 70 and 90 min and the area under the curve was between 20.2 and 40.8 ng h ml^{-1}. The authors of this study referred that the pharmacokinetics results indicated a large inter-individual variation (Lindenblatt et al. 1998). To investigate the elimination kinetics of psilocin a clinical study with eight volunteers was carried out. The participants received psilocybin in psychoactive oral doses of 212 ± 25 μg kg^{-1} body weight and the urine samples were collected 24 h after drug administration. The quantitative analysis was performed using a validated HPLC-ECD method. The results indicated that

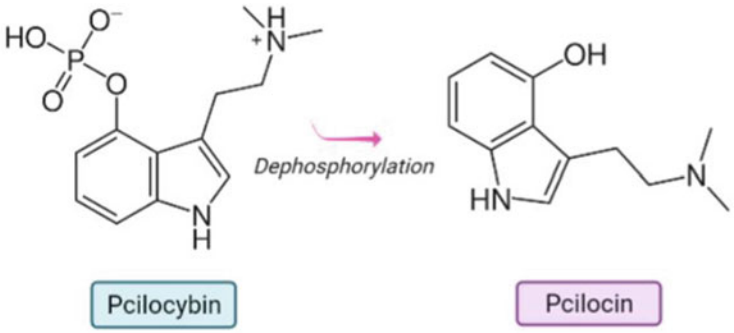

Fig. 16.3 Dephosphorylation of psilocybin (Created with BioRender.com)

3.4 ± 0.9% of the psilocybin administered was excreted as free psilocybin. Several studies have suggested that psilocin is highly excreted in urine as psilocin glucuronide (Grieshaber et al. 2001; Hasler et al. 2002; Kamata et al. 2003; Sticht and Käferstein 2000). To evaluate if psilocin can be excreted in a glucuronidated form, the urine samples of three volunteers were submitted to an enzymatic glucuronide cleavage procedure before analysis. The addition of glucuronidase to urine samples resulted in a twofold increase in psilocin concentrations, suggesting that psilocin is largely excreted as psilocin-*O*-glucuronide in humans (Hasler et al. 2002). These findings were later confirmed by Kamata et al. (2006). Direct analysis by liquid chromatography and mass spectrometry (LC–MS) of serum samples that were taken from a magic mushrooms user 5 h post-ingestion showed that up to 80% of the psilocin was present as the glucuronide conjugate (Kamata et al. 2006). The psilocybin tissue distribution has been analyzed in *vivo* studies. Kalberer et al. (1962) have reported that tissue ^{14}C-psilocin concentration decreases in the order of kidney > liver > brain > blood after intravenous administration of 10 mg kg^{-1} to male rats. These findings were further corroborated by Law et al. (2014). On the other hand, when ^{14}C-psilocin was orally administered at the same concentration (10 mg kg^{-1}) the amount decreases in the order of liver > kidney > adrenal > brain (Kalberer et al. 1962). Psilocin may also bypass the placental barriers of pregnant rats and diffuse swiftly to numerous fetal tissues via passive diffusion (Law et al. 2014).

As above mentioned, psilocybin is rapidly dephosphorylated to the pharmacologically active compound psilocin by alkaline phosphatase and unspecific esterases of the intestine mucosa (Fig. 16.3) (Eivindvik et al. 1989; Horita and Weber 1962; Kalberer et al. 1962).

Psilocybin has been shown that interacts mainly with serotonergic neurotransmission (5-HT$_{1A}$, 5-HT$_{1D}$, 5-HT$_{2A}$, and 5-HT$_{2C}$ receptor subtypes), acting as a serotonin agonist compound. Psilocin presents a high affinity for the 5-HT$_{2A}$ serotonin receptor in the brain and it binds less tightly to other serotonergic receptors 5-HT$_{1A}$, 5-HT$_{1D}$, and 5-HT$_{2C}$, where it mimics the effects of serotonin (Passie et al. 2002). Crystal structure of 5-HT2A complexed with psilocin is demonstrated in Fig. 16.4. Psilocybin through 5-HT$_{2A}$ can activate intracellular signaling pathways

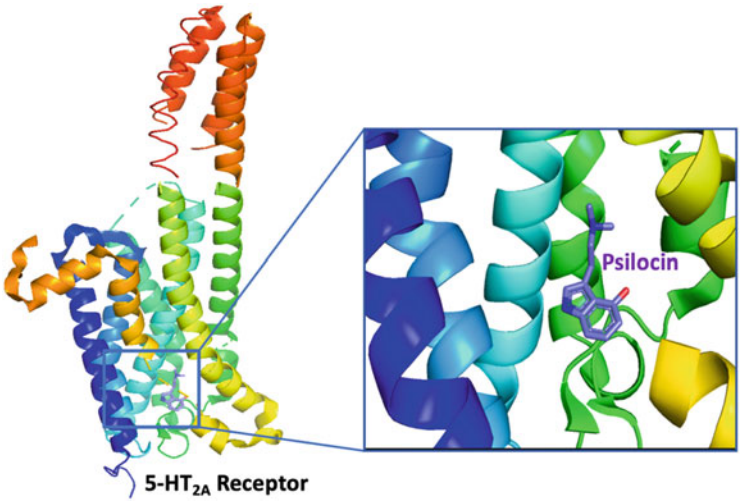

Fig. 16.4 Crystal structure of serotonin 2A receptor in complex with psilocin

in cortical pyramidal neurons, consequently, modulating downstream signaling proteins, such as early growth response protein 1 (EGR1) and EGR2 (González-Maeso et al. 2007). EGR1 has been reported to be controlled by a wide range of environmental events, indicating that it functions as an integrator and mediator of environmental impacts on neuronal activity. EGR1 can change the expression of genes associated with synaptic plasticity level, from vesicular transport and release of neurotransmitters to synaptic architecture, endocytosis, and protein degradation, without taking indirect effectors into account (González-Maeso et al. 2007). Converging lines of evidence indicate that psilocybin's psychotomimetic effects are mediated through the 5-HT_{2A}-receptor activation. Vollenweider et al. (1998) provide compelling evidence that psychosis induced by psilocybin in healthy human volunteers ($n = 15$; 0.25 mg kg^{-1} p.o.) is the result of specific activation of the 5-HT_{2A} subtype of serotonin receptors (Vollenweider et al. 1998). The authors also demonstrated that psilocybin-induced psychosis could be completely prevented by either atypical neuroleptics and mixed 5-HT_2/D_2 antagonist risperidone (1 mg p.o.) or by the 5-HT_2 antagonist ketanserin (20 mg/40 mg p.o.). This finding adds substantial evidence to the view that 5-HT_2 agonism is responsible for the psychological effects of psilocybin and, presumably, other indoleamine hallucinogens. Furthermore, the finding that ketanserin, with about 100-fold greater potency at the 5-HT_{2A} *vs* 5-HT_{2C} receptor, completely blocked psilocybin-induced psychosis which strongly indicates that the effects of psilocybin are mediated by 5-HT_{2A} rather than 5-HT_{2C} receptor activation (Vollenweider et al. 1998). Although the 5-HT_{2A} receptor is responsible for most of the effects of psilocin, various lines of evidence have shown that interactions with non-5-HT_{2A} receptors also contribute to the subjective and behavioral effects of the drug. In fact, the psychotomimetic effects of psilocybin also may

be explained by increased dopamine release through 5-HT receptor activation. An examination of the binding of [^{11}C]raclopride (antagonist of D2-dopamine receptors) to D2-dopamine receptors after placebo and a psychotomimetic dosage (0.25 mg kg^{-1} p.o.) of psilocybin on 7 healthy volunteers assessed the impact of psilocybin. Psilocybin administration produced changes in mood, disturbances in thinking, illusions, elementary and complex visual hallucinations, and impaired ego-functioning. This study demonstrated that psilocybin decreased [^{11}C]raclopride binding in the caudate nucleus and putamen in human subjects, reflecting an increase in striatal dopamine concentration. These findings suggested that stimulation of 5-HT, presumably 5-HT$_{2A}$ and 5-HT$_{1A}$ receptors, can lead to an increase in striatal dopamine efflux which may contribute to the psychotomimetic effects of psilocybin (Vollenweider et al. 1999).

Kometer et al. (2011) used psilocybin (125 and 250 g kg^{-1} vs. placebo) on normal volunteers (n = 17) to study the role of 5-HT$_{2A/1A}$ receptors in visual processing. The authors demonstrated that the 5-HT$_{1A}$ receptor may also play a role in the behavioral effects of the psilocybin (Kometer et al. 2011). Halberstadt et al. (2011) investigated the effects of psilocin administration on head twitch response (HTR), locomotor activity, and investigatory behavior in mice, as well as the role of 5-HT receptors in these behavioral effects (Halberstadt et al. 2011). Consistent with the fact that the HTR serves as a selective assay for 5-HT$_{2A}$ agonist activity in rodents, the authors found that psilocin administered subcutaneously (5 mL kg^{-1} body weight) was not able to induce the HTR in 5-HT$_{2A}$ KO mice. Furthermore, non-5-HT$_2$ receptors may potentially play a role in psilocybin's psychopharmacological activity, in addition to 5-HT$_{2A}$ receptors (Halberstadt et al. 2011; Mahapatra and Gupta 2017). The 5-HT$_{1D}$ and 5-HT$_{2C}$ receptor subtypes have also been involved in the biological activity of psilocybin and psilocin (Passie et al. 2002).

16.2.1.2 Analytical Methods

The Ehrlich test is a preliminary chemical test that may be used to evaluate if psilocybin and/or psilocin are present in a variety of matrices. The sample is treated with 1 w/v % p-dimethylaminobenzaldehyde in 10% HCl. Psilocin is represented by a blue-gray color, whereas psilocybin is represented by a red-brown color (Stuart 2013). However, this color test is not specific to psilocybin since it can also be used for LDS detection (Stuart 2013). Several analytical techniques have been used to quantify psilocybin levels in mushroom samples. The most extensively used method for quantifying psilocybin and psilocin is LC. Although gas chromatography has also been used it cannot be recommended since the high temperature for psilocybin evaporation causes the loss of the psilocybin phosphate group during injection (Elian et al. 2011). In mushrooms samples, all liquid chromatography methods described so far are based on HPLC with different detection methods such as electrochemical, ultraviolet (Wurst et al. 1992), fluorescence (Perkal et al. 1980; Saito et al. 2005), and electrospray ionization mass spectrometry (Saito et al. 2004). Several

chromatographic techniques have also been developed for the analysis of psilocybin and psilocin in biological samples. The REMEDi HS drug profiling system (Bio-Rad) that is based on HPLC proved to be effective for screening psilocin in urine (Sticht and Käferstein 2000). HPLC-ECD has been suitable for the detection of psilocin in human plasma (Lindenblatt et al. 1998) and rat urine (Kysilka 1990), while LC-MS (Kamata et al. 2003) and gas chromatography-mass spectrometry (GC-MS) (Grieshaber et al. 2001) shown to be more efficient for determination of psilocin in human urine.

16.2.1.3 The Biomedical Role of Psilocybin

In 1957, chemist Albert Hofmann discovered psilocybin from *P. mexicana*, and in 1958, it was synthesized for the first time. Pure synthetic psilocybin (Indocybin® Sandoz) had been used and commercialized for research and psychotherapy (Passie et al. 2002). Serotonin receptors are involved in a variety of biological, neurological, and neuropsychiatric processes, including anxiety and aggressiveness, cognition, learning, memory, and appetite (Lowe et al. 2021). Psilocybin is a serotonergic psychedelic molecule that has been used in studying the neurobiological basis of cognition and consciousness, and in psilocybin-assisted therapy for substance addiction, mental disorders, and chronic pain (Lowe et al. 2021). Increases in glial cell-derived neurotrophic factor and brain-derived neurotrophic factor, downregulation of serotonin receptors, changes in pyramidal cell dendritic spine architecture, and normalization of the default mode network are possible mechanisms of action (Shelton and Hendricks 2016).

Psilocybin has been investigated as a tool to treat nicotine addiction (Johnson et al. 2014; Noorani et al. 2018) and alcohol dependence (Bogenschutz et al. 2015). According to Johnson et al. (2014) psilocybin combined with a structured cessation program reduced cigarette smoking among dependent smokers. In all, 15 nicotine-dependent smokers were given moderate and high doses of psilocybin as part of a structured cognitive behavioral therapy program, and at the 6-month follow-up, 80% of smokers were abstinent, which is significantly more than what is expected from existing therapies (35%) (Johnson et al. 2014). In another study, Johnson and Griffiths (2017) found that in long-term follow-up periods, psilocybin, when used in association with a structured treatment program, has significant potential in establishing long-term smoking abstinence. Similarly, a single-group proof-of-concept study in alcohol-dependent participants performed by Bogenschutz et al. (2015) showed that psilocybin administration plus motivational enhancement therapy could reduce alcohol consumption in dependent drinkers. The abstinence increased following psilocybin administration when compared to the first 4 weeks of treatment (when participants had not yet received psilocybin). The percentage of drinking and heavy drinking days decreased significantly after psilocybin administration and remained low for up to 36 weeks (Bogenschutz et al. 2015).

Griffiths et al. (2006, 2008) evaluated psilocybin's psychological effects on healthy participants (Griffiths et al. 2006, 2008). Adults who had never experienced

hallucinogens were administered oral psilocybin or methylphenidate in two or three sessions. They were advised that at least one of the sessions would include a moderate or high dose of psilocybin. The patients showed significantly higher ratings of positive attitudes and mood during the psilocybin sessions compared to the methylphenidate sessions at both two months post-drug sessions and 14 months follow-up; revealing that psilocybin can induce mystically and spiritually experiences and lead to an improvement in well-being among participants. In addition, based on patient interviews, the 14-month follow-up revealed no evidence of deleterious consequences from psilocybin exposure (Griffiths et al. 2008).

With the global rate of mental disorders on the rise, which is being exacerbated by COVID-19, psychedelic-assisted psychotherapies may be able to alleviate some of the issues that traditional psychiatric care is facing (Lowe et al. 2021). According to behavioral and neuroimaging data, psychedelics not only modify brain networks (molecular and neural circuit mechanisms) implicated in mood and affective disorders but also can alleviate clinical symptoms of these illnesses. Psilocybin produces an increase in glucose metabolic rate in the brain, which has been linked to measures of psychological status and is compatible with neurobiological substrates of major mental disorders (Grob et al. 2011; Gukasyan et al. 2022). The effects of psychedelics as rapid-acting antidepressant drugs have been investigated in preclinical murine models. As an example, Hibicke et al. (2020) found that a single administration of psilocybin produces long-lasting antidepressant-like effects in rats (for 5 weeks after administration), in a time- and context-dependent way, using the forced swim test as a primary outcome measure, interpreting these parameters as behavioral despair and passive coping (immobility) (Hibicke et al. 2020). Recent data have shown that psilocybin administration leads to long-lasting modifications to the neural architecture *in vivo*, suggesting these behavioral effects may be due to changes in the density and strength of neuronal connections, promoting rapid structural plasticity in frontal cortex pyramidal neurons (Hesselgrave et al. 2021; Shao et al. 2021).

Furthermore, Hendricks et al. (2015) investigated the link between lifetime psilocybin use and recent psychological distress (Hendricks et al. 2015). Using the National Survey on Drug Use and Health, they also gathered past-year suicidal thinking, suicidal planning, and suicide attempts linked with psilocybin use among the adult population in the USA. Overall, participants were divided into four groups: psilocybin users only, psilocybin and other psychedelics, other non-psilocybin, or no psychedelic use in a lifetime. The odds of the outcomes were reduced in the psilocybin-only group compared to the no psychedelic use group. Past-year suicidal thinking and planning were lower in the psilocybin group compared to the psilocybin and other psychedelics group. These findings support the hypothesis that psilocybin may have a role in preventing suicidality and increasing mood, highlighting the potential safety of the substance in such a large population. Recently, Carhart-Harris et al. (2021) compared psilocybin to escitalopram, a selective serotonin reuptake inhibitor, in a 6-week phase 2 double-blind, randomized, controlled trial, enrolling patients with long-standing, moderate-to-severe major depressive disorder (MDD). A total of 59 patients were included in the study, of whom 30 received

psilocybin and 29 received escitalopram. At baseline, the psilocybin group had a 14.5 mean score, while the escitalopram group had a 16.4 mean score. The 16-item Quick Inventory of Depressive Symptomatology–Self-Report (QIDS-SR-16) was used to determine these scores (QIDS-SR-16; scores ranging from 0 to 27, with higher scores indicating greater depression). However, no significant difference in antidepressant effects between psilocybin and escitalopram was found (Carhart-Harris et al. 2021). In addition, Davis et al. (2021) conducted a randomized clinical experiment in which two psilocybin sessions (session 1: 20 mg.70 kg^{-1}; session 2: 30 mg.70 kg^{-1}) were given to 24 patients with MDD. The authors found that a clinically significant antidepressant response to psilocybin therapy persisted for at least 4 weeks, with 71% of the participants still showing a clinically significant response (≥50% reduction in GRID-Hamilton Depression Rating Scale score), suggesting substantial rapid and enduring antidepressant effects of psilocybin-assisted therapy among patients with MDD (Davis et al. 2021).

Besides the potential to treat mood and anxiety disorders, psilocybin has analgesic benefits, as demonstrated by various clinical trials on the treatment of cluster headache (Schindler et al. 2015; Sewell et al. 2006) and chronic pain (Whelan and Johnson 2018). One hypothesized mechanism of action is the coupling of this analgesic property with nociceptive and antinociceptive pathways (Whelan and Johnson 2018). Cluster headache is one of the most incapacitating pain-related syndromes, and a considerable number of patients are resistant to standard treatments. As so, Schindler et al. (2015) conducted a survey to characterize the effects of both conventional and alternative medications used in cluster headache, indicating that psilocybin's effect was comparable to or more efficacious than most conventional medications, with psilocybin being perceived to shorten/abort the cluster period and bring chronic cluster headache into remission more effectively than conventional therapies. In addition, non-hallucinogenic doses and occasional administration were found to be effective (Schindler et al. 2015). Recently, van Amsterdam and van den Brink (2022) performed a systematic review on the clinical effects of psilocybin in the treatment of a variety of mental disorders. Overall, 488 participants from randomized clinical trials were enrolled, with 333 receiving psilocybin and 155 receiving a placebo. The findings of included studies revealed that psilocybin has a favorable benefit–risk profile in the treatment of a variety of mental diseases, with an immediate and long-lasting effect after 1 to 3 doses (van Amsterdam and van den Brink 2022).

Anxiety and depression are clinically significant disorders that are linked to poor psychological and medical outcomes in cancer patients. As so, psilocybin may also be used in patients with advanced-stage cancer and cancer-related anxiety and depression (Griffiths et al. 2016; Grob et al. 2011). Grob et al. (2011) conducted a double-blind, placebo-controlled crossover research in which 12 patients with advanced-stage cancer were given a moderate dose of oral psilocybin (0.2 mg kg^{-1} oral) and an active placebo (niacin) in consecutive sessions. Despite the limited sample size, the study found nonsignificant trends for psilocybin to reduce depression more than placebo in the two weeks study. Furthermore, anxiety was reduced at

a 3-month follow-up compared to baseline screening, and depression outcomes were considerably reduced at a 6-month follow-up (Grob et al. 2011).

According to Griffiths et al. (2016) study, the effects of psilocybin were analyzed in 51 cancer patients with life-threatening diagnoses and symptoms of depression and/or anxiety. This randomized, double-blind, crossover trial investigated the effects of a very low (placebo-like) dose (1 or 3 mg 70 kg^{-1}) vs. a high dose (22 or 30 mg 70 kg^{-1}) of psilocybin administered for 5 weeks between sessions and a 6-month follow-up. High-dose psilocybin resulted in significant reductions in clinician- and self-reported lowered mood and anxiety, as well as improvements in quality of life, life purpose, and optimism. These effects were maintained at the 6-month follow-up, with >80% of participants endorsing moderately or greater increased well-being/life satisfaction (Griffiths et al. 2016). Similarly, in a double-blind, placebo-controlled crossover study, Ross et al. (2016) randomly assigned 29 patients with cancer-related anxiety and depression to receive treatment with single-dose psilocybin (0.3 mg kg^{-1}) or niacin, both in combination with psychotherapy. The outcomes were assessed between groups before the crossover at 7 weeks. Prior to the crossover, psilocybin led to improvements in anxiety and depression. Also, psilocybin was associated with persistent anxiolytic and antidepressant effects, sustained benefits in existential distress, and quality of life, at the 6.5-month follow-up. In combination with psychotherapy, single moderate-dose psilocybin exhibited fast, robust, and long-lasting anxiolytic and antidepressant effects (Ross et al. 2016).

Swift et al. (2017) evaluated an interview-based study to evaluate the experience of participants who completed a Phase 2 randomized controlled trial of psilocybin treatment for cancer-related psychological and existential distress. This study included 13 participants with clinically elevated anxiety associated with a cancer diagnosis who received a single dose of psilocybin under close clinical supervision. Semi-structured interviews were used to assess the subjective experiences of participants who received psilocybin therapy for cancer-related emotional distress. The authors concluded that psilocybin-assisted psychotherapy has the potential to complement medical and psychological treatment for cancer patients who are experiencing profound psychological and existential suffering as a result of their diagnosis (Swift et al. 2017).

Hallucinogenic alkaloids, particularly psilocybin and psilocin, have become increasingly important in pharmacological and medical research, both for their biological activity and for their role in the development of new medications. However, more research focusing on the molecular and pharmacologic mechanisms of psilocybin is warranted (Fig. 16.5).

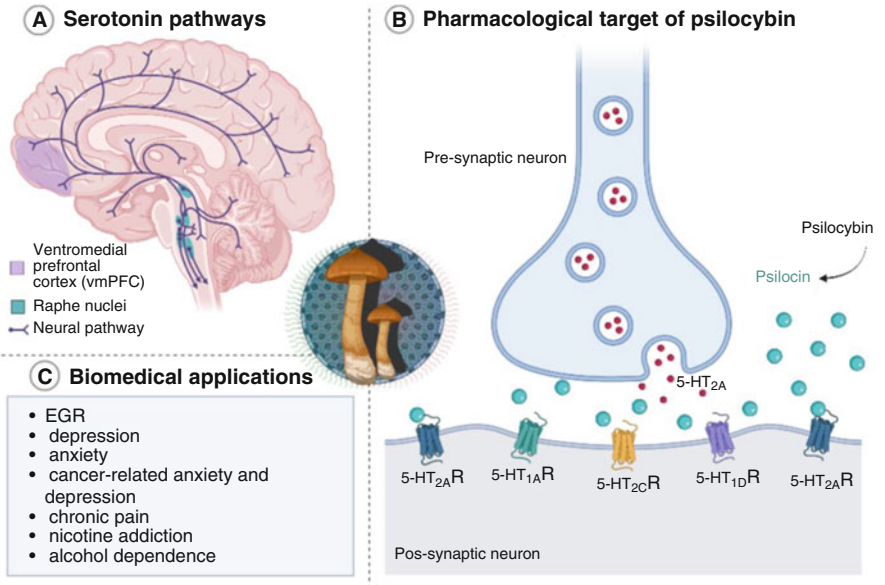

Fig. 16.5 Schematic overview of the serotonin pathways (a), main pharmacological targets of psilocybin (b), and several biological applications of psilocybin (c) (Created with BioRender.com)

16.3 Isoxazoles

Isoxazoles are a heterocyclic chemical family with a wide range of uses and have been shown to be particularly adaptable building blocks in organic synthesis. Isoxazoles are aromatic heterocycles with a weak nitrogen–oxygen interaction that might induce ring cleavage (Pinho e Melo 2005). Ibotenic acid and muscimol, two fungi isoxazoles that require special attention, will be explored in the next sections.

16.3.1 Ibotenic acid and Muscimol

The ibotenic acid and muscimol can be found in *A. muscaria*, *A. pantherina,* and *A. strobiliformis* mushrooms. The aqueous-soluble ibotenic acid ((S)-2-Amino-2-(3-hydroxyisoxazol-5-yl)acetic acid) is colorless, melts around 150–152 °C, and is unstable in solution. Muscimol (5-(aminomethyl)-3-hydroxyisoxazole) is less hydrophobic than ibotenic acid, although it may still be dissolved in cold water. Muscimol has a colorless crystalline structure with a melting point of 175 °C and it is produced by decarboxylation of ibotenic acid (Wieczorek et al. 2015).

16.3.1.1 Pharmacokinetics and Pharmacodynamics

Ibotenic acid and muscimol belong to the excitatory amino acid class of alkaloids being conformationally related to glutamic acid and gamma-aminobutyric acid (GABA), respectively (Johnson et al. 2014). As a result, ibotenic acid acts as an agonist on N-Methyl-D-aspartic acid (NMDA) and trans-1-Amino-1,3-dicarboxycyclopentane receptors (metabotropic quisqualate Qm receptors). Ibotenic acid's neurotoxicity, on the other hand, is mediated only by the activation of NMDA receptors (Stebelska 2013). Muscimol is a non-selective $GABA_A$ receptor agonist that activates both pre- and postsynaptic receptors, as well as a partial agonist of $GABA_c$ receptors. It has no effect on the GABA-metabolizing enzyme, $GABA_A$ transaminase, or the $GABA_A$ uptake systems, and it enters the brain via peripheral injection (Voynova et al. 2020).

Despite this, unlike glutamic acid and GABA, ibotenic acid and muscimol are believed to pass the blood–brain barrier via active transport, imitating natural neurotransmitters, and causing brain disorders (Li and Oberlies 2005).

In vivo, ibotenic acid is metabolized to muscimol, which is rapidly absorbed from the gastrointestinal tract and readily excreted in urine (Stebelska 2013). As reported by Stříbrný et al. (2012) both ibotenic acid and muscimol can be detected in urine, 3 to 8 h after ingestion of *A. pantherina* and *A. muscaria* poisonous mushrooms (Stříbrný et al. 2012). The least amount of muscimol required to produce symptoms of central nervous system intoxication is thought to be 6 mg. Ibotenic acid, which is significantly less active but far more dangerous, requires 30–60 mg to create a psychedelic effect (Stebelska 2013).

16.3.1.2 Analytical Methods

Various analytical techniques, and numerous biological, and mushroom matrices have been used for the detection and quantification of ibotenic acid and muscimol. LC with various detectors, such as MS or UV detector, is the most often used method for these alkaloids quantification. The evaluation of ibotenic acid and muscimol in *Amanita* mushrooms has been carried out using LC-tandem mass spectrometry (LC-MS/MS) (Gonmori et al. 2012). Tsujikawa et al. (2006) developed GC–MS (Tsujikawa et al. 2006) and LC–MS/MS (Tsujikawa et al. 2007) techniques for detecting ibotenic acid and muscimol in mushrooms samples. Stormer et al. (2004) employed LC–UV and LC–MS methods to determine ibotenic acid in *A. muscaria* spores and caps (Stormer et al. 2004), whereas Gennaro et al. (1997) used ion-interaction LC–UV to determine muscimol and ibotenic acid in *A. muscaria* mushrooms (Gennaro et al. 1997).

Concerning biological samples, the most often employed samples for detection of these alkaloids have been urine and serum (Garcia et al. 2015; Hasegawa et al. 2013; Merová et al. 2011, 2012). For the analysis of ibotenic acid and muscimol in urine, Stříbrný et al. (2012) developed a GC–MS methodology to quantify muscimol and

ibotenic acid in the urine of intoxicated people (Stříbrný et al. 2012). However, this GC–MS technique has rather low sensitivity, with a limit of detection (LOD) of 1 μg mL^{-1}. On the other hand, LC-MS/MS has been used to determine the levels of ibotenic acid and muscimol in human serum improved LODs with values of 0.3 ng mL^{-1} and 0.1 ng mL^{-1}, respectively (Xu et al. 2020). Capillary electrophoresis has been reported as another separation technique with high retention capacity for both compounds (Ginterová et al. 2014).

16.3.1.3 The Biomedical Role of Isoxazoles

Fungi-derived isoxazoles have been successfully converted into a variety of pharmaceutically significant compounds. Muscimol has been shown to have therapeutic potential in neurodegenerative disorders. Indeed, according to prior research performed by Levy et al. (2001) microinjections of muscimol and lidocaine into the subthalamic nucleus of humans result in a transitory improvement in akinesia, stiffness, and limb tremor in Parkinson's disease patients (Levy et al. 2001). Similar findings were obtained by Pahapill et al. (1999) who discovered that intrathalamic microinjections of muscimol reduced tremor in individuals with essential tremor (Pahapill et al. 1999). A study performed by Ludvig et al. (2009) showed that transmeningeally delivered muscimol can prevent the development of focal, acetylcholine-induced neocortical seizures in both rats and non-human primates, being a viable candidate compound for the transmeningeal pharmacotherapy of intractable focal epilepsy (Ludvig et al. 2009). Furthermore, Krogsgaard-Larsen et al. (2000) also discovered that lipophilic bioisosteres of muscimol and GABA may be produced and used as a therapeutic drug for epilepsy (Krogsgaard-Larsen et al. 2000).

Existing evidence suggests the GABAergic system is involved in the pathophysiology of Alzheimer's disease (AD) via inhibitory interneuron deficits and a decrease in functional $GABA_A$ receptors (Limon et al. 2012; Verret et al. 2012). *In vitro*, GABA and muscimol blocked neuronal death induced by β-amyloid peptides in rat hippocampal and cortical neurons (Paula-Lima et al. 2005). Additionally, Pilipenko et al. (2015) showed that muscimol improved rats' learning and memory abilities in both normal and AD-type *in vivo* models. Intra-hippocampal infusion of muscimol significantly increased the ratio of neurons active in *cornu ammonis*, improving learning and memory abilities, implying that intensification of GABAergic processes may be a useful pharmacotherapeutic strategy in early memory decline in AD (Pilipenko et al. 2015).

The anti-inflammatory activity of muscimol was also reported by Gharedaghi et al. (2013), revealing that muscimol (0.1 mg kg^{-1}) significantly decreased lipopolysaccharide-induced preterm delivery through modulating nitric oxide release (Gharedaghi et al. 2013).

Muscimol's anticancer properties have also been discovered. Indeed, long-term treatment of muscimol reduced the increase of N-methyl-N′-nitro-N-nitrosoguanidine-induced-gastric carcinogenesis in spontaneously hypertensive

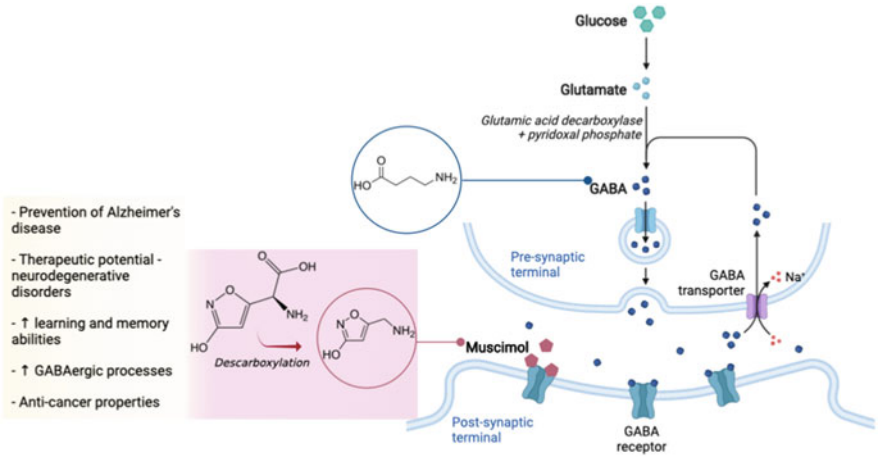

Fig. 16.6 Schematic overview of the GABA synthesis and uptake, the main pharmacological targets of muscimol, and several biological applications of muscimol (Created with BioRender.com)

rats (52 weeks) (Tatsuta et al. 1992). Furthermore, Ivashchenko et al. (2018) developed a gel formulation containing silver nanoparticles and *A. muscaria* extract (containing glucans and muscimol) as a capping agent, which significantly impacted anticancer properties. Moreover, a cytotoxicity study performed on 2D and 3D HeLa cell cultures pointed to a high potential for treating local cancer. However, cell response was found to be significantly different for 2D and 3D cell cultures, which was related to their different cytoarchitecture and gene expression profiles. The vitality of cellular spheroids revealed that 3D HeLa cell culture was more resistant to nanoparticle cytotoxicity and their gel formulation than 2D cell culture (Ivashchenko et al. 2018) (Fig. 16.6).

On the other hand, ibotenic acid, the main *Amanita* alkaloid, is a powerful neurotoxin more poisonous than muscimol. This compound has been labeled as a "brain-lesion" causing agent (Durmer and Rosenquist 2001), since can lead to ibotenate-induced seizures and damage to specific brain areas in animal studies, with relevance for AD-type neurodegeneration (Stebelska 2013). Ibotenic acid injections into the nucleus basalis magnocellularis of mice resulted in a substantial and highly reproducible decrease in cortical cholinergic activity (Contestabile et al. 2004; Winkler et al. 1998). As so, this excitotoxin is used to create animal models of AD-like neurodegeneration (Eleuteri et al. 2009; Lee et al. 2012).

Furthermore, the extract from the fruit body of *A. muscaria* is suggested to be poisonous for some fly species. As so, according to Narahashi et al. (2007) ibotenic acid exerted an inhibitory neurotransmitters action (effective concentration 50 of 42 mM) in insects due to the exclusive presence of glutamate-gated chloride channels in invertebrates (Narahashi et al. 2007). The activity of ibotenic acid is mediated through insect muscle fibers. This compound stimulates chloride channels distributed on the nonsynaptic membrane and increases the coxal adductor muscle

fiber conductance (Lea and Usherwood 1973). Also, a study by Yokoi et al. (1977) showed the inhibitory activity of ibotenic acid and other structurally similar compounds on a snail (*Achatina fulica*) periodically oscillating neuron (Yokoi et al. 1977).

16.4 Muscarine

Muscarine is a parasympathomimetic alkaloid found in the various mushroom genus, including *Inocybe*, *Clitocybe*, and *Omphalotus*. These species include but are not limited to *Clitocybe dealbata*, *Clitocybe rivulosa*, *Clitocybe dilatata*, *Inocybe agglutinata*, *Inocybe fastigiata*, *Inocybe geophylla*, and *Omphalotus illudens*. Muscarine was first discovered and isolated in *A. muscaria* in 1869 (Michelot and Melendez-Howell 2003) and the structure was elucidated in 1957 through X-ray diffraction analysis (Kögl et al. 1957). Several enantiomers of muscarine are present in muscarine-containing mushrooms (e.g., *allo*-muscarine, *epi*-muscarine, *epi-allo*-muscarine), however, only L-(+)-muscarine contributes to the cholinergic effects of muscarine-containing mushrooms. The remaining enantiomers have been found in several mushroom species, but their toxicological relevance is negligible due to their low percentages and minimal biological activity. L-(+)-muscarine is a colorless, thermostable, and water-soluble compound. Cooking muscarine-containing mushrooms for an extended period does not affect their toxicity (Barceloux 2008).

16.4.1 Pharmacokinetics and Pharmacodynamics

Limited data are available concerning to pharmacokinetics of muscarine. Muscarine's biological activity is due to a structural resemblance between it and the neurotransmitter acetylcholine. As a result, muscarine binds to acetylcholine receptor sites in the central and peripheral nervous systems, stimulating muscarinic autonomic and postganglionic receptors (White et al. 2019). As clinical signs developed rapidly it is reasonable to assume that muscarine is quickly distributed throughout the body (Puschner 2012). Muscarine does not pass the blood–brain barrier because of its ionic nature and quaternary configuration, hence its cholinergic actions are exclusively peripheral (Rumack and Spoerke 1994). A portion of the ingested muscarine is eliminated unchanged in urine (Merová et al. 2011; Tomková et al. 2015). Pharmacokinetics and gamma scintigraphy was used to investigate the biological pharmacokinetics of muscarine in New Zealand white rabbits (Sai Latha et al. 2020). Within 5 min of injection, roughly 42% of muscarine reached rabbit blood, which confirms that muscarine is a quick-acting toxin. The biodistribution immediately after muscarine administration revealed that the majority of muscarine diffused into the animal's organs was localized in the thoracic area (lungs, heart,

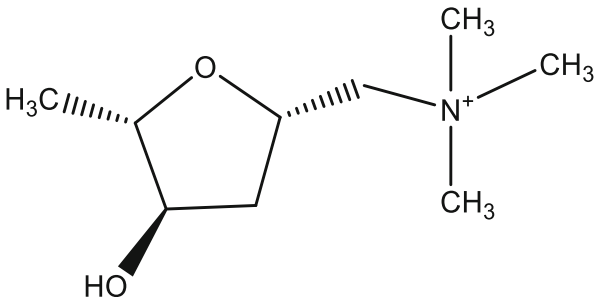

Fig. 16.7 Chemical structure of L-(+)-Muscarine

liver, and stomach) (Sai Latha et al. 2020). More detailed pharmacokinetic studies are needed to be performed (Fig. 16.7).

16.4.2 Analytical Methods

Several analytical methods have been developed for the analysis of muscarine in mushrooms and biological samples. Chung et al. (2007), used hydrophilic interaction LC separation in combination with electrospray ionization (ESI)-MS/MS to develop a reliable and sensitive method for the simultaneous determination of highly potent hydrophilic mushroom toxins, ranging from bicyclic oligopeptides to small-charged toxins such as muscarine (Chung et al. 2007).

Muscarine has also been analyzed in biological samples such as urine (Merova et al. 2008, 2011). Muscarine was extracted from urine using a Strata X-CW column and a solid-phase extraction procedure. For the first time, this LC-MS approach provides an objective analytical tool for isolating, identifying, and determining muscarine in human urine at concentrations ranging from 0.3 ng mL^{-1} to 2 μg mL^{-1} (Merova et al. 2011). In addition, capillary electrophoresis with electrospray tandem mass spectrometry has been used to isolate and determine muscarine. This approach was successfully used in human urine that had been spiked with muscarine, implying that it might be used for regular muscarine analysis following mushroom intoxication (Ginterova et al. 2014). Solid-phase extraction and ultra-high-performance liquid chromatography combined with ultra-high-resolution time-of-flight mass spectrometry have also been used to evaluate muscarine in human urine (Tomková et al. 2015).

16.4.3 The Biomedical Role of Muscarine

Muscarine has been used to explore the role of muscarinic receptors in the induction of antinociceptive behaviors in the spinal cord and anterior cingulate cortex

(Bartolini et al. 1992; Ghelardini et al. 1996, 2000; Koga et al. 2017; Naguib and Yaksh 1997). Indeed, in diabetic rats, intrathecal injection of muscarine showed a stronger antinociceptive effect than normal rats, according to a prior study (Chen and Pan 2003). As a result of this research, spinally delivered cholinergic medications may have some specific therapeutic utility in the treatment of diabetic neuropathic pain. This muscarinic action will need to be further investigated.

16.5 Conclusion

Research on alkaloids has increased noticeably in importance and has been given a special role in their biological activity and novel therapeutics.

Tryptophan indole-based alkaloids (psilocybin, psilocin) are among the alkaloids that have been found in a variety of mushrooms, particularly those of the genus *Psilocybe*, although further research is needed to determine their therapeutic potential in disorders other than psychiatry. As so, our chapter underlines the key role of the 5-HT_{2A} receptor in the psychedelic activity of psilocybin, unveiling synaptic plasticity and behavioral events. However, basic and translational research focused on understanding the molecular mechanisms mediating the clinical effectiveness of psychedelic compounds is needed. The genetic background, namely the epigenomic indicators could also be a step ahead in clarification of the psychedelics' mechanism of action used in psychopharmacological therapies. Therefore, additional large randomized clinical trials are needed to confirm the potential role of psilocin on depression, anxiety, and stressor-related disorders.

The compounds found in *Amanita* species mushrooms have received far less attention including muscimol and ibotenic acid. In a wide range of therapeutic fields, isoxazole derivatives have been linked to key biological functions such as anti-neurodegeneratives and anticonvulsants. Anticancer studies are also being conducted using these drugs. Although more research on the therapeutic effect of isoxazoles is required.

Muscarine has been performed to explore the agonistic effects on muscarinic receptors, although additional biological aspects should be investigated to elucidate potential therapeutic properties.

We hope that this chapter will open the path for future studies on these fungal alkaloids and enhance understanding and progress in this field.

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Chapter 17
Polyketides from Fungi

Christiane Contigli, Marcelo Siqueira Valle, Sílvia Catarina Salgado Oloris, Lúcia Pinheiro Santos Pimenta, and Jacqueline Aparecida Takahashi

Abstract Fungal secondary metabolites present a huge scaffold of chemical entities. Many of these substances have played important roles not only in nature, but also in human life, as lead compounds of immunosuppressants, antibiotics, anticancer drugs, and cholesterol-lowering medicines, along with other industrial uses. Among fungal natural products, polyketides represent a large group of metabolites defined by their biosynthetic origin, highly programmed by iterative multifunctional proteins. This chapter brings some novelties, especially from 2020 onward, focusing on the main classes of polyketides, new isolated compounds, innovative strategies for synthetic and biosynthetic production. Furthermore, we discuss the current and future biological applications of fungal polyketides in different fields.

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17.1 Historical Overview of the Discovery of Fungal Polyketides

Polyketides comprise a large and structurally diverse group of molecules that constitute one of the most important classes of secondary metabolites originated from bacteria, fungi, plants, and marine organisms. The term polyketide stems from their biosynthetic origin, which derives from the acetate or similar units coupling to malonyl-CoA units to provide long poly-β-keto chains. These chains can afford compounds with a huge structural diversity as macrolide antibiotics and many aromatic compounds, e.g., anthraquinones, tetracyclines, furochromones, pyrones, and benzophenones derivatives, after reactions such as aldol- and Claisen reactions, C- and O-alkylation, enolization, reduction, and decarboxylation. Most fungal metabolites originate from this metabolic pathway and display interesting biological activities or functionalities. However, it is hard to correlate one specific chemical core to a particular bioactivity. The most diverse biological activities have been associated with natural polyketides, supporting the development of a variety of commercial products. Koskinen and Karisalmi (2005) suggested that 1% of the secondary metabolites of polyketide origin may be of interest due to potential drug activities. In the same year, 20% of the blockbuster pharmaceutical drugs were polyketide-derived, according to Weissman and Leadlay (2005).

The first fungal polyketide identified that found industrial application was, probably, mycophenolic acid (**1**) (Fig. 17.1), an immunosuppressive agent which exerts its effect by inhibiting the growth of B and T cells of the immune system. It was first isolated by Bartolomeo Gosio in 1896, from *Penicillium brevicompactum*, and rediscovered and named later by Alsberg and Black (1913), who isolated it from cultures of *Penicillium stoloniferum*. Mycophenolic acid (**1**) can be produced by indigenous strains of *Penicillium glabrum* (Mahmoudian et al. 2021) and is available commercially as the ester prodrug mycophenolate mofetil (**2**) (CellCept®, Roche) or an enteric-coated form as mycophenolate sodium (**3**) (Myfortic®, Novartis) (Budde et al. 2004). Studied since the 1970s, mycophenolate mofetil (**2**) was approved for medical use in the USA in 1995, as an immunosuppressant to prevent organ transplantation rejection, and later, to treat psoriasis (Strathie Page and Tait 2015). Mycophenolic acid (**1**) is also an antimicrobial agent, and, over the years, several other biological properties have been attributed to it, such as antitumor, anti-inflammatory, and more recently, as an antiviral against SARS-CoV-2 (Wang et al. 2021a).

In the middle 1970s, an antimycotic agent named echinocandin B (**4**) (peptide-polyketide mixed biosynthesis) (Fig. 17.1) was concomitantly isolated from *Aspergillus delacroxii* (former *Aspergillus nidulans* var. *echinolatus*) and *Aspergillus rugulosus* (Hüttel 2021). The problems associated to solubility, toxicity, and industrial production were overcome in 2001, with the approval of caspofungin acetate (**5**) (Cancidas®, Merck Sharp & Dohme), a semi-synthetic derivative of the natural product (Fig. 17.1). Later, two other derivatives, micafungin (Mycamine®, Astellas Pharma) (**6**) and anidulafungin (Eraxis™, Pfizer) (**7**) (Fig. 17.1) were approved by

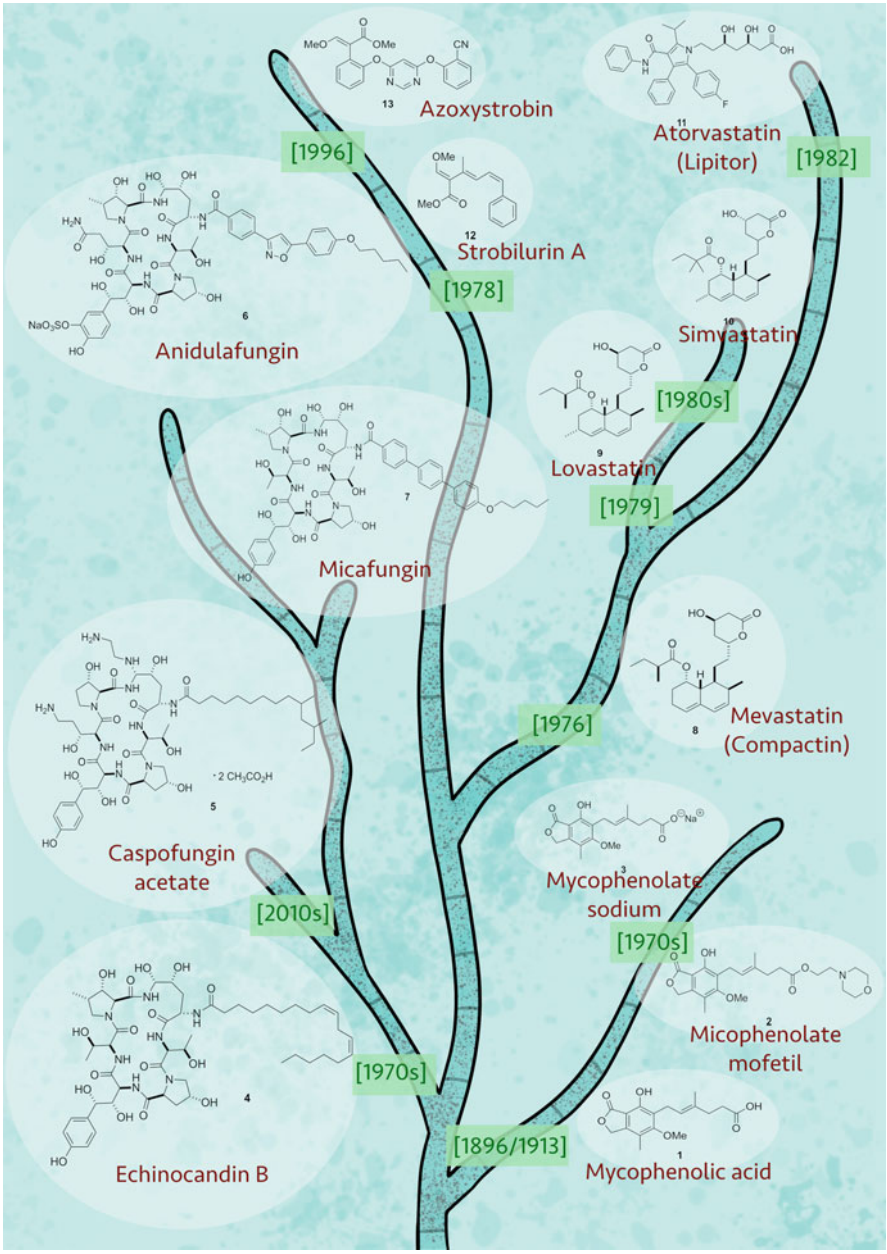


Fig. 17.1 Timeline of the discovery of some fungal polyketides and derivatives with expressive industrial importance: mycophenolic acid (**1**), mycophenolate mofetil (**2**), mycophenolate sodium (**3**), echinocandin B (**4**), caspofungin acetate (**5**), micafungin (**6**), anidulafungin (**7**), mevastatin (compactin) (**8**), lovastatin (mevinolin) (**9**), simvastatin (**10**), atorvastatin (**11**), strobilurin A (**12**), azoxystrobin (**13**)

American and European regulatory agencies (Hüttel 2021). Caspofungin is a prescription drug for several fungal infections, including candidiasis, as well as empirical antifungal therapy in patients with neutropenic fever of unknown origin, because it competitively inhibits 1,3-β glucan synthase, an enzyme that is essential for the synthesis of β-glucan in the fungal cell wall. This metabolite is also efficient in the treatment of invasive aspergillosis in patients that were refractory to amphotericin B (Heinz et al. 2016). According to a report from Transparency Market Research (TMR 2019), the global caspofungin market was valued at US$ 484.20 million in 2018 and is projected to expand at a compound annual grow rate (CAGR) of 0.18% from 2019 to 2027.

Among the most prominent fungal polyketides and their derivatives, the statins currently used to lower serum cholesterol deserve a highlight. Mevastatin (**8**) was the first statin drug, isolated from the ascomycete fungus *Penicillium citrinum* by Endo in the early 1970s, and described as an hypolipidemic agent capable of competitively inhibiting the 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMG-CoA reductase), critical in the biosynthetic pathway of cholesterol (Endo 2017). The clinical trial on mevastatin (**8**) (Fig. 17.1) to reduce cholesterol levels was done in the late 1970s in Japan. At the same time, this compound was isolated in Britain, as an antifungal, and designated as compactin. The C-12 methylated derivative of mevastatin (compactin) (**8**), lovastatin (mevinolin) (**9**) (Fig. 17.1), was independently isolated from *Aspergillus terreus* by Endo and Merck researchers in 1979, and it was the first pharmacological agent able to reduce LDL-cholesterol to unprecedented levels, without serious side effects. Lovastatin (**9**) was approved for commercial use in the USA in 1987 as Mevacor® (Merck) (Endo 2017; Dehnavi et al. 2020). It was followed by the development of simvastatin (**10**), a semi-synthetic version of the natural product, launched in 1992 as Zocor® (Merck). Atorvastatin (**11**) was synthetized in 1985, and clinical trials revealed it as more effective than simvastatin (**10**). Marketed as Lipitor® (Pfizer) (Fig. 17.1) since 1986, atorvastatin (**11**) has been reported as the best-selling drug in the last two decades (Newman and Cragg 2020). Statins have saved millions of lives in patients with coronary problems and nowadays it has been considered as a repurposing drug to fight major human diseases such as cancer (Jiang et al. 2021) and as an adjuvant therapy to treat patients with COVID-19 (Vuorio and Kovanen 2021).

In addition to the use of fungal polyketides in the pharmaceutical industry, another outstanding industrial application can be pointed. Strobilurins are polyketide-derived natural products, usually synthesized by species of the genus *Strobilurus* (basidiomycetes), that consist in important fungicides of broad agricultural use. These metabolites are very efficient in the control of ascomycetes, basidiomycetes, and oomycetes, being applicable in different crops like rice, coffee, wheat and vines, fruits like bananas, and several other vegetables to control and prevent fungal diseases such as water molds, downy mildews, leaf spotting and rusts (Balba 2007; Selim and Khalil 2021). The first natural compound isolated from this class was strobilurin A (**12**) (Fig. 17.1), isolated from *Strobilurus tenacellus* by Schramm et al. (1978). The basic structure of these fungicides contains a methyl (E)-3-methoxy-2-(5-phenylpenta-2,4-dienyl) acrylate moiety, and the derivatives have

different patterns of substituents in the aromatic ring (Balba 2007; Wang et al. 2021b). Despite the great applicability of strobilurins, the natural products were photo-unstable, leading the pesticide industry to synthesize more stable semi-synthetic derivatives, which have reached the world market in 1996. In 2016, strobilurins already accounted for a quarter of the world's fungicide market (US $15.3 billion), with 11 different commercial derivatives (Selim and Khalil 2021). The world's biggest selling fungicide, azoxystrobin (**13**) (Fig. 17.1), is a semi-synthetic derivative of strobilurins, produced and patented by Syngenta (formerly Zeneca Agrochemicals) (Balba 2007; Wang et al. 2021b).

In this chapter, some remarkable fungal polyketides and derivatives will be presented, and their routes of synthesis and applications will be discussed, along with several examples of compounds with pharmacological activities, such as antimicrobial, antiparasitic, anticancer, immunological and metabolism regulators, neuro- and cardioprotectors, as well as polyketides useful in agriculture and environment, such as anti-phytopathogen, and in the food industry, such as pigments. Furthermore, a summary of the new bioactive compounds will be reported, along with their chemical structures, the progress in their biosynthesis research, and their biotechnological potential.

17.2 Polyketides from Fungi: A Chemical Perspective

Although fungal polyketides display a huge structural diversity, it is possible to classify them among three main classes: aromatic polyketides—products from Diels–Alder reactions, macrolides, and polyethers (Simpson and Cox 2008; Crawford and Townsend 2010; Pavesi et al. 2021). Aromatic polyketides from the acylpolymalonate pathway can be as simple as orsellinic acid (OA) (**14**) or highly complex as aflatoxins or tetracyclines. The examples grow geometrically with more interesting compounds if polyketides of mixed biogenetic origins are considered. Diels–Alder reactions are part of many metabolic pathways and can occur in different steps of the biosynthesis. For instance, statins, cytochalasans, and sorbicillinoids, which exhibit diverse biological activities, are fungal polyketides displaying a cyclic aliphatic system arising from enzymatic Diels–Alder reactions (Hantke et al. 2020; Kahlert et al. 2020). Macrolides, macrolactones, and polyesters are known for their large range of biological activity as antibacterial, antifungal, antitumor, and plant-growth regulating (Karpiński 2019). There are other interesting polyketides, that will be presented in this chapter, which did not fit in those three classes, being generally classified as miscellaneous polyketides.

17.2.1 Aromatic Polyketides

Among the simplest aromatic polyketides, orsellinic acid (**14**), 6-methylsalicylic acid (6-MSA) (**15**), and phloracetophenone (**16**) (Fig. 17.2) are the most illustrative.

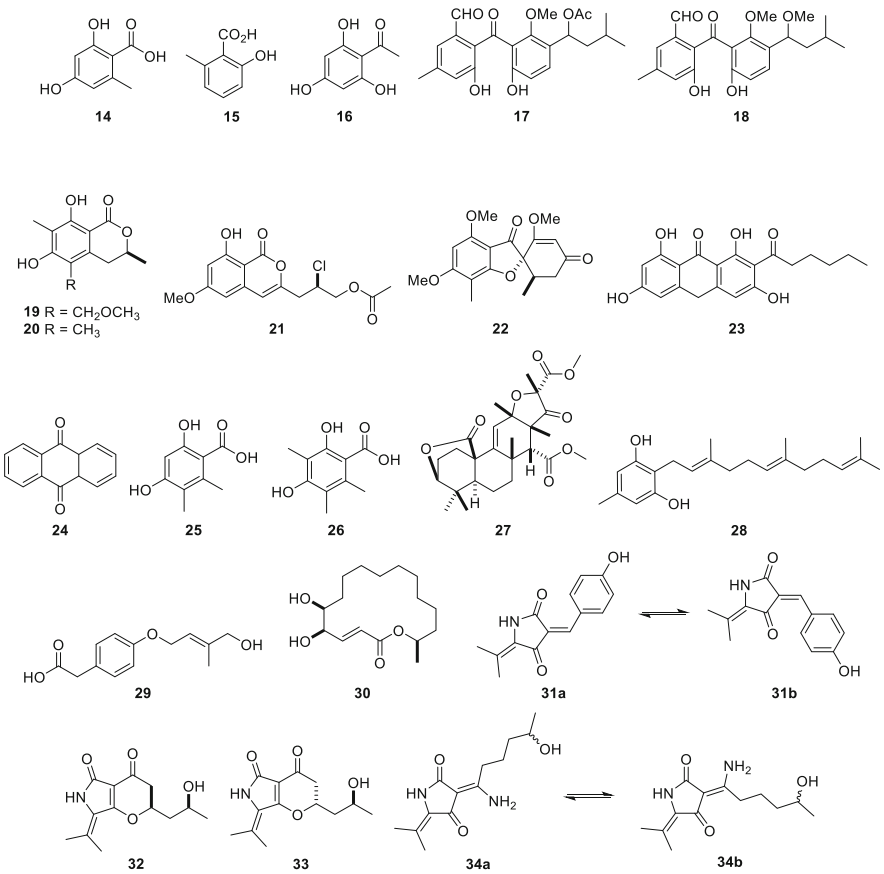


Fig. 17.2 Chemical structures of some aromatic fungal polyketides: orsellinic acid (OA) (**14**), 6-methylsalicylic acid (6-MSA) (**15**), phloracetophenone (**16**), penibenzones A (**17**) and B7 (**18**), (S)-6,8-dihydroxy-5-(methoxymethyl)-3,7-di-methylisochroman-1-one (**19**), (S)-6,8-dihydroxy-3,5,7-tri-methylisochroman-1-one (**20**), (R)-2-chloro-3-(8-hydroxy-6-methoxy-1-oxo-1H-isochromen-3-yl) propyl acetate (**21**), griseofulvin (**22**), norsolorinic acid anthrone (**23**), anthraquinone (**24**), 5-methyl orsellinic acid (5-MOA) (**25**), 3,5-dimethyl orsellinic acid (DMOA) (**26**), aspergillactone (**27**), grifolin B (**28**), 12-hydroxyhomovalencic acid (**29**), (5R,6S,16R,3E)-5,6-dihydroxy-16-methyloxacyclohexadec-3-en-2-one (**30**), and cladosins L−O (**31**–**34**)

They can be produced by several fungi or be part of hybrid polyketides, such as meroterpenes. Endophytic fungi of *Penicillium* genus have provided some examples of new aromatic polyketides as benzophenones [e.g., penibenzones A (**17**) and B (**18**)] and isocoumarins derivatives [e.g., (S)-6,8-dihydroxy-5-(methoxymethyl)-3,7-di-methylisochroman-1-one (**19**), (S)-6,8-dihydroxy-3,5,7-tri-methylisochroman-1-one (**20**), and (R)-2-chloro-3-(8-hydroxy-6-methoxy-1-oxo-1H-isochromen-3-yl) propyl acetate (**21**)] (Xia et al. 2020; Anh et al. 2021). Griseofulvin (**22**) is another benzophenone derivative produced by *Penicillium* species and administered orally as an antibiotic against skin fungal infections (Samuelsson and Bohlin 2009).

Furthermore, four polyketide dimers characterized by 6/6/8 tricycle carbon skeleton, incorporating an unusual bicycle [5.3.1] hendecane core, were identified in *P. canescens* ATCC 1049. Although these structures showed modest antitumor activity as compared to taxol or cisplatin, they are examples of the extremely enriching chemical diversity of fungal polyketides (Zang et al. 2020).

Anthrone, anthraquinone, and naphthopyrone are very representative of fungal polyketide metabolites. They are biosynthesized by several genera, including *Aspergillus,* known producers of highly carcinogenic compounds on foodstuffs such as cereals, beans, peanuts, coconuts, and almonds. Norsolorinic acid anthrone (**23**) and anthraquinone (**24**) (Fig. 17.2) are intermediates in the biosynthetic pathway of aflatoxins B1 and B2 (Samuelsson and Bohlin 2009).

Meroterpenoids (Fig. 17.2) are hybrid partially originated from terpenoids and display a broad range of structural diversity and biological activity. One of the largest meroterpenoids subgroups is composed of polyketide-terpenoid hybrid molecules. For instance, OA (**14**), 5-methyl orsellinic acid (5-MOA) (**25**), and 3,5-dimethyl orsellinic acid (DMOA) (**26**) are common polyketide parts of different meroterpene structures. Moreover, the genus *Aspergillus* is a great contributor to marine-derived fungal meroterpenoids. Aspergillactone (**27**), a DMOA-based meroterpene, exhibiting antibacterial activity against drug-resistant clinical isolates of *Helicobacter pylori* and *Staphylococcus aureus*, was isolated from *Aspergillus* sp. CSYZ-1 (Cen et al. 2021). *Aspergillus unguis* 158SC-067 and *A. flocculosus* 01NT-1.1.5 afforded a new resorcinol-meroterpene called grifolin B (**28**), a new homovalencic acid derivative, 12-hydroxyhomovalencic acid (**29**), and (5R,6S,16R,3E)-5,6-dihydroxy-16-methyloxacyclohexadec-3-en-2-one (**30**), a cytotoxic compound isolated from a natural source for the first time, along with seven known compounds (Anh et al. 2021).

Cladosin is a class of hybrid polyketides also produced by endophytic fungi. These compounds originated from the fusion of amino acids and polyketide units, and commonly isolated from the endophytic fungus *Cladosporium sphaerospermum* (Pan et al. 2020). *C. sphaerospermum* WBS017 afforded four new hybrid polyketides of cladosins L−O (**31–34**), which showed moderate antibacterial and antifungal activity (Pan et al. 2020) (Fig. 17.2).

17.2.2 Polyketides by Enzymatic Diels–Alder Reactions

Aromatic and cyclohexane rings can be built not only by Claisen and aldol reactions but also by enzymatic Diels–Alder reactions. Among the polyketides whose scaffold was originated from a [4 + 2] cycloaddition, the statins, such as lovastatin (**9**) (Fig. 17.1) and simvastatin (**10)** are good examples (Fig. 17.3). They arise from two polyketide chains, joined through an ester linkage with the decalin ring, formed by the Diels–Alder reaction, displaying a γ-lactone ring and other functional groups substituents (Campbell and Vederas 2010; Wang et al. 2021c).

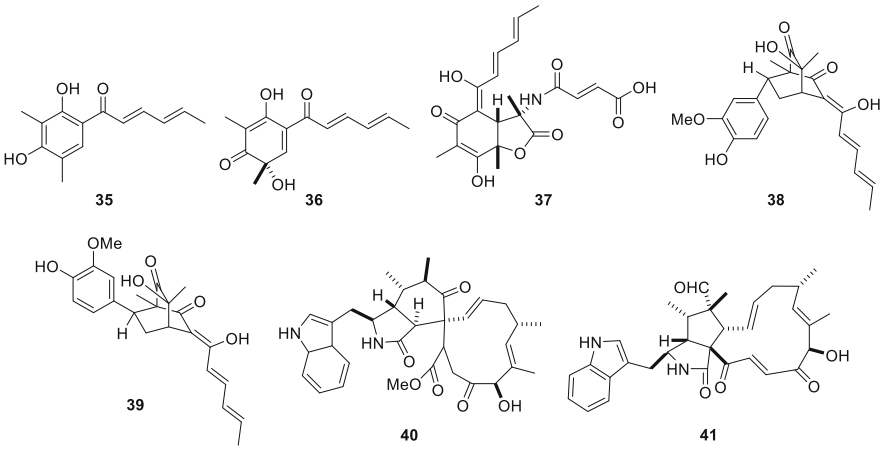


Fig. 17.3 Chemical structures of some fungal polyketides with Diels–Alder origin: sorbicillin (**35**), sorbicillinol (**36**), sorbicillactone A (**37**), sorbicatechol A (**38**) and B (**39**), spiropchaeglobosin A (**40**), and pchaeglobosal B (**41**)

Hybrid sorbicillinoids are another interesting class of biologically active and structurally diverse fungal polyketides arising from enzymatic Diels–Alder reaction. Sorbicillinoids are hexaketides derived from a cyclization on the carboxylate terminus. The first representative sorbicillin (**35**) was isolated from *Penicillium notatum* by Cram and Tishler in 1948 (Fig. 17.3). Up to now, more than 90 sorbicillinoids produced by terrestrial and marine fungi are known (Kahlert et al. 2020) and classified into four sub-classes. The hybrid sorbicillinoids are biosynthesized from either a Diels–Alder reaction or a Michael reaction of a monomeric sorbicillinoid diene and a second non-sorbicillinoid dienophile (Meng et al. 2016; Kahlert et al. 2020). The sorbicillinoids display a variety of biological activities including cytotoxic, antioxidant, antiviral, and antimicrobial. For instance, sorbicillinol (**36**) and sorbicillactone A (**37**) (Fig. 17.3) demonstrated a great cytotoxicity against cancer cell lines, and sorbicatechol A and B (**38–39**) displayed antiviral activities (Meng et al. 2016).

Finally, cytochalasans represent an interesting class of hybrid tricyclic polyketides containing an 11- to 16-membered macrocycle ring fused to an octahydro-isoindole derived from amino acids (e.g., phenylalanine, tyrosine, tryptophan, leucine, or alanine). These compounds exhibit a wide range of activities such as phytotoxic, antitumor, antiviral, antiangiogenic, immunomodulatory, and nematicidal (Peng et al. 2020). *Chaetomium globosum* P2-2-2 afforded four novel chaetoglobosins with an unprecedented system of fused rings. Among them spiropchaeglobosin A (**40**) was remarkable for its unique spiro [5.10]hexadecane system, which distinguished it from all known cytochalasans. Moreover, the new compound pchaeglobosal B (**41**) exhibited significant cytotoxicity against cancer cell lines (Peng et al. 2020) (Fig. 17.3).

17.2.3 Macrolides and Polyesters

Macrolides (Fig. 17.4) are an interesting group of 10–22-membered lactones originated from acetate or propionate polyketides and produced by bacteria and fungi. The polyketide chain can have a portion which might cyclize to an aromatic ring originating olivetolic acid (**42**), a 6-alkyl-substituted 2,4-dihydroxybenzoic acid, also known as a β-resorcylic acid, frequently present in fungal polyketide macrolactones, including the well-known zearalenone (**43**), besides hypothemycin (**44**) and radicicol (**45**) (Okorafor et al. 2021). Compound **43** is a 14-membered β-resorcylic lactone containing all types of oxidation levels seen during the fatty acid extension cycle. Also, this polyketide is a toxin produced by the fungus *Gibberella zeae* and several *Fusarium* species, displaying a high antifungal activity (Cox et al. 2018).

Another interesting macrolide groups are those which lack the β-resorcylic moiety forming the lactone. They are often biosynthesized by endophytic marine fungi of genera *Cladosporium*, *Curvularia*, *Hypoxylon*, *Paraphaeosphaeria*, *Penicillium*, *Phomopsis*, and *Sphaeropsidales* (Karpiński 2019; Zhang et al. 2020a). Besides, these macrolides presented antimicrobial, anticancer, chemopreventive, antibacterial, and/or antifungal activities. The mangrove-derived fungus *Cladosporium cladosporioides* MA-299 provided the new macrolide cladocladosin A (**46**), with an unprecedented bicycle 5/9 ring system, along with the two new sulfur-containing macrolides thiocladospolides F and G (**47–48**) (Fig. 17.4). The three compounds demonstrated antimicrobial activity against human and aquatic pathogenic bacteria (Zhang et al. 2020a).

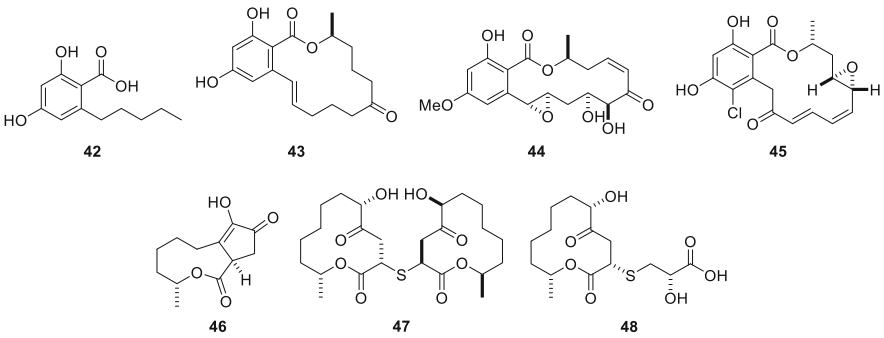


Fig. 17.4 Chemical structures of some fungal macrolide and polyester polyketides: olivetolic acid (**42**), zearalenone (**43**), hypothemycin (**44**), radicicol (**45**), cladocladosin A (**46**), thiocladospolides F (**47**) and G (**48**)

17.3 Routes of Synthesis of Polyketides Derived from Fungi

17.3.1 Fungal Polyketide Biosynthesis: The Most Recent Insights

The biosynthesis of fungal polyketides was previously reviewed by Simpson and Cox (2008) and recently considered by Cox et al. (2018). The genes encoding enzymes of a given biosynthetic pathway are frequently found adjacent in the genome and co-regulated, being denominated biosynthetic gene clusters (BGC). The biosynthesis of polyketides is catalyzed by modular polyketide synthases (PKS), which are enzymes with many domains. A set of ketoacylsynthase (KS), acyltransferase (AT), and acyl carrier protein (ACP) domains are mandatory in PKS to catalyze all chain extension cycles (iterative PKS). Besides, several additional domains, such as ketoreductase (KR), dehydratase (DH), enoyl reductase (ER), thioesterase (TE), and C-methyl transferase (CMeT) can be involved in a β-ketothioester processing, which performs specific operations in the chain-elongating process, usually acting in an iterative way, and the degree of reduction can vary into different units.

Genetic engineering provided tools to determine the PKS cluster genes, and their sequencing allowed to group them into three different classes, according to their architecture and mechanism of action (Fig. 17.5). Fungi present only types I and III PKS. Iterative type I modular PKS, present in most fungi, display modules which catalyze two-carbon linear extension, carrying out different actions in the β-keto group during the different cycles of chain elongation. In this sense, they are classified according to the degree of reduction of their products into non-reducing (NR) PKS, partially reducing (PR) PKS, and highly reducing (HR) PKS. HRPKS perform a cryptic biosynthetic pathway to selectively reduce β-keto moieties after each extension step into a β-alcohol, alkene, or alkane through KR, DH, and ER domains (Xu et al. 2020). Additionally, a hybrid synthetase composed by an HR-PKS fused to a non-ribosomal peptide synthetase (NRPS) leads to polyketides such as congeners of the benzendiol lactones, polylactones, sorbicillinoids, and some azaphilones (Xu et al. 2020). Moreover, these HRPKS-NRPS systems also produce polyketides fused to an amino acid by an amide bond, as found in the cytochalasans. Even though fungal type III PKS are structurally the simplest PKS, they present very complex mechanisms, producing chalcones, stilbenes, and small aromatic

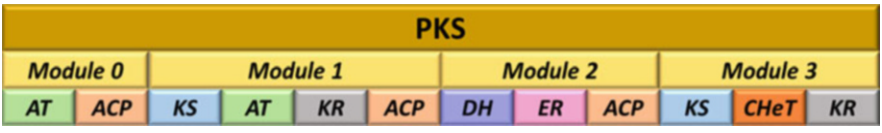


Fig. 17.5 Generic model for a polyketide synthase (PKS) and its modules and domains: acyltransferase (AT), acyl carrier protein (ACP), ketoacylsynthase (KS), ketoreductase (KR), dehydratase (DH), enoyl reductase (ER), and C-methyl transferase (CMeT) (Simpson and Cox 2008; Cox et al. 2018)

compounds (Schuemann and Hertweck 2009; Cox et al. 2018). The largest structural diversity of polyketides is also due to an impressive set of post-PKS modifications performed by cytochrome P450 monooxygenases, dehydrogenases, methyltransferases, as well as pathway regulators and transporters (Schuemann and Hertweck 2009).

The advance of the sequenced genome allows identifying BGC and comparing the PKS genes with known ones linked to natural products or to a specific biosynthetic step. Normally, most filamentous fungi can contain between 10 and 50 PKS genes (Cox et al. 2018), although a description of the fungal genome can reach up to 80 BGC (Meng et al. 2022). Moreover, this gene can be cloned and introduced in a heterologous host, in which not only the biosynthetic pathway can be deeply investigated, but also provides a convenient platform to generate a supply of useful secondary metabolites. Gene knockout, transcriptional activation, epigenetic regulation of fungal BGC, and combinatorial biosynthesis have also been revolutionizing the conventional fungal natural products discovery and production (Xu et al. 2020; Du and Li 2021).

Subcellular compartmentalization of secondary metabolism pathway enzymes also plays an important role in the biosynthesis of fungi. In fact, an isolated reaction occurs in an organelle where the substrates are highly concentrated, providing an optimal physiochemical environment for enzymatic catalysis. Moreover, the efficiency of precursor supply and intermediate channeling might be improved, preventing the loss of intermediates by off-target pathways, and the negative effects of toxic intermediates/products on the other cellular networks (Du and Li 2021). Therefore, more attention should be paid to the compartmentalization of biosynthetic steps to better understand the physiological and biochemical mechanisms of fungal biosynthesis.

Heterologous and engineering expression, and genome mining strategies were used by Zhang et al. (2020b) to investigate the biosynthesis of thermolides, fungal polyketide-nonribosomal peptide (PK-NRP) hybrid macrolactones produced by extreme thermophilic fungi *Talaromyces thermophilus* NRRL 2155. The study showed that the *Thm* gene cluster (*Thm*ABCE) is critical for the synthesis. Two separate single-module HRPKS (*Thm*A) and NRPS (*Thm*B) enzymes collaborate to synthesize the core macrolactone backbone type PK-NRP. Also, the NRPS *Thm*B-C_T domain catalyzes the key macrocyclization step in PK-NRP intermediate release via ester bond formation, representing a novel function of fungal NRPS C domains. These strategies not only dramatically enhanced the yields of thermolides, but also significantly increased its structural complexity and diversity, such as the synthesis of the first *L*-phenylalanine-based thermolides A (**49**) and B (**50**) (Fig. 17.6).

Co-expression, feeding experiments, knockout of genes, and heterologous expression are other successful tools for understanding the biosynthesis of dimeric or hybrid sorbicillinoids. The study of Kahlert et al. (2020) in *Trichoderma reesei* QM6a identified the gene SorD, recognized for encoding the first flavin-dependent monooxygenase enzyme operating in the biosynthesis of dimeric, hybrid, and epoxy sorbicillinoids in vivo. This monooxygenase acts in the dimerization of highly

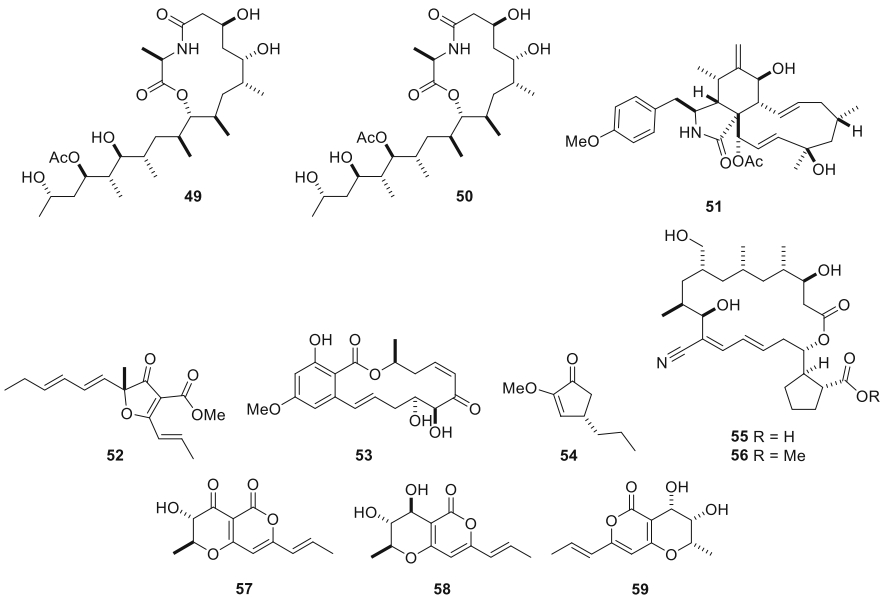


Fig. 17.6 Some polyketides elucidated by novel biosynthetic strategies: thermolides A (**49**) and B (**50**), pyrichalasin H (**51**), gregantin A (**52**), (5*Z*)-7-oxozeaenol (**53**), asperterrein (**54**), borrelidins J (**55**) and K (**56**), radicinin (**57**), radicinol (**58**), and 3-epiradicinol (**59**)

reactive sorbicillinoids (**36–39**), catalyzing an intermolecular Diels–Alder e/or Michael-addition, as well as in the epoxidation of sorbicillinol (**36**) (Fig. 17.3).

Directed gene knockout and complementation strategy were also carried out by Hantke et al. (2020) to investigate the function of the gene PyiF in the formation of cytochalasins, as well as to study if the closely related homologous protein ORF3 (62% identity to PyiF protein), from cryptic gene cluster ACE1 BGC in *P. oryzae* Guuy11, was also able to produce these polyketides. Their results strongly suggested that PyiF protein and ORF3 catalyze the long-proposed intramolecular Diels–Alder reaction, forming the tricyclic core structure of pyrichalasin H (**51**) (Fig. 17.6).

Another interesting approach to elucidating the biosynthetic mechanism to furnish the intriguing molecular skeleton of the alkylated furanone gregantin A (**52**) (Fig. 17.6) was reported by Wang et al. (2020a). Genome sequence of *Penicillium* sp. sh18 identified the gene cluster *grg* as responsible for the biosynthesis. Heterologous expression of the five enzyme-coding genes in *Aspergillus oryzae* successfully reconstituted the gregantin A (**52**) biosynthesis. Isotope-incorporation experiments indicated that compound **52** was biosynthesized by fusion of two polyketide chains synthesized by a single PKS, GrgA. In vitro enzymatic reactions confirmed that hydrolase GrgF was responsible for the fusion of the C11 and C4 carbon chains to produce strobilurin A (**12**) (Fig. 17.1). Finally, X-ray structural analysis and mutational experiments using GrgF provided a plausible mechanism for the chain fusion reaction (Wang et al. 2020a).

17.3.1.1 Enhancing the Biosynthesis of Fungal Polyketides

Fungi, as well as any other living being, tend to adapt to the conditions imposed by their natural habitats and develop tools to ensure their survival in that specific environment. These tools are basically metabolic alterations, which, for example, activate the biosynthesis of defense metabolites when dispute for space or food with other microorganisms takes place (Oliveira et al. 2021). These defense mechanisms become unnecessary to the extent that fungi are isolated and stored ex situ, in culture media containing enough amounts of carbon, nitrogen, macro, and microelements, as well as water (Oliveira et al. 2019). In vitro, fungal metabolism tends to decrease, with the deactivation of unnecessary biosynthetic routes. However, fungal metabolism is adjustable, due to the susceptibility of these organisms to the different conditions to which they are subjected. Therefore, physical, chemical, and biological modifications during the fermentation process can reactivate silenced biosynthetic routes (Peng et al. 2021). The various processes used for metabolic activation result, in general, from biotic or abiotic stress imposed on the fungus during its development. Biotic stress consists of the addition of microbial genetic material to the culture medium and the response tends to be the production of antimicrobial metabolites (Oliveira et al. 2021). Abiotic stress consists of physical (luminosity, agitation) or chemical (pH, nutrient type) alterations of the culture medium or during fungal development. In this case, the metabolites produced are not necessarily antimicrobials. Biotic and abiotic stress are relatively ways to activate metabolic production without the need for complex genetic modifications (Peng et al. 2021).

The general term used to describe improvement in the fermentation parameters is OSMAC (One Strain Many Compounds). This strategy was utilized to produce large amounts of the macrolactones hypothemycin (**44**) and (5*Z*)-7-oxozeaenol (**53**) (Fig. 17.6), using selected strains of the fungus *Setophoma* sp., to be used as feedstock for preparing synthetic analogs of resorcyclic acid lactones (RAL) in an economic way (Al Subeh et al. 2021). These fungal secondary metabolites are RAL with a *cis*-enone moiety, which show irreversible inhibitory activity against protein kinases, with selectivity toward transforming growth factor beta-activated kinase 1 (TAK1).

The secondary metabolites produced by fungi under stress are not normally produced by the species, under non-stressful conditions, as reported for the co-cultivation of *Aspergillus terreus* and *Paecilomyces lilacinus*. The interaction of these two fungi led to the production of asperterrein (**54**), a new antibacterial substance not detected in extracts where the two species developed individually (Li et al. 2020). In the same way, co-culture of a sponge-derived actinomycete *Streptomyces rochei* and a gorgonian-derived fungus *Rhinocladiella similis* was shown to induce the production of borrelidins J (**55**) and K (**56**), another antibacterial compounds, only expressed in the co-culture (Yu et al. 2019).

Abiotic stress has also been reported as a useful tool to enhance the biosynthesis of fungal polyketides. The chemical stress caused to the phytopathogenic fungus *Curvularia lunata* by the addition of sodium and calcium chloride, and

glycyrrhetinic acid, led to enhanced production of the polyketides radicinin (**57**), radicinol (**58**), and 3-epiradicinol (**59**) in up to 30 times. The salts were more effective stressing agents, probably because they trigger osmotic stress and, consequently, water uptake, leading to imbalance ionic environment inside the cells (Srivastava et al. 2021).

17.3.2 Synthetic Preparation of Polyketides and Derivatives

There are many strategies being applied to synthetically obtain polyketides with industrial and environmental interest. The advantage of exploring these routes of synthesis mainly relies in the search for improved yields and quicker processes for scaling-up.

The natural polyketide 6-pentyl-2*H*-pyrone-2-one (**60**) and other natural products are generated from the marine-derived fungi *Trichoderma reesei* (TR–13–01) and *T. atroviride* (TA–13–01), which were isolated from the marine sponge *Niphates* sp. (Khan et al. 2021). The high antimicrobial activity of this pyranone encouraged Khan et al. (2021) to synthesize this natural product and some analogs (**61** and **62**), seeking to prepare compounds to prevent marine biofouling, a widespread phenomenon in oceans worldwide. The development of nontoxic antifouling paint, in contrast of organotin and copper-based paints, reduces the environmental impact caused by increasing human activities in open and coastal waters. The activity of the synthesized compounds was evaluated in barnacle cyprid settlement assays, biofilm formation, and antimicrobial assays against marine bacteria, indicating 6-pentyl-2*H*-pyrone-2-one (**60**) as a good prototype entity with antifouling potential. The synthesized pyranone derivatives **61** and **62** demonstrated higher antifouling potential. The pyrone analogs were synthesized using two different alkynes, through a gold-catalyzed coupling reaction employing silver trifluoromethane sulphonate (AgOTf) as a catalyst in dichloromethane, during 12 h (Fig. 17.7).

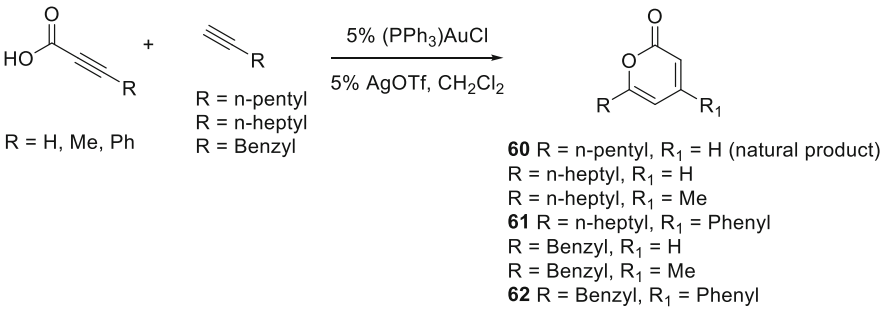
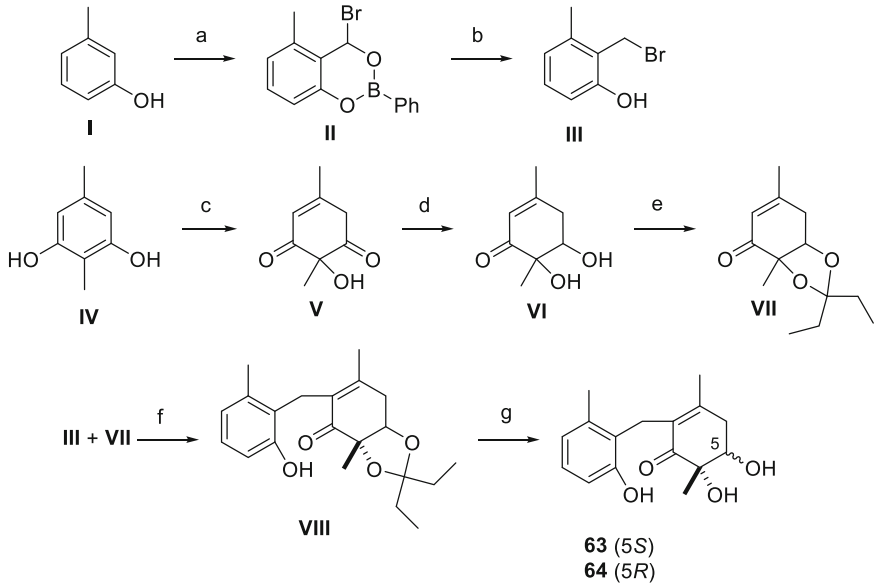


Fig. 17.7 Synthesis of the natural polyketide 6-pentyl-2*H*-pyrone-2-one (**60**) and its derivatives (**61** and **62**) (modified from Khan et al. 2021)

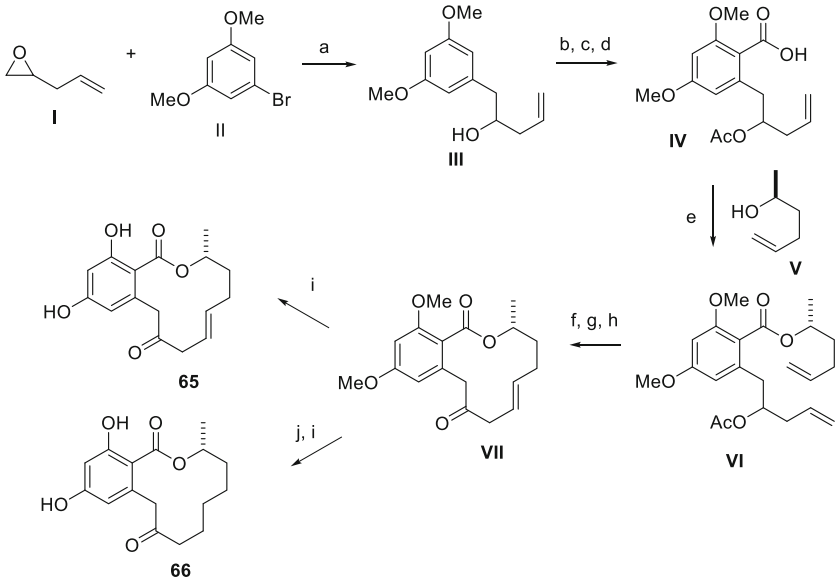


a) $PhB(OH)_2$, BrCHO; b) *terc*-$BuNH_2.BH_3$, $AlCl_3$; c) SIBX; d) [(*S*) or (*R*)-BINAP-Ru], H_2; e) 3-pentenone, TsOH, THF; f) $PhS(CH_2)_2OH$, $CsCO_3$, CH_3CN/*tert*-BuOH (1:1), 24h, 80 °C; g) TFA, H_2O, THF.

Fig. 17.8 Synthesis of the natural microketides A (**63**) and B (**64**) (modified from Wu 2021)

The C-11 epimeric polyketides microketides A (**63**) and B (**64**) were originally isolated from the marine gorgonian-derived fungus *Microsphaeropsis* sp. RA10-14. Both presented antimicrobial activity against several fungi and bacteria (Liu et al. 2020), motivating their synthesis through a convergent strategy, from two precursors (Fig. 17.8) (Wu 2021). The first precursor (III) was prepared by an *ortho*-specific alkylation of phenols, and the second precursor (VII) was synthesized oxidizing and then reducing the phenols. After using Noyori asymmetric hydrogenation to reduce intermediate V producing compound VI, which was protected as an acetonide (VII), a Morita–Baylis–Hillman reaction between the precursors III and VII provided microketides A (**63**) and B (**64**) after deprotection of compound VIII.

The macrocyclic lactones of RAL class are polyketides isolated from a range of fungal strains like *Lasiodiplodia theobromae*, *Penicillium* sp., and *Syncephalastrum racemosum* (Das and Reddy 2021). RAL were synthetically obtained, since this class of metabolites exhibits a large spectrum of biological activities (Das and Reddy 2021). The synthesis of these compounds was described (Fig. 17.9) starting with a Grignard reagent, obtained from aryl bromide (II), which opened the epoxide (I), gave the corresponding secondary alcohol (III), that was protected before reacting with a benzoic acid, prepared via Vilsmeier–Haack formylation, followed by Pinnick oxidation giving intermediate IV. The latter was coupled with the chiral alcohol (V) under Mitsunobu reaction condition. Using Grubb's II catalyst, a ring-closing



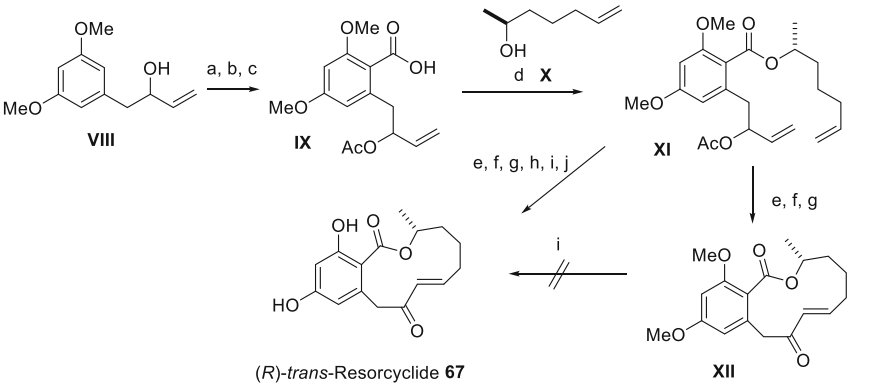
a) Mg, THF, 1h, CuI, -78 °C to rt, 12h, 86%; b) Et_3N, Ac_2O, DMAP, CH_2Cl_2, 1h; c) $POCl_3$, DMF, 4h; d) NaH_2PO_4, $NaClO_2$, DMSO, H_2O, 5 °C to rt, 53% (two steps); e) PPh_3, DIAD, THF, 0 °C to rt, 12h; f) Grubbs' II, CH_2Cl_2, reflux, 30 min, 65%; g) K_2CO_3, MeOH, 0 °C to rt, 1h; h) DMP, CH_2Cl_2, 0 °C to rt, 4h, 63% (two steps); i) AlI_3, TBAI, phloroglucinol, benzene, 5-7 °C, 34%; j) H_2 (ballon), 10% Pd/C, EtOH, 5h.

Fig. 17.9 Synthesis of (*R*)-penicimenolide (**65**) and (*R*)-dihydroresorcyclide (**66**) (modified from Das and Reddy 2021)

metathesis allowed the obtaining of macrolactone (VII) from diene (VI). After deprotection of compound VII, a Dess–Martin oxidation and removal of the methyl groups, the (*R*)-penicimenolide (**65**) was achieved (34% yield). The macrolactone (VII) was hydrogenated and demethylated, to furnish RAL12 fungal polyketide (*R*)-dihydroresorcyclide (**66**).

The polyketide (*R*)-trans-resorcyclide (**67**) was also prepared synthetically (Fig. 17.10), starting from 3,5-dimethoxybenzaldehyde, which was converted in the allyl alcohol (VIII) (Gualandi et al. 2014). After protection of the hydroxyl group, formylation (Vilsmeier–Haack reaction), followed by oxidation (Pinnick reaction), gave the compound IX. Esterification of IX using the alcohol (X), under Mitsunobu conditions, provide the diene (XI). A further sequence of metathesis, deprotection, and oxidation steps furnished the dimethylated macrolactone (XII). To overcome the problem of removing the methyl groups from intermediate XII, the double bond was masked though the formation of a selenide intermediate, which was totally demethylated using AlI_3, and then the selenide was oxidated and eliminated in a one-pot reaction, to finally furnish (*R*)-trans-resorcyclide (**67**).

The polyketide 5′-methoxy-6-methyl-[1,1′-biphenyl]-3,3′,4-triol (**68**) (Fig. 17.11) was isolated from *Ulocladium* sp., *Nigrospora sphaerica*, *Phialiphora* sp., *Penicillium pinophilum* SD-272, *Alternaria* sp., and from the endophytic fungus



a) Et_3N, Ac_2O, DMAP, CH_2Cl_2, 1h; b) $POCl_3$, DMF, 4h; c) NaH_2PO_4, $NaClO_2$, DMSO, H_2O, 5 °C to rt, 5h, 58% (two steps); d) PPh_3, DIAD, THF, 0 °C to rt, 12h, 62%; e) Grubbs' II, CH_2Cl_2, reflux, 45 min, 61%; f) K_2CO_3, MeOH, 0 °C to rt, 1h; g) DMP, CH_2Cl_2, 0 °C to rt, 4h, 60% (two steps); h) Ph_2Se_2, $NaBH_4$, EtOH, AcOH, THF, rt, 10 min, 84%; i) AlI_3, TBAI, phloroglucinol, benzene, 5-7 °C; j) H_2O (30%), AcOH, THF, H_2O, 0 °C, 2h, 54% (two steps).

Fig. 17.10 Synthesis of (*R*)-trans-resorcyclide (**67**) (modified from Gualandi et al. 2014)

Botryosphaeria dothidea in *Melia azedarach*. It shows relevant DPPH radical scavenging activities, with determined half maximal inhibitory concentration (IC_{50}) values of 18.7 ± 0.2 μM and 148 ± 3 μM, respectively, and exhibits inhibitory activity against three tyrosine kinases (EGFR, VEGFR-1, and c-Met) (Warmuth et al. 2021). For the chemical preparation of **68**, a convergent synthesis was conceived, employing a Suzuki cross coupling to join two intermediates, a boronate and an arylbromide. For the synthesis of the first intermediate (Fig. 17.11a), it was envisaged the use of a silyl protecting group, *tert*-butyldimethylsilyl group (TBS), suitable for all steps. The 4-methylcatechol was used as starting material which was protected with TBSCl in the presence of 4-(dimethylamino)pyridine and imidazole to furnish the disilylated compound (I). Using *N*-bromosuccinimide (NBS) in acetonitrile, the bromide (III) was obtained in excellent yield. The formation of boronate (V) was finished treating III with butyllithium and trapping with 2-isopropoxy-4,4,5,5-tetramethyl-1,3,2-dioxaborolane. The other electrophilic intermediate necessary to couple with boronic compound (IX) was prepared from the commercial 1-bromo-3,5-dimethoxybenzene (VII), which was mono-demethylated by using BBr_3 to give the phenol (VIII), that, in its turn, protected with TBS group to give IX (Fig. 17.11b). Finally, the Suzuki coupling led to the product XI with 98% yield (Fig. 17.11c). However, the desilylation of XI failed giving a mixture of products hard to separate. Because of this, several issues post-reaction had to be addressed before reaching the final product, changing the protect group (silyl to benzyl), allowing to obtain product **68** from compound XII, in the last step, with 88% yield.

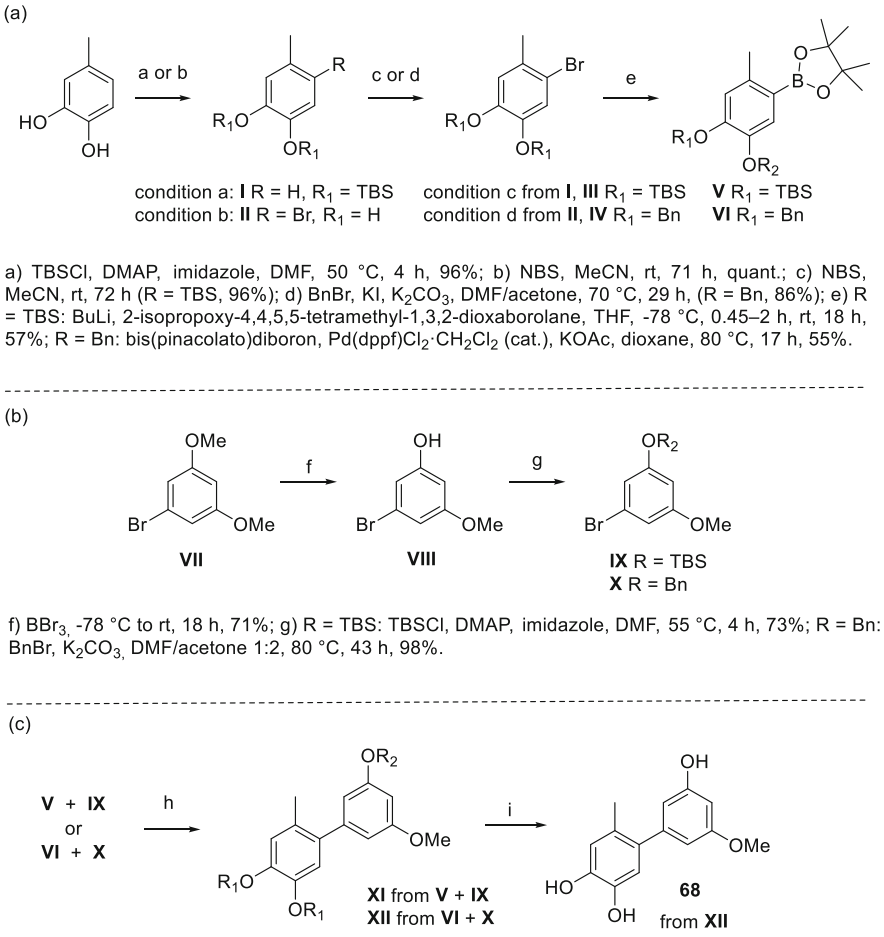


Fig. 17.11 Synthesis of 5′-Methoxy-6-methyl-[1,1′-biphenyl]-3,3′,4-triol (**68**): (**a**) preparation of intermediates boronates (V and VI); (**b**) preparation of intermediate aryl bromide (IX and X); (**c**) obtention of polyketide (**68**) from a Suzuki reaction between the boronate (V and VI) and aryl bromide (IX and X) after deprotection of (XII) (modified from Warmuth et al. 2021)

17.4 Overview of Fungi Polyketides as Lead Compounds for Biotechnological Applications

Polyketides originated from fungi and their synthetic/semi-synthetic derivatives have biological properties as antibiotic, antifungal, antiparasitic, immunosuppressant, anti-cholesterol, antitumoral, antidiabetic, insecticide, among other applications. Many fungal polyketides also have coloring properties that can be explored as

food pigments. In this topic, several biologically active polyketides from fungal origin are presented, and the scope of their biological action, source, biotechnological potential, and recent achievements are discussed.

17.4.1 Antimicrobial

17.4.1.1 Antibacterial

Fungi of the genus *Aspergillus* are prolific in the production of polyketides with antimicrobial activity. A strain of *Aspergillus niger*, isolated in Egypt as an endophytic from the marine alga *Laurencia obtuse*, produced six metabolites, including a new pentacyclic polyketide named RF-3192C (**69**), active against three bacteria species (*Pseudomonas aeruginosa*, *Bacillus subtilis*, and *S. aureus*) and the yeast *Saccharomyces cerevisiae*, in a trial using disc diffusion method (Mahmoud et al. 2021). Although the crude extract was active against *Candida albicans*, the metabolites isolated were inactive against this yeast and *C. albicans* inhibition may be related to a non-isolated metabolite or to synergic interactions (Table 17.1, Fig. 17.12). On the other side, the crude extract was unable to inhibit the growth of *P. aeruginosa*, while the activity of isolated RF-3192C (**69**) (inhibition zone, IZ, 15 mm) against this bacterium was comparable to positive control gentamycin (IZ 18 mm) (Mahmoud et al. 2021). The polyketide glycoside isotorachrysone 6-O-α-d-ribofuranoside (**70**), recovered from a culture of another Egyptian *Aspergillus* species, *A. ochraceopetaliformis*, showed broad antimicrobial activity in both disc diffusion and microdilution assays against *S. aureus, Staphylococcus epidermidis, P. aeruginosa, Klebsiella pneumoniae, Enterobacter cloacae, Escherichia coli, C. tropicalis,* and *C. glabrata* (IZ 11–17 mm; Minimum inhibitory concentration, MIC, 0.09–0.90 mg/mL) (Asmaey et al. 2021). *Aspergillus unguis* PSU-MF16, isolated from the sponge *Dysidea* sp. in Thailand, was reported to produce several metabolites, including six new polyketides. The diphenyl ether (**71**) was active against *S. aureus*, *Microsporum gypseum*, and a methicillin-resistant *S. aureus* (MRSA) strain (MIC 16 μg/mL) (Saetang et al. 2021). From the fifteen polyketides recovered from *Aspergillus versicolor*, isolated from Chinese deep-sea sediment, the metabolite 3,7-dihydroxy-1,9-dimethyldibenzofuran (**72**) stood out for being able to inhibit *S. aureus* and *Aeromonas salmonicida* (MIC 13.7 μM) (Yang et al. 2020) (Table 17.1, Fig. 17.12).

Two new 3,4-dihydroisocoumarins, 3 *R*-8-methoxy-3-(4-oxopentyl) isochroman-1-one (**73**), and 3 *R*-7-hydroxy-8-methoxy-3-(4-oxopentyl) isochroman-1-one (**74**) isolated from a *Penicillium* sp., collected from the Xinren coal area of Guizhou province in China, probably biosynthesized from malonyl-CoA and acetyl-CoA via a linear polyketide synthesis pathway, with further functionalization, were active against *C. albicans* and *S. epidermidis* (50–100 μg/mL). Other two 3,4-dihydroisocoumarins isolated from the same species, 5,6-dihydroxy-3*R*-(4S-hydroxypentyl)-isochroman-1-one (**75**) and 3 R-(7,8-dihydroxy-1-

Table 17.1 Fermentation parameters and scope of antibacterial activity of some fungal polyketides

Metabolite (#) [Activity]	Fungal species [Fermentation parameters]	Reference
Cladosin L (**31**) [*S. aureus* MIC 25–50 μM]	*Cladosporium sphaerospermum* WBS017 [100 g rice in 100 mL demineralized water; static conditions]	Pan et al. (2020)
RF-3192C (**69**) [*P. aeruginosa*: IZ 15 cm; *B. subtilis*: IZ 12 cm; *S. cerevisiae*: IZ 9 cm; *S. aureus*: IZ 7 cm]	*Aspergillus niger* SB4 [Rice medium: 100 g commercial rice in 100 mL 50% seawater; 14 days, 37 °C]	Mahmoud et al. (2021)
Isotorachrysone 6-O-α-d-ribofuranoside (**70**) [*S. aureus*, *S. epidermidis*, *P. aeruginosa*, *K. pneumoniae*, *E. cloacae*, *E. coli*, *C. tropicalis,* and *C. glabrata*: IZ 11–17 mm, MIC 0.09–0.90 mg/mL]	*Aspergillus ochraceopetaliformis* ASAI [100 g commercial rice in 150 mL 50% seawater;14 days, 35 °C]	Asmaey et al. (2021)
Diphenyl ether (**71**) [*S. aureus*, MRSA, and *Microsporum gypseum*: MIC 16 μg/mL]	*Aspergillus unguis* PSU-MF16 [Potato dextrose broth; 4 weeks, room temperature]	Saetang et al. (2021)
3,7-Dihydroxy-1,9-dimethyldibenzofuran (**72**) [*S. aureus* MIC 13.7 μM; *Aeromonas salmonicida* MIC 13.7 μM]	*Aspergillus versicolor* SH0105 [80 g rice in 80 mL seawater; 60 days, 25 °C]	Yang et al. (2020)
3 *R*-8-methoxy-3-(4-oxopentyl) isochroman-1-one (**73**) 3 *R*-7-hydroxy-8-methoxy-3-(4-oxopentyl) isochroman-1-one (**74**) [*C. albicans* MIC 50 μg/mL; *S. epidermidis* MIC 100 μg/mL]	*Penicillium* sp. XR046 [Solid rice medium]	Xu et al. (2019)
5,6-dihydroxy-3*R*-(4S-hydroxypentyl)-isochroman-1-one (**75**) 3 R-(7,8-dihydroxy-1-oxoisochroman-3-yl) propanoic acid (**76**) [*B. subtilis* MIC 100 μg/mL]		
Pseudophenone A (**77**) [*X. citri* MIC_{50} 36.16–44.19 μM; *S. aureus* MIC_{50} 47.44 μM; *A. salmonicida* MIC_{50} 36.90 μM; *P. fulva* MIC_{50} 35.64 μM]	*Pseudogymnoascus* sp. HSX2#-11 [Potato Dextrose Agar; 45 days, 16 °C]	Shi et al. (2021)
Pseudophenone A benzoic acid derivative (**78**) [*X. citri* MIC_{50} 40.83 μM; *S. aureus* MIC_{50} 56.93 μM; *A. salmonicida* MIC_{50} 26.86 μM; *P. fulva* MIC_{50} 37.61 μM]		
Arthproliferin A (**79**) [*S. aureus* MIC 78 μg/mL]	*Stachybotrys chartarum* SCSIO41201 [rice 200 g, NaCl 0.5 g, distilled water 200 mL; static conditions and daylight, 50 days, 25 °C]	Yang et al. (2021)

(continued)

Table 17.1 (continued)

Metabolite (#) [Activity]	Fungal species [Fermentation parameters]	Reference
(2*S*)-2,3-dihydro-5,6-dihydroxy-2-methyl-4H-1-benzopyran-4-one (**80**) [*B. cereus* MIC 12.5 μg/mL]	*Colletotrichum gloeosporioides* [50 g rice in 50 mL seawater; 30 days, static conditions, room temperature]	Luo et al. (2019)
4-ethyl-3-hydroxy-6-propenyl-2H-pyran-2-one (**81**) [*S. aureus* and *S. albus* MIC 12.5 μg/mL]		
Daldispone B (**82**) [*S. aureus* MIC 32 μg/mL; *E. faecalis* MIC 16 μg/mL; *B. cereus* MIC 32 μg/mL]	*Daldinia* sp. CPCC 400770 [80 g rice in 120 mL distilled water; 40 days, 30 °C]	Gu et al. (2021)
Koninginin W (**83**) [*E. coli* MIC 128 μg/mL, *B. subtilis* MIC 128 μg/mL; *S. typhimurium* MIC 64 μg/mL]	*Trichoderma koningiopsis* YIM PH30002 [Fermentation parameters not provided]	Wang et al. (2021d)

Notes: IZ inhibition zone (disc diffusion assay), MIC Minimum inhibitory concentration (microdilution assay), MRSA methicillin-resistant *S. aureus*

oxoisochroman-3-yl) propanoic acid (**76**), were the only metabolites active against *B. subtilis*. The activity of the latter was related to the ortho-dihydroxy function in the benzene ring, which is not present in the structures of (**73** and **74**) (Xu et al. 2019) (Table 17.1, Fig. 17.12).

A broad screening with five phytopathogenic bacteria (*Xanthomonas citri* pv. *malvacearum*, *X. citri*, *Pseudomonas syringae*, *Dickeya chrysanthemi*, and *Erwinia amylovora*), four animal pathogenic bacteria (*E. coli*, *S. aureus*, *P. aeruginosa*, and *B. subtilis*), and eight marine fouling bacteria (*P. fulva*, *Aeromonas hydrophila*, *A. salmonicida*, *Vibrio anguillarum*, *V. harveyi*, *Photobacterium halotolerans*, *P. angustum*, and *E. cloacae*) was conducted with pseudophenone A (**77**) and its benzoic acid derivative (**78**) (Table 17.1, Fig. 17.12), both isolated from the psychrophilic pathogenic Antarctic fungus *Pseudogymnoascus* sp. Interestingly, these compounds did not show cytotoxicity against five human cancer cell lines (Shi et al. 2021).

Antimicrobial polyketides with complex structural features have been frequently reported from fungi associated to marine organisms. A comprehensive screening of more than 200 strains of symbiotic and epiphytic fungi isolated from the soft coral *Sinularia* sp., collected in the South China Sea, led to the isolation of the fungal strain *Stachybotrys chartarum* (Yang et al. 2021). This species furnished four new polyketide derivatives, arthproliferins A–D, along with other thirteen metabolites. Arthproliferin A (**79**) was inactive against *Acinetobacter baumannii*, *K. pneumoniae*, *E. coli*, *Aeromonas hydrophila*, and *Enterococcus faecalis*, but displayed moderate inhibitory activity against *S. aureus* (MIC 78 μg/mL) (Table 17.1, Fig. 17.12).

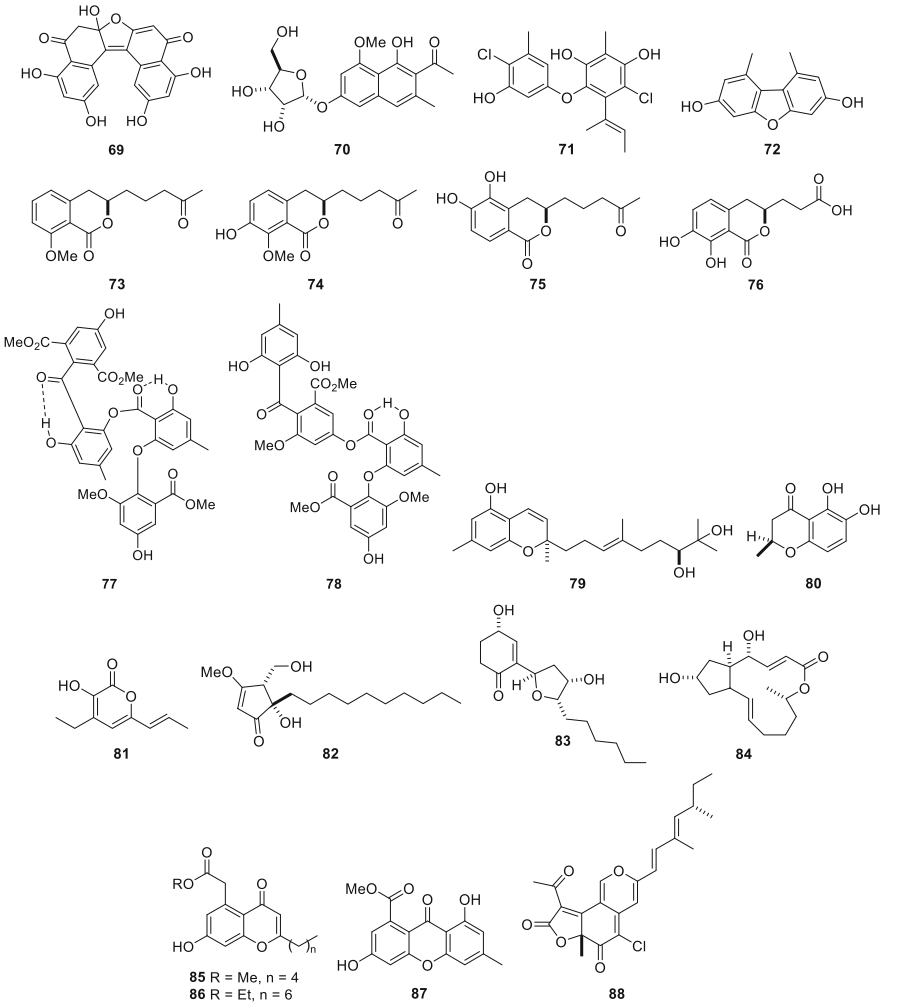


Fig. 17.12 Fungal polyketides and derivatives with antimicrobial applications: RF-3192C (**69**), isotorachrysone 6-O-α-d-ribofuranoside (**70**), diphenyl ether (**71**), 3,7-dihydroxy-1,9-dimethyldibenzofuran (**72**), 3 *R*-8-methoxy-3-(4-oxopentyl) isochroman-1-one (**73**), 3 *R*-7-hydroxy-8-methoxy-3-(4-oxopentyl) isochroman-1-one (**74**), 5,6-dihydroxy-3*R*-(4S-hydroxypentyl)-isochroman-1-one (**75**), 3 R-(7,8-dihydroxy-1-oxoisochroman-3-yl) propanoic acid (**76**), pseudophenone A (**77**) and its benzoic acid derivative (**78**), arthproliferin A (**79**), (2*S*)-2,3-dihydro-5,6-dihydroxy-2-methyl-4H-1-benzopyran-4-one (**80**), 4-ethyl-3-hydroxy-6-propenyl-2H-pyran-2-one (**81**), daldispone B (**82**), koninginin W (**83**), brefeldin A (**84**), pestalotiopsone F (**85**), pestalotiopsone B (**86**), 3,8-dihydroxy-6-methyl-9-oxo-9*H*-xanthene-1-carboxylate (**87**), and 5-chloroisorotiorin (**88**)

The already mentioned polyketide asperterrein (**54**), obtained in special conditions of co-culture (see Sect. 17.3.2), was able to inhibit the bacteria *Alternaria brassicae*, *E. coli*, *Physalospora piricola*, and *S. aureus* (MIC 4–64 μg/mL) (Li et al. 2020). Another example is borrelidin J (**55**), which demonstrated significant activity against methicillin-resistant *S. aureus* (MIC 0.195 μg/mL) (Yu et al. 2019). Radicinin (**57**), obtained under stressing culture conditions, was also identified as an antibacterial agent active against the phytopathogenic *Xylella fastidiosa* (Aldrich et al. 2015).

Other fungal species capable of producing antibacterial polyketides include *Cladosporium sphaerospermum* (Pan et al. 2020), *Colletotrichum gloeosporioides* (Luo et al. 2019), *Daldinia* sp. (Gu et al. 2021), *Trichoderma koningiopsis* YIM PH30002 of *Panax notoginseng* (Wang et al. 2021d) (Table 17.1, Fig. 17.12).

17.4.1.2 Antifungal

Griseofulvin (**22**) (Fig. 17.2) and echinocandins (Fig. 17.1) are, respectively, polyketide and peptide-polyketide mixed drugs acting on fungi. Griseofulvin (**22**) was first discovered by Oxford et al. in 1939 and tested as an antifungal agent in humans in the 1950s. Since then, more than 400 analogs have been synthesized (Petersen et al. 2017). It is a secondary metabolite of the fungus *Penicillium griseofulvum* with selective inhibitory activity in the assembly of microtubules, and thus the first example of a product from one fungus being used to attack another (Odds 2003). Griseofulvin (**22**) (GRIS-PEG®, Bausch Health) is indicated for the treatment of dermatophyte infections tinea corporis (ringworm of the body), tinea pedis (athlete's foot), tinea cruris (ringworm of the groin and thigh), tinea barbae (barber's itch), tinea capitis (ringworm of the scalp), and tinea unguium (onychomycosis, ringworm of the nails).

Echinocandin B (**4**) was first discovered in 1974 by a group from Ciba-Geigy AG (Switzerland) as a metabolite of *Aspergillus delacroxii* (former *Aspergillus nidulans* var. *echinulatus*) and *Aspergillus rugulosus* (Hüttel 2021). Currently, strains of more than 20 fungal species are reported to produce echinocandins, of which echinocandin B (**4**) from diverse *Aspergillus* (Emericella) is the most common product. Echinocandins are cyclic non-ribosomal hexapeptides equipped with a lipophilic side chain. They are produced by filamentous fungi (Ascomycota) of the classes Leotiomycetes (mostly Helotiales) and Eurotiomycetes (Aspergillaceae). Echinocandins are potent antifungal compounds, due to their strong inhibitory effect on 1,3-β-D-glucan synthase, an enzyme required for the biosynthesis of β(1,3)-D-glucan, one of the main polysaccharides of the fungal cell wall. A fungistatic effect is observed as result of limited cell growth due to inhibition of cell wall synthesis. While a fungicidal effect is the result of the cellular destruction due to changes in the integrity of fungal cell wall, leading to loss of mechanical strength and failure to maintain intracellular osmotic pressure (Hashemian et al. 2020).

The semi-synthetic derivatives of echinocandins include caspofungin acetate (**5**) (Cancidas®), micafungin (**6**) (Mycamine®), and anidulafungin (**7**) (Eraxis™) and

they are first-line antimycotics for the treatment of invasive mycosis (Denning 2002; Patil and Majumdar 2017). Caspofungin acetate (**5**) is the first member of echinocandins that was approved by the U.S. Food and Drug Administration (FDA) against fungal infection in 2001. It is indicated in adults and pediatric patients for treatment of invasive candidiasis, aspergillosis, and invasive aspergillosis in patients that were refractory to amphotericin B (Heinz et al. 2016; Fisher et al. 2019). Caspofungin (**5**) may have fungicidal effects on *Candida* spp. and fungistatic effect against filamentous fungi such as *Aspergillus* species (Horan-Saullo and Alexander 2016).

Micafungin sodium (**6**) is a semi-synthetic lipopeptide echinocandin, synthesized by a chemical modification of a fermentation product of *Coleophoma empetri* F-11899. It is indicated for treatment of esophageal candidiasis, candidemia, and prophylaxis of *Candida* infections in patients undergoing hematopoietic stem cell transplantation. Micafungin (**6**) has also strong inhibitory effects against both adhesion and biofilm formation of different *Candida* species, like *C. guilliermondii*, *C. tropicalis*, and *C. parapsilosis* (Zuo et al. 2021). These *Candida* species had varied biofilm-forming capabilities and are very important pathogens in catheter-related candidemia patients.

Anidulafungin (**7**) is indicated for the treatment of candidemia and other forms of *Candida* infections (intra-abdominal abscess and peritonitis) in adults and pediatric patients.

17.4.1.3 Antivirals

Fungal secondary metabolites are likewise a potential and valuable source in drug screening for the development of antiviral agents. Many fungal polyketides are under in vitro screening with promising results. For instance, mycophenolic acid (**1**) (Fig. 17.1) became a promising drug candidate against HIV-1 (Chapuis et al. 2000), vesicular stomatitis Indiana virus, hepatitis C virus (Henry et al. 2006), Dengue virus (Takhampunya et al. 2006), Zika virus (Barrows et al. 2016), and SARS-CoV-2 (Kato et al. 2020).

Likewise, brefeldin A (**84**), isolated from *Penicillium* sp. FKI-7127, has shown antiviral activity against Dengue virus (subtypes 1–4) and Zika virus, besides Japanese encephalitis virus, demonstrated by focus reduction assay in Vero cell (Raekiansyah et al. 2017). It is also a potent inhibitor of poliovirus RNA replication (Crotty et al. 2004).

Among 28 aromatic polyketides from the mangrove-associated fungus *Diaporthe* sp. SCSIO 41011, pestalotiopsone F (**85**), pestalotiopsone B (**86**), 3,8-dihydroxy-6-methyl-9-oxo-9*H*-xanthene-1-carboxylate (**87**), and 5-chloroisorotiorin (**88**) displayed pronounced anti-IAV activities against three virus subtypes, including A/Puerto Rico/8/34 H274Y (H1N1), A/FM-1/1/47 (H1N1), and A/Aichi/2/68 (H3N2) (Luo et al. 2018).

17.4.2 Antiparasitic

Three new polyketides active against malaria were recently isolated from the basidiomycete fungus *Favolaschia* sp. BCC 18686 [X (**89**), Y (**90**) and Z (**91**), Fig. 17.13)] (Kornsakulkarn et al. 2020). Other 14 new compounds, in addition to another nine known, were also isolated from the same strain. These polyketides were subjected to biological assays for antiplasmodial activity using *Plasmodium falciparum* (strain K1) and showed discrete values of IC_{50} (X > 45 μM, Y > 38 μM, and Z > 43 μM).

Fungal polyketide-nonribosomal peptide (PK-NRP) hybrid macrolactones, such as the thermolides A (**49**) and B (**50**) (Fig. 17.6), are produced by extreme thermophilic fungi and exhibit strong nematocidal activity (Zhang et al. 2020b). Structurally, thermolides are thirteen-membered lactam-bearing macrolactones that possess a polyketide chain featuring eleven chiral centers and an unnatural amino acid D-alanine or D-valine. The activities of thermolides A (**49**) and B (**50**) are comparable to those of commercial avermectins (Guo et al. 2012), and they are therefore considered valuable nematocidal agents for biological control in agriculture (Degenkolb and Vilcinskas 2016; Zhai et al. 2016).

The polyketide ravenelin (**92**) (Fig. 17.13) was isolated from the biomass extracts of *Exserohilum rostratum* fungus, and its antiplasmodial and trypanocidal activities were evaluated (Pina et al. 2021). Compound **92** was tested in vitro against cultures of *P. falciparum* 3D7, a chloroquine-sensitive strain, and against the epimastigote and intracellular amastigote forms of *Trypanosoma cruzi*. The compound showed antiplasmodial (IC_{50} 3.4 μM) and trypanocidal activities (IC_{50} 5.0 μM and 9.0 μM, respectively, for epi and amastigote forms) in the low micromolar range.

17.4.3 Antidiabetic/Hypoglycemic and Hypolipidemic

Polyketides are promising in the development of antidiabetic drugs. A major effect of polyketides in diabetes-associated conditions occurs via protein tyrosine phosphatase PTP1B, a protein that is also a target for developing anti-obesity and

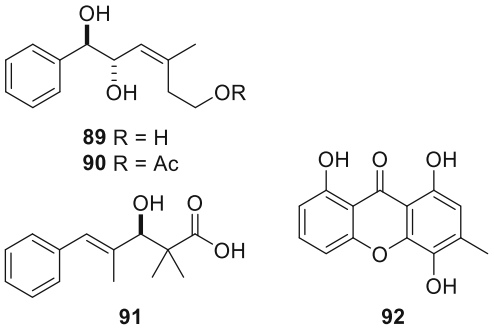


Fig. 17.13 Antiparasitic polyketides from fungi: compounds X (**89**), Y (**90**), and Z (**91**) from *Favolaschia* sp. BCC 18686, and ravenelin (**92**)

antitumor drugs. The inhibition of PTP1B has a positive influence in alleviating insulin resistance, therefore compounds able to inhibit this enzyme may have positive results in patients with type 2 diabetes.

Many polyketides produced by fungi of *Penicillium* genus, able to inhibit PTP1B have been described (Fig. 17.14), such as penipyrol C (**93**), a molecule with a rare γ-butyrolactone moiety linked to α-pyrone ring. This metabolite, isolated from the mangrove species *Penicillium* sp. HDN-11-131, induced the regeneration of pancreatic β cells in a zebrafish model; decrease of total glucose level was also detected for this metabolite (Wang et al. 2021e). Penicanesin D (**94**), an acyl phloroglucinol pyrone, is a highly oxygenated aromatic polyketide produced by the soil species *Penicillium canescens* CGMCC 3.79658. This metabolite, elicited by OSMAC approach, presented modest PTP1B inhibition at 50 μM (58.7%) (Zang et al. 2022). The polyketides 5-((*R*,1*Z*,3*E*)-6-hydroxy-1,3-heptadien-1-yl)-1,3-benzenediol (**95**), 4-carboxy-5-((*R*,1*Z*,3*E*)-6-hydroxy-1,3-heptadien-1-yl)-1,3-benzenediol (**96**), and 4-carboxy-5-((1*Z*,3*E*)-1,3-heptadien-1-yl)-1,3-benzenediol (**97**) were isolated from the marine fungal species *Penicillium* sp. TW58-16. These metabolites were suggested to be further studied as antidiabetic agents due to their strong α-glucosidase inhibitory activity (73.2%, 55.6%, and 74.4%, respectively, at 400 μM) (Gou et al. 2021).

Fungal species from other genera have also been described as PTP1B inhibitors (Fig. 17.14), as is the case of the marine-derived strain *Aspergillus* sp. SF-5929, from where the polyketides (±)-tylopilusin D (**98**) and funalenone (**99**) were recovered (IC_{50} 8.1 and 6.1 μM, respectively) (Kim et al. 2020). The hypoglycemic activity of butyrolactone I (**100**), a polyketide produced by several strains of *Aspergillus terreus,* led to the synthesis of butanolide derivatives, after modification of the butyrolactone side chain. Studies using molecular docking simulation compared to experimental results over PTP1B inhibition of the butanolide derivatives pointed that the chiral center at C-4 has deep influence in PTP1B inhibition (Hong et al. 2020).

Neglectine A (**101**), a metabolite bearing a bis-tetrahydrofuran ring, was isolated from endophytic fungus *Pestalotiopsis neglecta*, was able to inhibit PTP1B (IC_{50} 6.7 μg/mL) (Gao et al. 2019a). Other interesting PTP1B inhibitors comprise (+)-palitantin (**102**), isolated from the Antarctic fungus *Geomyces* sp. 3-1 (IC_{50} 7.9 μM) (Yu et al. 2021), and chrysopyrones A (**103**) and B (**104**), metabolites of the deep-sea fungus *Penicillium chrysogenum* SCSIO 07007 (IC_{50} 9.32 and 27.8 μg/mL, respectively) (Han et al. 2020) (Fig. 17.14).

A comprehensive review on antidiabetic fungal agents, with focus on fungal metabolites able to inhibit PTP1B, is available at Nazir et al. (2021), while the review of Hussain et al. (2021) emphasizes the α-glucosidase inhibitors.

Another group of drugs associated to the treatment of diabetes consists of competitive inhibitors of 3-hydroxy-3-methyl-glutaryl coenzyme A (HMG-CoA) reductase, which converts HMG-CoA to mevalonate (Endo 2017). This mechanism is associated with statins, the most effective and best-tolerated agents for treating dyslipidemia, highly prescribed to diabetic individuals. As pointed before, statins are a group of exceptionally successful drugs that lower cholesterol levels in blood,

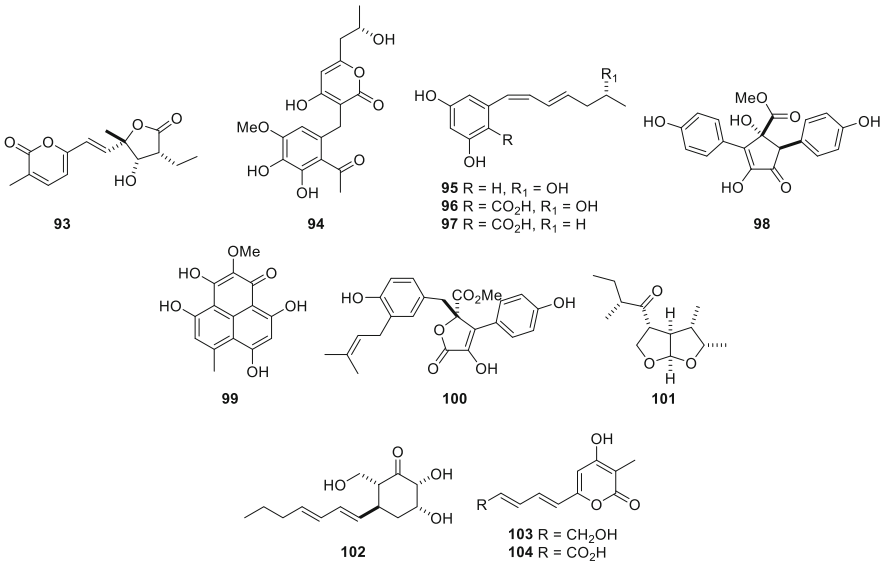


Fig. 17.14 Fungal polyketides with antidiabetic/hypoglycemic effect: penipyrol C (**93**), penicanesin D (**94**), 5-((R,1Z,3E)-6-hydroxy-1,3-heptadien-1-yl)-1,3-benzenediol (**95**), 4-carboxy-5-((R,1Z,3E)-6-hydroxy-1,3-heptadien-1-yl)-1,3-benzenediol (**96**), 4-carboxy-5-((1Z,3E)-1,3-heptadien-1-yl)-1,3-benzenediol (**97**), (±)-tylopilusin D (**98**), funalenone (**99**), butyrolactone I (**100**), neglectine A (**101**), (+)-palitantin (**102**), chrysopyrones A (**103**) and B (**104**)

widely used in the prevention and management of cardiovascular disease, decreasing the risk of heart attack or stroke (Barrios-González et al. 2020). The remarkable safety of statins derives from their unique mechanism of action. When statin is ingested, the drug is routed primarily to the liver, where it inhibits HMG-CoA reductase, lowering cholesterol production. This decrease in liver cholesterol triggers a compensatory feedback loop that increases the number of receptors for low density lipoprotein (LDL), displayed on the hepatocyte membrane. These LDL receptors grab onto LDL and remove it from blood (Endo 2017).

Natural statins, like compactin (**8**) and lovastatin (**9**) (Fig. 17.1), are produced as secondary metabolites, by direct fungal fermentation following a polyketide pathway, predominantly by *Aspergillus* and *Penicillium* species (Subhan et al. 2017). Pravastatin (**105**) (Fig. 17.15) is derived from compactin by biotransformation, and simvastatin (**10**) (Fig. 17.3) is a semi-synthetic derivative of lovastatin (Tobert 2003). Chemically synthesized statins include atorvastatin (**11**) (Fig. 17.1), rosuvastatin, fluvastatin, and pitavastatin (Endo 2017).

Other polyketides are being proposed for hypercholesterolemia treatment, for example, zaragozic acid A (squalestatin) (**106**), B (**107**) (Fig. 17.15), C, D and D2, isolated from different fungi sources, such as *Sporormiella intermedia*, *Leptodontium elatius*, and *Cladosporium cladosporioides* (Lebe and Cox 2019). They are characterized by a 2,8-dioxobicyclic[3.2.1]octane-3,4,5-tricarboxyclic acid core connected to two lipophilic polyketides or fatty acid derived chains (Liu et al. 2017). Their structure comprises two benzoic acids extended with acetate-derived

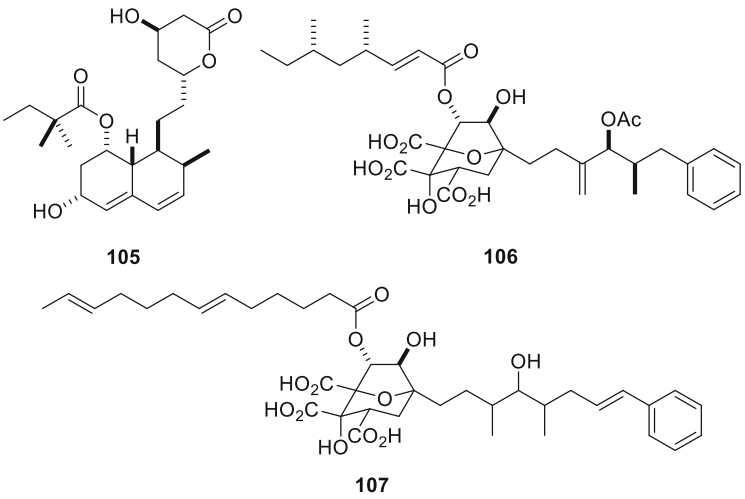


Fig. 17.15 Chemical structure of some fungal polyketides with hypolipidemic activity: the semi-synthetic statin pravastatin (**105**), and zaragozic acids A (**106**) and B (**107**). Other statins are presented on Figs. 17.1 and 17.3

chains and an oxaloacetate unit from the Krebs cycle. Zaragozic acids are inhibitors of fungal and mammalian squalene synthase, the first committed enzyme in sterol synthesis, and an alternative potential target to control cholesterol blood levels (Dufresne et al. 1993; Bergstrom et al. 1995; Rimondi et al. 2021).

17.4.4 Anti-Inflammatory and Immunosuppressant

Besides the effect of lowering LDL and cholesterol, statins (Figs. 17.1, 17.3 and 17.15) present cholesterol-independent pleiotropic effects. This topic remains controversial, however, there is consistent evidence that statins affect downstream steps of the mevalonate pathway, thus influencing inflammation, immunomodulation, the nitric oxide production, the coagulation cascade, and other biochemical processes (Steffens and Mach 2004; Undas et al. 2005; Kagami et al. 2009; Oesterle et al. 2017; Rimondi et al. 2021). The anti-inflammatory effects of statins make them promising candidates for the future treatment of inflammatory and immune-mediated disorders.

In fact, statins were recommended as a first-line therapy for atherosclerotic cardiovascular disease (ASCVD) risk reduction, based on high-quality data regarding the consistent reduction of events with their regular use, in primary and secondary prevention of atherosclerosis (Adhyaru and Jacobson 2018). Chronic inflammation has been recognized as one of the most important pathophysiological mechanisms of initiation and progression of atherosclerosis, thus, the suppression of inflammatory response by statins contributes to their generally positive action in

atherosclerosis and represents an important part of the vasculo- and athero-protective effect of this drug class (Satny et al. 2021).

The pleiotropic effects proposed for statins, that may contribute to reduce ASCVD, are plaque stabilization with increased fibrous cap thickness and macrocalcification, reduced platelet aggregation, decreased oxidative stress by reducing reactive oxygen species, improved vascular tone by increasing nitric oxide, and anti-inflammatory effects with reduction of interleukins and decreased leukocyte-endothelial adhesion (Almeida and Budoff 2019). The cytokines secreted by adipose tissue, so-called adipokines, have been proved to play a critical role in inflammation, and atherosclerosis development and vulnerability. Up to now, robust evidence implicates a significant statin-induced reduction of pro-inflammatory adipokines IL-6, TNF-α and visfatin (Kadoglou et al. 2021).

Additionally, due to their anti-inflammatory and immunomodulatory features, statins have been studied as complementary therapy for various autoimmune diseases, like multiple sclerosis, systemic lupus erythematosus, rheumatoid arthritis, Hashimoto's thyroiditis, psoriasis, and antiphospholipid syndrome (Dehnavi et al. 2020). The underlying mechanisms, the precise doses required to exert immunomodulatory effects, as well as the doses that trigger a statin-mediated autoimmune response as a side effect of dyslipidemia therapy, remain unclear.

Atorvastatin (**11**) and simvastatin (**10**) are among the most studied drugs to treat autoimmune diseases. Simvastatin (**10**) has shown beneficial effects on reducing the rate of brain atrophy and slowing the deterioration of disability in progressive multiple sclerosis, independently of serum cholesterol reduction (Eshaghi et al. 2019). Evidence suggests this may be due to its effect on vascular function and cell protection. Several clinical trials have shown benefits or no significant difference in clinical outcomes of patients treated with statins alone or in combination with other drugs (Dehnavi et al. 2020). Although, the USA Food and Drug Administration (FDA) has not yet approved statins for multiple sclerosis treatment.

Several other fungal polyketides have anti-inflammatory and immunosuppressive properties. Mycophenolic acid (**1**), already cited due to its historical role as an immunomodulatory agent, has also anti-inflammatory properties. Its ester derivative, mycophenolate mofetil (**2**) (Fig. 17.1) is a prodrug with improved bioavailability, efficacy, and has better side-effect profile. This ester is mostly hydrolyzed in the upper gastrointestinal tract, to the active drug, mycophenolic acid, a selective, noncompetitive, and reversible inhibitor of inosine monophosphate dehydrogenase (IMPDH). IMPDH is an important enzyme in the de novo pathway of guanine nucleotide synthesis (Ransom 1995). Mycophenolate mofetil (**2**) depletes guanosine nucleotides preferentially in T and B lymphocytes to inhibit their proliferation. Considering that clonal expansion of lymphocytes is a hallmark of the adaptive immunity, mycophenolate mofetil inhibits cellular and humoral immune response. It also inhibits the glycosylation and expression of adhesion molecules, and the recruitment of lymphocytes and monocytes into sites of inflammation (Allison 2005).

Mycophenolate mofetil (**2**) is recommended for prophylaxis of solid organ transplant rejection, typically used in combination with glucocorticoids, as part of

the immunosuppressive induction therapy for transplanted patients. It is also a mainstay of the therapy in lupus nephritis and a second-line immunosuppressive agent in various immunological diseases (e.g., renal vasculitis, idiopathic nephrotic syndrome, scleroderma, and myasthenia gravis) (Morren and Li 2020; Walters et al. 2020; Lugani et al. 2021; Martini et al. 2021). Mycophenolate mofetil (**2**) and enteric-coated mycophenolate sodium (**3**) (EC-MPS) are drugs approved for the immunosuppressive therapy in solid organ transplantation, but the use is off label for all immunological diseases (Ehren et al. 2021).

Moreover, evidence is accumulating that mycophenolate mofetil (**2**) has clinical utility to treat other autoimmune diseases, like systemic lupus erythematosus (Trindade et al. 2021). This compound has been also trialed for a first-line treatment of immune thrombocytopenia, in addition to a glucocorticoid, and results showed greater response, besides a lower risk of refractory or relapsed immune thrombocytopenia, in comparison to the standard glucocorticoid monotherapy (Bradbury et al. 2021).

In addition, experimental models have confirmed that mycophenolic acid (**1**) reduces the production of lymphocyte-derived cytokines, such as interferon-gamma and tumor necrosis factor alpha, pro-inflammatory cytokines produced by monocytes, along with the inhibition of primary humoral responses. In turn, mycophenolate mofetil (**2**) is an effective treatment for induction and maintenance of remission of antineutrophil cytoplasmic antibody-associated vasculitis (AAV) (Koukoulaki and Iatrou 2019).

17.4.5 Neuroprotective

Neurodegenerative diseases are currently a serious and complex public health problem. Humanity has reached new levels of health and well-being, along with an increase in life expectancy. However, well-being in old age has been greatly affected by neurodegenerative diseases that limit various cognitive functions, along with several other progressive symptoms that contribute to the withering of the individual as the age advances (Takahashi et al. 2020). Awareness of the severity of these diseases has prompted a large portion of the population to create healthy habits to delay the onset of degenerative diseases. New drugs for the treatment of patients with different degrees of degeneration are still needed, even with palliative effects. Neuroprotective compounds, to be ingested as medicines or nutraceuticals, are also necessary. These agents can work as hormones, neurotransmitter modulators, neurotrophic factors, antioxidants, or anti-inflammatory agents. In this way, a significant number of fungal polyketides are under study to understand their role in several aspects of neuroprotection (Tong et al. 2021; Wu et al. 2021).

Currently, virtual screening has been widely used in the search for new acetylcholinesterase inhibitors, with the advantage of allowing screening of large chemical databases, including synthetic derivatives, in the search for novel scaffolds (David et al. 2021). Diketopiperazine alkaloids are classical fungal polyketide, produced by

a variety of species, such as the mangrove-sediment-derived fungus *Aspergillus* sp. SCSIO41407 (Cai et al. 2021), and the Antarctic fungus *Penicillium* sp. SCSIO 05705 (Hu et al. 2021). In some studies, acetylcholinesterase inhibition is confirmed by molecular docking studies, as in the study of Sallam et al. (2021), working with metabolites isolated from a marine endophyte species *Cladosporium cladosporioides*, isolated from the leaves of the mangrove *Avicennia marina*. Endophyte fungal species isolated from plants from *Huperzia* sp. have been described as good sources of acetylcholinesterase inhibitors, since these herbs, used in the traditional Chinese medicine, produce huperzine A (**108**), a metabolite known due to its protective brain effects (Cao et al. 2021; Xiao et al. 2022) (Fig. 17.16).

However, metabolites with other mechanisms of neuroprotective activity have also been described (Fig. 17.16). The cytoprotective effect of terphenyllin (**109**), 3″-hydroxyterphenyllin (**110**), and 3′-hydroxyterphenyllin (**111**) against rotenone- and paraquat-induced oxidative stress in neuro-2a cells was reported by Yurchenko et al. (2021). The hydroxyl groups at C-3 and C-4 positions in the structures of these *p*-terphenyl polyketides, isolated from *Aspergillus candidus* KMM 4676, were claimed to modulate the activity, which is also influenced by the strong activity in ROS scavenging. Phomopsol (**112**), an unusual N-oxide alkaloid isolated from a mangrove endophyte species of *Phomopsis* sp., was also reported as a neuroprotective agent against corticosterone-induced injury in PC12 cells, as determined by MTT assays (Li et al. 2019).

Hispidin (**113**) (Fig. 17.16), produced by various mushrooms from the genus *Phellinus* and *Inonotus*, is another good candidate as a neuroprotective compound. This antioxidant compound was reported to activate the JNK-pathway and Nrf2 signaling. In addition, **113** can act in Parkinson and Alzheimer diseases, inhibiting BACE 1, which is responsible for stimulating the release of β-amyloid, a toxic

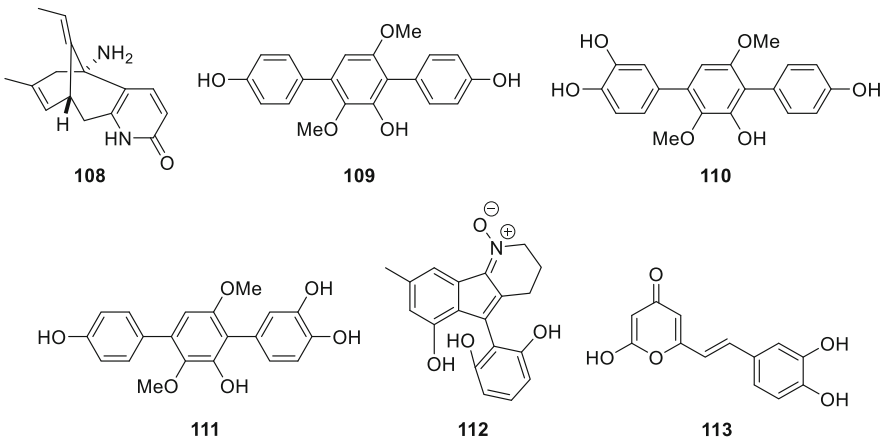


Fig. 17.16 The neuroprotective fungal polyketides huperzine A (**108**), terphenyllin (**109**), 3″--hydroxyterphenyllin (**110**), 3′-hydroxyterphenyllin (**111**), phomopsol (**112**), and hispidin (**113**)

peptide, in the brain. Several other effects of hispidin (**113**) were reviewed by Palkina et al. (2021).

17.4.6 Anticancer

For decades, fungal metabolites have been researched as lead compounds for the development of chemotherapeutics directed toward many types of cancer diseases. Considering the diversity of structures and biological properties presented by polyketides, this group offers new chemotherapeutical approaches to overcome issues that commonly affect the success of cancer treatment, such as drug resistance, insufficient selectivity to tumor cell, and side effects.

Marine fungi are revealed as a rich source of bioactive polyketides, including against cancer. Among polyketides of the 12-membered macrolide class, obtained by Sun et al. (2013) from the marine fungus *Dendrodochium* sp., isolated from the South China Sea *Holothuria nobilis* Selenka, ten structurally diverse dendrodolides were able to elicit in vitro cytotoxic effects against human tumor cell lines from hepatoma origin [dendrodolides A-D, H, I, and K (**114–117, 120–122**)] or colorectal carcinoma [dendrodolides C, E, G, I, K, and L (**116, 118, 119, 121–123**)] (Table 17.2, Fig. 17.17).

Emodin (**124**) and questin (**125**) are polyketones and quinones, isolated from the sea-cucumber-associated fungus *Aspergillus terreus*, which demonstrated cytotoxic effects on human oral epithelial cancer cells (KB) and multidrug-resistant KB cells (KBv200) (Xia et al. 2014a). Found in another sea-cucumber-associated fungus of the genus *Fusarium*, javanicin (**126**), norjavanicin (**127**), and fusarubin (**128**) were also able to inhibit KB and KBv200 cell lines (Xia et al. 2014b) (Table 17.2, Fig. 17.17).

Penicitrinone A (**129**), a polyketide derivative of citrinin, isolated from marine-derived *Penicillium purpurogenum* (G59 strain) by Wu et al. (2015), demonstrated cytotoxicity to human cancer cell lines from leukemias, uterus, and gastric carcinomas. The same compound was later obtained from *Penicillium citrinum* (SCSIO 41017) by Salendra et al. (2021), eliciting inhibitory effects toward the human breast cancer cell line MCF-7, comparable to taxol positive control (Table 17.2, Fig. 17.17).

Among the anticancer anthraquinones, one example is the polyketide 1,3-dihydroxy-6-methyl-7-methoxyanthraquinone (**130**), isolated from the marine thermophilus fungus *Thermomyces lanuginosus*. According to Sobolevskaya et al. (2021), it was responsible of reducing cell viability of a human drug-resistant prostate cancer cell line, with lower effects on non-tumoral control cell line. The compound (**130**) also elicited the reproductive death of those cancer cells, as revealed in colony formation assay (Table 17.2, Fig. 17.17).

Sulochrin (**131**) is another marine-derived fungal metabolite with in vitro anticancer activities. It was isolated from *Aspergillus falconensis* by El-Kashef et al. (2021), using the OSMAC approach, among other 10 polyketide compounds.

Table 17.2 Fungal polyketides with anticancer effect

Metabolite	Fungal Source	Activity	Reference
Pchaeglobosal B (**41**)	Endophytic *Chaetomium globosum* (P2-2-2)	Cytotoxicity to MCF-7, HepG2, HT29, A549, and CT26 (IC_{50} 1.04–9.90 μM) Apoptosis induced on CT26 (at 3-4 μM) and HT29 cells (at 12–15 μM), with cell cycle arrest at S phase on CT26 cells, and at G0/G1 phase on HT29 cells	Peng et al. (2020)
Zaragozic acid A (**106**)	*Sporormiella intermedia*, *Exserohilum khartoumense*	Inhibition of squalene synthase (IC_{50} 0.5 nM) and Ras FPTase (IC_{50} 250 nM)	Dufresne et al. (1993)
		Blockage of cholesterol synthesis and proliferation inhibition on LNCaP (40 μM)	Brusselmans et al. (2007)
Zaragozic acid D, and D_2	*Amauroascus niger* MF5683	Inhibition of squalene synthase (IC_{50} 6 nM and 2 nM, respectively) and Ras FPTase (IC_{50} 100 nM)	Dufresne et al. (1993)
Hispidin (**112**)	Fungus *Phellinus linteus* and *Inonotus* sp	Dose-dependent cytotoxicity to human cancer cell lines SCL1, Capan-1, BxPC-3, AsPC-1, A549, SGC-7901, HepG2, and HCT116, and to murine cancer cell line CMT-93	Gonindard et al. (1997), Lim et al. (2014), Nguyen et al. (2016), Chandimali et al. (2018)
		Dose-dependent increase of apoptosis (25.23–40.4% at 50–200 μg/mL) in CMT-93 cells, with cell cycle arrest at sub-G1 phase, increased expression of apoptotic proteins (intrinsic and extrinsic pathways), and high levels of ROS	Lim et al. (2014)

(continued)

Table 17.2 (continued)

Metabolite	Fungal Source	Activity	Reference
		Dose-dependent increase of apoptosis on BxPC-3 and AsPC-1. Suppressive effects on BxPC-3 $CD44^+$ cells (CSC), enhanced with gemcitabine: cell cycle arrest at G1 phase, apoptosis, inhibition of cell proliferation, migration and invasion, low expression of stem cell markers	Chandimali et al. (2018)
		Selective induction of autophagic and necrotic pathways in cancer cells (SGC-7901, A549, and HepG2 cells), through microtubule depolymerization and lysosomal membrane permeabilization	Lv et al. (2017)
		Low cytotoxic effects on non-tumoral cells MRC5 and GES-1	Gonindard et al. (1997), Lv et al. (2017)
		Protective effect against H_2O_2-induced apoptosis in non-tumoral H9c2, with ROS reduction, inhibition of pro-apoptotic proteins, and activation of Akt/GSK-3β and ERK1/2 signaling pathways	Kim et al. (2014)
Dendrodolides A-D, H, I, and K (**114–117, 120–122**)	Marine *Dendrodochium* sp.	Cytotoxicity to SMMC-7721 (IC_{50} 14.7–24.8 μg/mL)	Sun et al. (2013)
Dendrodolides C, E, G, I, K, and L (**116, 118, 119, 121–123**)		Cytotoxicity to HCT116 (IC_{50} 5.7–26.5 μg/mL)	
Emodin (**124**)	Marine *aspergillus terreus*	Cytotoxicity to KB (IC_{50} 32.97 μg/mL) and multidrug-resistant KBv200 (IC_{50} 16.15 μg/mL)	Xia et al. (2014a)

(continued)

Table 17.2 (continued)

Metabolite	Fungal Source	Activity	Reference
Questin (**125**)		Cytotoxicity to KB and multidrug-resistant KBv200 ($IC_{50} > 50$ μg/mL for both)	
Javanicin (**126**)	Marine *fusarium* sp.	Cytotoxicity to KB (IC_{50} 2.9 μg/mL) and multidrug-resistant KBv200 (IC_{50} 5.91 μg/mL)	Xia et al. (2014b)
Norjavanicin (**127**)		Cytotoxicity to KB (IC_{50} 10.6 μg/mL) and multidrug-resistant KBv200 (IC_{50} 12.12 μg/mL)	
Fusarubin (**128**)		Cytotoxicity to KB (IC_{50} 9.61 μg/mL) and multidrug-resistant KBv200 (IC_{50} 6.74 μg/mL)	
Penicitrinone A (**129**)	Marine *penicillium purpurogenum* (G59)	Cytotoxicity to K562, HL-60, HeLa, and BGC-823 (IC_{50} 34.8–61.5 μM)	Wu et al. (2015)
	Marine *Penicillium citrinum* (SCSIO 41017)	Cytotoxicity to MCF 7 (IC_{50} 1.3 μM); positive control taxol (IC_{50} 1.4 μM)	Salendra et al. (2021)
1,3-dihydroxy-6-methyl-7-methoxyanthraquinone (**130**)	*Thermomyces lanuginosus* KMM 4681	Selective cytotoxicity to drug-resistant 22Rv1 (35% viability at 100 μM) in comparison to non-cancer PNT-2 (65% viability at 100 μM) Inhibition of colony formation of 22Rv1 (70% at 50 μM)	Sobolevskaya et al. (2021)
Sulochrin (**131**)	*Aspergillus falconensis*	Affinity to CDK-2 ($\Delta G = -25.03$ kcal/Mol), TOP-2 (−12.11 kcal/Mol), and MMP-13 (−33.83 kcal/Mol), indicated by molecular docking experiments Cytotoxicity to L5178Y (IC_{50} 5.1 μM) Inhibition of cell	El-Kashef et al. (2021)

(continued)

Table 17.2 (continued)

Metabolite	Fungal Source	Activity	Reference
		migration to MDA-MB-231 (70 μM)	
4,5-dihydroxy-6-(6′--methylsalicyloxy)-2-hydroxymethyl-2-cyclohexenl-one (**132**)	*Epicoccum sorghinum* from sorghum	Cytotoxicity to A549, HepG2, and MDA-MB-231 (IC_{50} 1.86–18.31 μM); doxorubicin positive control (IC_{50} 0.2–0.47 μM)	Chang et al. (2021)
Gentisyl alcohol (**133**)			
6-(hydroxymethyl)-benzene-1,2,4-triol (**134**)			
Cytochalasin E (**135**)	*Aspergillus clavatus*	Inhibition of angiogenesis and autophagy on A549 cells, when associated to bortezomib chemotherapy	Takanezawa et al. (2018)
Myceliothermophin F (**136**) (polyketide-amino acid hybrid)	*Thermothelomyces thermophilus*	Cytotoxicity to DLD-1, Hep3B, HepG2, and HGC-27 (IC_{50} 0.33–0.89 μg/mL)	Gao et al. (2019b)
Globosuxanthone F (**137**)	Marine *Pleosporales sp.* NBUF144	Cytotoxicity to CCRF-CEM (IC_{50} 0.46 μM)	Zhou et al. (2021)
Alterperylenol (**138**)	Endophytic *Alternaria* sp. MG1	Cytotoxicity to NCI-H1299 (98%) and HT-1080 (96.2%) at 20 μM	Tian et al. (2021)
Fusaketide A (**139**) and B (**140**)	Entomogenous *fusarium equiseti*	Cytotoxicity to MCF-7, MGC-803, HeLa and Huh-7 (IC_{50} 2.4–69.7 μg/mL) Dose-dependent inhibition of migration and invasion, and apoptosis induction by fusaketide B on MGC-803	Liu et al. (2021)

Notes: **Human cancer cell lines:** KB—oral epidermal carcinoma; KBv200—multidrug-resistant derived from KB; K562—chronic myelogenous leukemia; HL-60—acute promyelocytic leukemia; CCRF-CEM—acute lymphatic leukemia; HeLa—uterus adenocarcinoma; SGC-7901—endocervical adenocarcinoma; LNCaP, 22Rv1—prostate carcinoma; BGC-823, MGC-803—gastric carcinoma; MCF-7, MDA-MB-231—triple-negative breast carcinoma; A549—lung adenocarcinoma; HepG2, SMMC-7721, Huh-7—hepatocarcinoma; HCT116—colorectal carcinoma; HT29—colorectal adenocarcinoma; A549—lung carcinoma; SCL1—skin squamous cell carcinoma; Capan-1, BxPC-3, AsPC-1—pancreatic adenocarcinoma;. **Murine cancer cell lines:** CT26, CMT-93—colorectal carcinoma; L5178Y—lymphoma. **Non-tumoral cell lines:** MRC5—human pulmonary fibroblasts; H9c2—rat embryonic cardiomyoblasts; PNT-2—human prostate cells; GES-1—human gastric epithelial cells. **Enzymes**: CDK-2—human cyclin-dependent kinase 2; TOP-2—human DNA topoisomerase II; MMP-13—matrix metalloproteinase 13. CSC—cancer stem cells. ROS—reactive oxygen species

Molecular docking studies indicated favorable interactions of sulochrin (**131**) to human cyclin-dependent kinase 2 (CDK-2), DNA topoisomerase II (TOP-2), and matrix metalloproteinase 13 (MMP-13), enzymes directly related to tumorigenesis pathways. In addition, in vitro assays revealed that sulochrin (**131**) was cytotoxic to mouse lymphoma cells and inhibit the migration of triple-negative breast cancer cells (Table 17.2, Fig. 17.17).

Exploring other biological effects of secondary metabolites from the sorghum contaminant fungus *Epicoccum sorghinum*, already known to produce potent mycotoxins, Chang et al. (2021) isolated and identified the chemical structures of three polyketides, 4,5-dihydroxy-6-(6′-methylsalicyloxy)-2-hydroxymethyl-2-cyclohexenl-one (**132**), gentisyl alcohol (**133**), and 6-(hydroxymethyl)benzene-1,2,4-triol (**134**), which demonstrated cytotoxicity to human cancer cell lines from lung adenocarcinoma, hepatoma, and breast carcinoma (Table 17.2, Fig. 17.17).

The biological mechanisms of action of some polyketides on cancer are very diverse and reveal potential new targets and strategies for cancer chemotherapy. For example, zaragozic acid A (**106**) (Fig. 17.15, Table 17.2), as well as zaragozic acids D and D2, inhibits the human enzyme Ras farnesyl-protein transferase (FPTase), responsible for the first step of post-translational processing of Ras, a protein expressed by the *ras* oncogene, related to tumorigenesis (Dufresne et al. 1993). Zaragozic acids are also potent inhibitors of squalene synthase, an enzyme with a critical role in de novo synthesis of cholesterol on cancer cells. In fact, zaragozic acid A (**106**) was able to inhibit cholesterol synthesis and membrane exposure, as well as proliferation of androgen-responsive prostate cancer cells (Brusselmans et al. 2007).

Among the fungal PKS-NRPS hybrid cytochalasans, several compounds have been related to anticancer activities. Cytochalasin E (**135**), isolated from *Aspergillus clavatus*, exhibited a potent angiogenesis inhibitory activity, as well as the ability to prevent autophagy, enhancing the effect of the chemotherapeutic bortezomib on human lung cancer A549 cells (Takanezawa et al. 2018). Pchaeglobosal B (**41**) (Table 17.2, Fig. 17.3), a cytochalasan isolated from endophytic *Chaetomium globosum* (P2-2-2), exhibited significant cytotoxic activity against cell lines representative from human breast, liver, colorectal, and lung cancer, and murine colorectal carcinoma, with IC_{50} ranging from 1.04 to 9.90 μM. Moreover, the compound induced death by apoptosis on CT26 and HT29 cell lines, by eliciting cell cycle arrest at early phases of mitosis (Peng et al. 2020).

Nevertheless, hispidin (**113**), already cited in previous sections (Table 17.2, Fig. 17.16), is probably one of the most promising fungal polyketides with anticancer activity, as well as other interesting biological properties such as antioxidant, hypoglycemic, anti-inflammatory, neuroprotective, and antiviral, besides strong evidences of low toxicity to humans (Palkina et al. 2021). Several cancer cell lines from different origins were found to be sensitive to hispidin (**113**), like those from carcinoma of human keratinocytes, pancreatic ductal adenocarcinoma, lung carcinoma, and endocervical adenocarcinoma, as well as human and murine colon carcinoma (Gonindard et al. 1997; Lim et al. 2014; Nguyen et al. 2016; Lv et al. 2017). In counterpart, **113** showed low cytotoxicity on non-tumoral cell lines, such as human pulmonary fibroblasts and gastric epithelial cells (Gonindard et al. 1997;

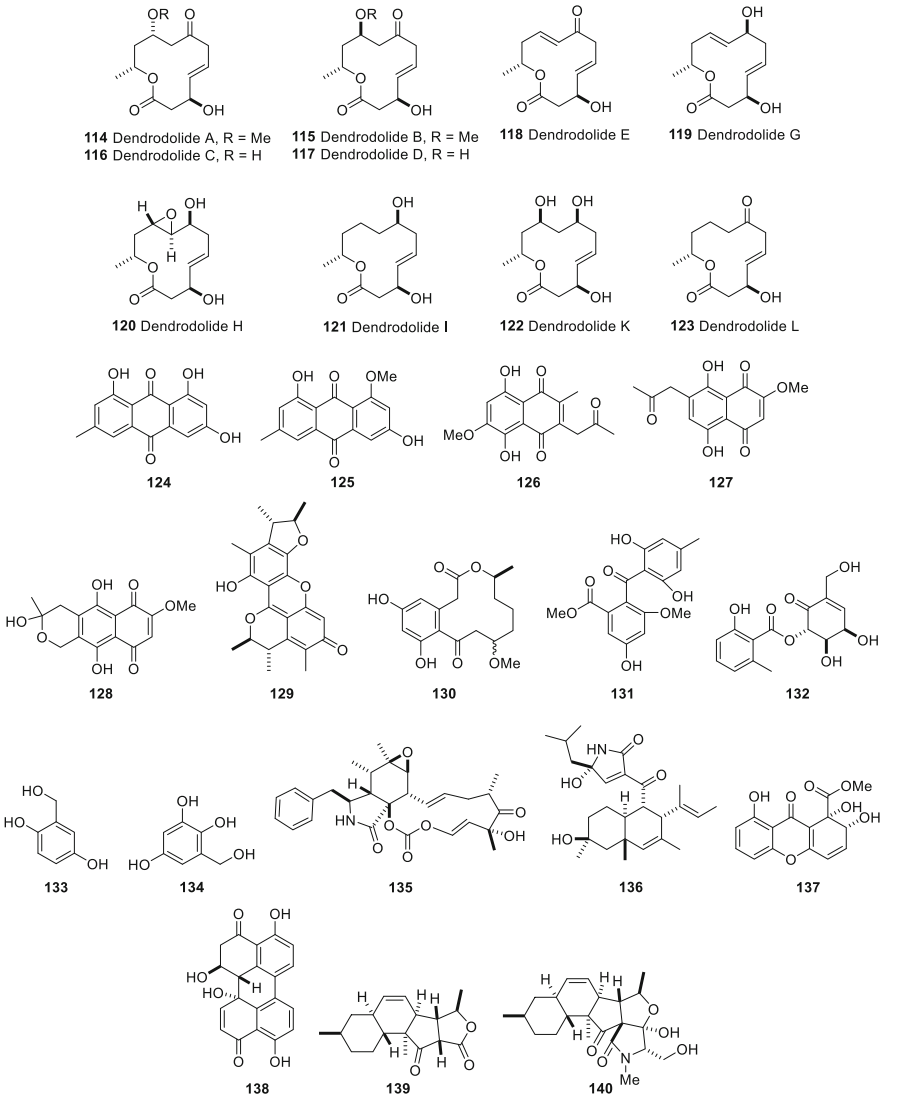


Fig. 17.17 Some prominent fungal polyketides with anticancer potential: dendrodolides A-D, E, G, H, I, K, and L (**114–123**), emodin (**124**), questin (**125**), javanicin (**126**), norjavanicin (**127**), fusarubin (**128**), penicitrinone A (**129**), 1,3-dihydroxy-6-methyl-7-methoxyanthraquinone (**130**), Sulochrin (**131**), 4,5-dihydroxy-6-(6′-methylsalicyloxy)-2-hydroxymethyl-2-cyclohexenl-one (**132**), gentisyl alcohol (**133**), 6-(hydroxymethyl)benzene-1,2,4-triol (**134**), cytochalasin E (**135**), myceliothermophin F (**136**), globosuxanthone F (**137**), alterperylenol (**138**), Fusaketide A (**139**) and B (**140**)

Lv et al. 2017), and even protective effects against oxidative stress and apoptosis on rat embryonic cardiomyoblasts (Kim et al. 2014). The selective mechanisms of action of hispidin (**113**) on cancer cells have been related to a potent inhibition of protein kinase C (IC_{50} 2 x 10^{-6} mol/L), which is involved on signal-transduction pathways (Gonindard et al. 1997), and to the induction of intrinsic and extrinsic apoptosis pathways, mediated by the generation of reactive oxygen species (ROS) (Lim et al. 2014), as well as autophagic and necrotic pathways (Lv et al. 2017). Other promising results were presented by Chandimali et al. (2018), studying cancer stem cells (CSC), known to be responsible for metastasis and chemoresistance in pancreatic adenocarcinoma and other aggressive cancer types. Hispidin (**113**) was able to significantly inhibit stemness of BxPC-3 $CD44^+$ CSC, enhancing the effects of gemcitabine-associated treatment.

Other examples of polyketides with anticancer activities, such as the polyketide-amino acid hybrid metabolite myceliothermophin F (**136**), globosuxanthone F (**137**), alterperylenol (**138**), fusaketide A (**139**) and B (**140**), are indicated on Table 17.2.

17.4.7 Other Biotechnological Uses of Polyketides

Fungal polyketides have other important uses in diverse areas, herein exemplified in the control of phytopathogens and in food industry. These are very different applications, but the outcomes are very promising. Starting with their use in agriculture to control several organisms, natural polyketides are eco-friendly alternatives for biological control of organisms that causes global losses of over 20% of many crops worldwide (Xu et al. 2021). The chemical structures of some fungal polyketides active against some major phytopathogens are shown in Fig. 17.18 and the corresponding spectrum of activity are presented in Table 17.3.

Regarding the use of fungal polyketides as pigments, the azaphilones (Fig. 17.19) play an important role in this field, standing out for their yellow [monascin (**150**)], orange [rubropunctatin (**151**)], and red [rubropunctamine (**152**)] colors. These polyketides contain a pyrone–quinone core, a chiral quaternary center, and hydroxyl groups as substituents. Orange-colored azaphilones, such as rubropunctatin (**151**), usually possess a heterocycle containing a pyranyl oxygen that is susceptible to aminophilic reactions, where the pyran oxygen atom is exchanged for a nitrogen atom derived from peptides, nucleic acids, proteins, and others. This exchange alters the absorption of the pigment, that goes from orange to red, as occurs on rubropunctamine (**152**), frequently also altering its biological properties (Pimenta et al. 2021).

Other biological activities include nitric oxide (NO) production, antioxidant activity, inhibition of biofilm formation, phytotoxicity, insecticidal action, and the inhibition of several enzymes committed to distinct metabolic pathways, as extensively reviewed by Wang et al. (2020b), Zheng et al. (2021) and others.

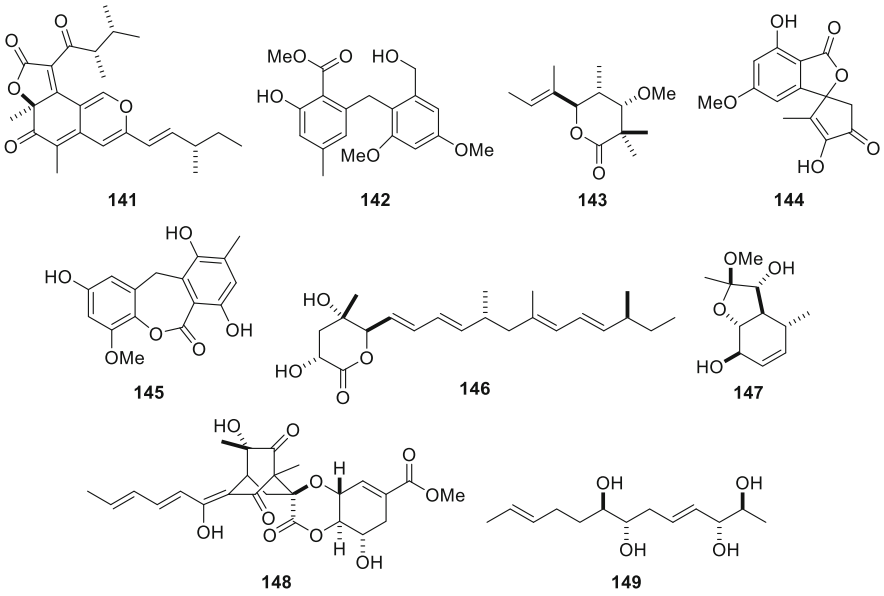


Fig. 17.18 Structures of fungal polyketides with anti-phytopathogenic activities: chaetoviridin A (**141**), epicoccether K (**142**), helicascolide F (**143**), isotalaroflavone (**144**), methyleurotinone (**145**), nafuredin C (**146**), paraverrucsin A (**147**), spirosorbicillinol D (**148**), and triharzianin B (**149**)

17.5 Final Considerations

Polyketides have been widely produced as bioactive fungal metabolites, such as cytochalasans, isoindole macrocyclic derivatives, hybrid peptide-polyketide cyclic depsipeptides, and other uncommon compounds with unprecedented carbon skeletons. Among the fungi that stand out as natural sources of these remarkable products, terrestrial endophytic fungi have been extensively studied, but marine fungi are also prominent contributors. In the review of Zheng et al. (2021), 69 new polyketides, produced by endophytic fungi isolated from different host plants, were reported only in the period of 2017–2019. According to Wang et al. (2020b), 221 new polyketides with specific antimicrobial activities have been isolated from marine fungi, accounting for 81.2% of all the natural products from these organisms, reported from 1998 to 2019.

The pharmaceutical use of fungal polyketides has been recognized early and some of them conquered a prominent position on the world stage, increasing human life expectancy with applications such as dyslipidemic agents, antibiotics, immunosuppressants, antiparasitic, as well as other biotechnological uses, such as anti-phytopathogens and insecticides. Moreover, polyketides have demonstrated to inhibit a range of cancer cells, showing good prospects for the development of new anticancer drugs. The chemistry and biosynthesis of this class have been widely

Table 17.3 Fungal polyketides with anti-phytopathogenic activities

Metabolite	Fungal Source	Phytopathogen Target	Reference
Strobilurin A (**12**)	*Strobilurus tenacellus*, *Oudemansiella mucida*, and *Bolinea lutea*	Ascomycetes, basidiomycetes, and oomycetes	Wang et al. (2021b)
Dendrodolide E (**118**)	*Plenodomus influorescens* and *Pyrenochaeta nobilis*	*Xanthomonas campestris* and *Phytophthora infestans*	Oppong-Danquah et al. (2020)
Chaetoviridin A (**141**)	*Chaetomium globosum*	*Sclerotium rolfsii, Macrophomina phaseolina, Sclerotinia sclerotiorum,* and *Fusarium oxysporum*	Kumar et al. (2021)
Epicoccether K (**142**)	*Epicoccum sorghinum* derived from *Myoporum bontioides*	*P. italicum* and *F. graminearum*	Junjie et al. (2021)
Helicascolide F (**143**)	*Talaromyces assiutensis* JTY2	*Alternaria brassicicola, Phytophthora parasítica var. nicotianae, Colletotrichum capsici, Bipolaris oryzae, Diaporthe medusaea* Nitschke, and *Ceratocystis paradoxa* Moreau	Li et al. (2021)
Isotalaroflavone (**144**)	*Alternaria alternata ZHJG5* derived from *Cercis chinensis*	*Xanthomonas oryzae* pv. Oryzicola (Xoc) and Rs.	Zhao et al. (2021)
Methyleurotinone (**145**)	*Eurotium rubrum*	*Pectobacterium carotovorum subsp. Carotovorum, Pseudomonas syringae pv. Syringae, Rhizobium radiobacter*, and *Ralstonia solanacearum*	Saad et al. (2021)
Nafuredin C (**146**)	*Trichoderma harzianum* D13	*Botrytis cinérea, Magnaporthe grisea, Phytophthora parasítica, Pestalozzia theae,* and *Valsa mali*	Zhao et al. (2020)
Paraverrucsin A (**147**)	*Paraphaeosphaeria verruculosa*	*C. gloeosporioides, D. glomerata, N. oryzae,* and *P. verruculosa*	Hu et al. (2020)
Spirosorbicillinol D (**148**)	*Trichoderma longibrachiatum*	*Phytophthora infestans*	Ngo et al. (2021)
Triharzianin B (**149**)	*Trichoderma harzianum*	*Aspergillus fumigatus, Trichoderma edulis,* and *Tricholoma matsutake*	Wang et al. (2021f)

studied, and the advances in genetic engineering have allowed the manipulation of biosynthetic pathways, providing a convenient platform to supply new and useful lead compounds. New semi-synthetic and synthetic derivatives have been prepared to increase therapeutic capacity or develop industrial-scale production. This scenario is encouraging, especially given the novelties in the biotechnological exploration of fungi specimens and secondary metabolites from unexpected ecological niches.

By the other side, as demonstrated in this review, a variety of newly identified fungal polyketides, with unprecedent biological mechanisms of action, are still on

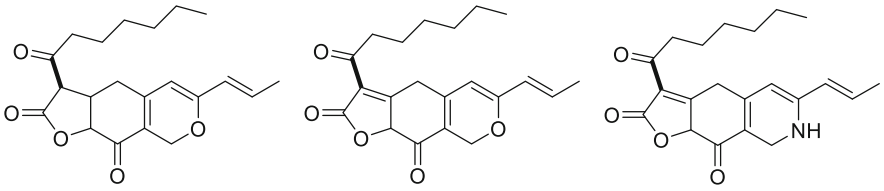


Fig. 17.19 Representative fungal polyketide azaphilones used as pigments: monascin (**150**), rubropunctatin (**151**), and rubropunctamine (**152**)

pre-clinical studies. Attempts should be done to evaluate the in vivo therapeutical effects of these natural compounds and derivatives, as well as the absence of significant side effects, to move forward to clinical trials and to the market as commercial products.

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Chapter 18
Fungal Quinones: Benzo-, Naphtho-, and Anthraquinones

Víctor López and Francisco Les

Abstract This chapter summarizes and reviews the main chemical, biological, and pharmacological aspects of fungal benzo-, naphtho-, and anthraquinones, with a particular emphasis in their potential applications. These compounds represent a class of natural metabolites present in fungi but also in certain groups of plants, lichens, and other microorganisms. Considering that benzo, naphtho-, and anthraquinones are in many fungi of the Basidiomycota and Ascomycota divisions, their presence is mainly reported in the genus *Alternaria*, *Aspergillus*, *Fusarium*, *Penicillium*, and *Talaromyces*. The chemical diversity due to the high number of different producer organisms makes this class of metabolites a very interesting source of bioactive compounds with antioxidant/pro-oxidant, antibacterial, antifungal, antiviral, and cytotoxic properties. Certain compounds are also used and proposed as pigments with dying properties, but the main applications could be in the field of microbial disease or cancer. However, the pro-oxidant and toxic effects of certain compounds, due to ROS production and DNA damage, is a critical point to be addressed before its use in pharmacological applications.

18.1 Introduction

18.1.1 Definition, Chemical Aspects, and Origin

Quinones are an important group of natural secondary metabolites of polyketide origin generally synthesized from acetyl and malonyl-CoA (Christiansen et al. 2021). The biosynthesis of quinones is characterized by the diversity of metabolic pathways of the different living organisms. In this sense, other pathways different

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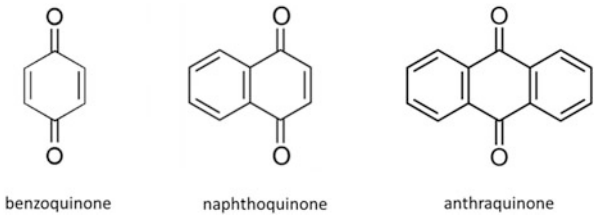


Fig. 18.1 Basic core structures of quinones

from polyketide synthases, like mevalonic or hydroxybenzoic acids, are also involved.

From a structural point of view, quinones are oxygenated compounds derived from aromatic rings characterized by a six-carbon conjugated ring with keto-groups in *ortho* or *para* positions. The most basic structure is the benzoquinone, but other frequent structures are naphtho- and anthraquinones, with one or two merged aromatic rings, respectively (Fig. 18.1). Other less frequent core structures are the four-ring quinones known as tetracenequinones and perylenequinones. Depending on the functional groups (methyl, hydroxyl, amino, etc.) attached to the core structures, a wide chemical diversity can be observed, even dimeric structures.

More than 1000 quinoid structures have been described in nature, being naphthoquinones and anthraquinones the most common. Quinones have been described in plants, fungi, lichens, and algae but also in microorganisms, invertebrates, and vertebrate animals. Fungi represent an important source of biologically active metabolites, and quinones may represent an example. Many of these compounds are also present in marine fungi associated to aquatic organisms. In fact, endophytic fungi can produce alkaloids, terpenoids, and phenolic compounds such as coumarins, flavonoids, or phenylpropanoids but also quinones (Singh et al. 2021). Endophytes can be defined as a group of fungal microorganisms residing in internal healthy living tissues of the plants with positive effects in their hosts establishing relationships between them and their hosts. This relationship can be very important because certain works have demonstrated that many plant-derived compounds are produced by endophytes instead of their hosts.

Free quinones (aglycones) are insoluble in water and can be extracted with non-polar common organic solvents such as hexane or chloroform. The separation of the same is recommended to be done by chromatographic techniques. If the quinones are in the form of glycosides, the extraction could be carried out with water or hydroalcoholic solutions (Bruneton 2001).

Among all filamentous fungi, *Aspergillus*, *Penicillium*, *Talaromyces*, *Fusarium*, *Arthrinium,* and *Alternaria* may be the most important genera producing a wide diversity of quinones and its derivates (Christiansen et al. 2021). Many of them are known for their ecological role but also new future industrial and pharmacological applications are being studied.

18.1.2 Ecological and Biological Significance

Quinones seem to have an important role on electron transfer reactions of oxidative processes. For example, ubiquinones (also known as coenzyme Q), which are class of benzoquinones, are involved in electron transport during cellular respiration of eukaryotic and procaryotic cells.

From an ecological and biological perspective, quinones are considered protective compounds for the producer organism constituting a chemical defense with toxic or antimicrobial properties, also known as allelopathic activity; for example, certain beetles produce vaporized quinones to combat predators and many fungi increases their virulence by producing quinones. The fungus *Beauveria bassiana* produces a benzoquinone known as oosporein that represses the immune response of insects (Mc Namara et al. 2019). Other plant pathogenic fungi produce photosensitizing quinones causing damage to the plant, which could be in relation with degradation of plant material (Daub et al. 2013). Quinones might exert toxic effects using different mechanisms such as redox reactions, reactive oxygen species production, arylation of proteins, DNA breaks or alkylations via ortho- or para-quinone methides (Futuro et al. 2018).

Quinones have also been proposed to protect the organisms from sunlight exposure as physcion, an anthraquinone produced by many fungal species, is induced under UV radiation in some lichens.

Another hypothesis has confirmed the biological role of these compounds in fungal survival as quinones produced by fungi of the Basidiomycota group could be involved in the degradation of plant material by producing reactive oxygen species (Kerem et al. 1999).

18.1.3 Industrial and Pharmaceutical Applications

The potential pharmaceutical and industrial applications of these compounds are extremely high, although many of the revealed properties and activities have only been screened using basic models and *in vitro* bioassays. Apart from their biological and pharmacological activities, quinones have been proposed as colorants but also electrolytes in redox flow batteries.

Natural colorants and dyes are generally presented as safer and more eco-friendly products compared to synthetic compounds. Fungi can produce pigments like melanins, carotenoids, monascins, flavins, phenazines, and quinones. Within the group of quinones, anthraquinones are the most investigated as colorants, with the pigment Arpink Red ™ obtained from *Penicillium oxalicum* as a well-known example. Fungal pigments can be obtained in industrial fermentation bioreactors improving the process without being exposed to seasonal variations that can occur with plants or other producer organisms that are in nature. However, certain

challenges should be solved for fungal quinoid pigments as the cost of production and the chemical stability.

From a pharmacological perspective, plant quinones have traditional been used in the pharmaceutical industry to produce medicines and food supplements with laxative properties. The better-known laxative plants with quinones are alder buckthorn (*Frangula alnus*), Indian senna (*Senna alexandrina*) and aloe (*Aloe spp*). These quinone glycosides reach the colon, are hydrolyzed by intestinal microbiota, and may induce the secretion of water and electrolytes by altering certain ATP-ases of the large intestine; nevertheless, none of the fungal quinones, which are generally in form of aglycone, are currently used for this purpose.

Many works have revealed the antimicrobial and cytotoxic properties of this class of secondary metabolites. Due to the cytotoxic properties in relation with ROS release and DNA or protein damage, these compounds have been presented as potential anticancer agents. For instance, plumbagin, found in *Plumbago zeylanica* but also in endophytes such as *Cladosporium delicatum,* is a naphthoquinone with anticancer properties inducing apoptosis via ROS generation, caspase activity and JNK pathways (Singh et al. 2021). However, most of these properties have only been validated using in vitro methodologies and more studies are needed to use them clinically.

Other published works demonstrated that many quinoid compounds exert antibacterial, antifungal, antiparasitic, and antiviral properties being a chemical group with potential applications as chemotherapeutic and antibiotic agents. The naphthoquinone plumbagin has also been tested for antifungal properties against *Candida albicans,* while the anthraquinone rhein, found in plants like *Rheum palmatum* and fungi like *Fusarium solani,* has also demonstrated antimicrobial properties; more quinones will be discovered soon for their antimicrobial effects and therefore potential pharmacological applications.

18.2 Benzoquinones

Benzoquinones are the simplest structures in the quinone group. They consist of a conjugated six carbon ring with two carbonyl groups in the positions ortho or para. There are several types of benzoquinones in mycelial fungi but also in mushrooms.

For example, aculeatusquinones and anserinones are classical examples of benzoquinones present in the genus *Penicillium* and *Aspergillus;* while anserinones consist of a methylated benzoquinone ring, aculeatusquinones are methylated benzoquinones linked to another benzene ring. **Anserinones A** and **B** have been reported as antibacterial and antifungal compounds since the 90s (Wang et al. 1997).

Asterriquinones are probably the most different subgroup of benzoquinones due to the presence of nitrogen-containing rings in their structure; they are dihydroxibenzoquinoid structures linked to two indol rings derived from the aminoacid tryptophan. Asterriquinones found in *Aspergillus* and *Penicillium* have shown neuroprotective properties in neuronal-like cells acting as activators of the

nerve growth factor receptor (Lin et al. 2007; Girich et al. 2020). A very similar group to asterriquinones are atromentins but they contain tyrosine residues instead of tryptophan; these atromentins.

Citrinin H1 and citriquinones have been detected in *Penicillium citrinum,* being citrinin H1 a compound with anti-inflammatory activity (Ngan et al. 2017) but also antibacterial properties against methicillin-resistant *Staphylococcus aureus* (Wang et al. 1997).

Coenzyme Q10, also known as CoQ 10, ubiquinone or 2,3-dimethoxy, 5-methyl, 6-decaprenyl benzoquinone, is probably one of the most popular quinones in the pharmaceutical industry for its applications as an antioxidant and anti-aging ingredient (Varela-López et al. 2016). This compound can be obtained by chemical synthesis, extraction from animal or plant tissues or by microbial fermentation. It can be found in animal tissues such as heart, liver, or kidney but also in plant sources like soy oil or peanuts; ubiquinone can also be obtained by fermentation using bacteria or fungi such as those of the genus *Aspergillus*, *Candida, Neurospora,* and others (Pierrel et al. 2022). Coenzyme Q10 plays a central role in mitochondria because it is involved in energy production and ATP synthesis. Due to its antioxidants and protective benefits against free radicals and ROS, this compound has an interesting role in cardiovascular and degenerative diseases. In fact, in recent years, this compound has been included as an ingredient of many food supplements and vitamin-based products, it has also been suggested that coenzyme Q10 could have a protective effect in statin-treated patients to prevent them from rhabdomyolysis.

Although **cycloepoxydon** is not a quinone from a strict structural point of view, it can be considered a quinone derivative found in mushroom species such as the *Xylaria* genus. This compound has revealed NF-κB inhibitor activity demonstrating potential antitumor activity. Another epoxy-related compound with similar activity is **panepoxydone** from *Panus conchatus* and *Lentinus crinitus*. In relation with antitumor potential of wild mushrooms, **clavilactones** from *Clitocybe clavipes* can inhibit protein tyrosine kinases, involved in cell proliferation (C.F.R. Ferreira et al. 2010).

The benzoquinone known as **490 quinone** (γ-L-glutaminyl-4-hydroxy-2,5-benzoquinone), due to its maximal wavelength at 490 nm, was isolated from the edible mushroom *Agaricus bisporius* and acts as a cytotoxic compound in certain cells due to its ability to inhibit DNA polymerase (Zaidman et al. 2005).

Fumigatins and **toluquinones** are simple benzoquinones observed in *Aspergillus;* although some scientific debate has been created about toluquinones considered or not as in the same group of fumagatins there is not much evidence about applications of this group.

Other more complex structures of benzoquinones found in *Penicillium* are **macrophorins**, the dimer structures **phenicins**, and **spathullins** with heterocyclic nitrogen and stemphones; spathullins, which can be classified as isoquinoline alkaloids better than benzoquinones, have shown antibacterial activity (Nord et al. 2019). Finally, **variecolorquinone B** and **yanuthones** from *Aspergillus* have revealed cytotoxic and antifungal properties, respectively (Wang et al. 2007; Petersen et al. 2015).

Benzoquinones exhibit a wide range of biological activities, some of them summarized with the main naphthoquinones structures in Table 18.1.

18.3 Naphthoquinones

Naphthoquinones are one of the most important groups of quinones, as they are some of the most common types of these compounds found in nature, particularly in fungi, microorganisms, plants, and lichens. More than 100 naphthoquinones have been described in fungi and in general, they exert antimicrobial and cytotoxic properties due to its ability to induce DNA breaks and ROS. Between them we can find a wide diversity of naphthoquinone structures: aspetritones, griseusins, juglones, lapachol, lawsone, naphthgeranines, plumbagin, purpurogenone, shikonin, thysanone and dimeric structures such as xanthomegnins, xanthoviridicatins, and xylindein.

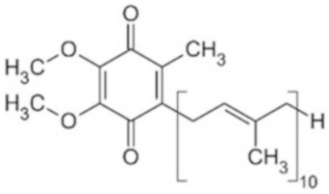
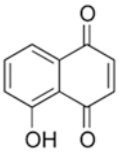
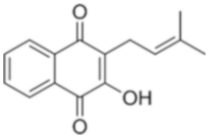
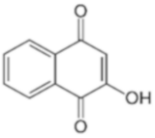
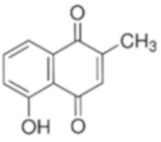
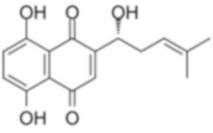
According to aspetritones, they are tricyclic naphthoquinones produced by *Aspergillus* that could apparently be confused with anthraquinones. **Aspetritone A**, which is produced by the coral-derived fungus *A. tritici*, has been shown as a cytotoxic compound against the human cancer cell lines HeLa, A549, and Hep G2 and as an antibacterial agent against methicillin-resistant strains of *S. aureus* (Wang et al. 2017).

Griseusin C, a naphthoquinone derivative from a marine-derived fungus *Penicillium* sp., has not been studied in terms of bioactivity (Li et al. 2006); nevertheless, other griseusins have been tested as antibacterial and cytotoxic compounds.

Juglone (5-hydroxy-1, 4-naphthalene diketone) and its derivatives such as 6-ethyl-7-methoxy-juglone are some of the simplest and well-known naphthoquinones. Juglone and related-derived compounds are present in different fungi (*Aspergillus*, *Penicillium*, *Talaromyces*) but also in different plant parts of walnut trees (plants of the genus *Juglans*). Juglone and its derivatives are very interesting from a pharmaceutical perspective as they have shown antibacterial, antiviral, hemostatic, and pro-oxidative effects (dos S. Moreira et al. 2021). Due to this pro-oxidative effect, juglone is used in experimental biology and pharmacology to induce oxidative stress and cell death in preclinical studies in relation with ROS production. Although juglone has never been used as an anticancer drug, this molecule has inhibited cell proliferation-inducing autophagy and apoptosis of bladder, cervical and breast cancer cells (Tang et al. 2022). Other studied mechanisms involved in the anticancer activity could be in relation to the inhibition of mammalian DNA polymerase and peptidyl proline isomerase (Pin1). Juglone has also been found to act as a Pin1 inhibitor reducing the expression of acetyl CoA carboxylase 1 (ACC1). Other suggested mechanisms of the anticancer activity of juglone are IL-6/STAT3 and AMPK signaling or the promotion of ROS and the p53 pathways.

Lapachol (2-hydroxy- 3-prenyl-1,4-naphthoquinone) and **lawsone** (2-hydroxy-1,4-naphthoquinone) are quite similar naphthoquinones with a prenylated residue in the case of lapachol. Lapachol can be found in endophytic fungi such as *Alternaria*

Table 18.1 Chemical structure, source, and biological effects of some interesting benzo- and naphthoquinones of fungal origin

Structures	Origin	Biological effect	References
Coenzyme Q10 (ubiquinone) 	*Aspergillus*, *Candida*, *Neurospora*	Antioxidant, anti-ageing, cardioprotection.	Varela-López et al. (2016), Pierrel et al. (2022)
Juglone 	*Aspergillus*, *Penicillium*, *Talaromyces*	Pro-oxidative, antibacterial, antiviral, hemostatic, anticancer	dos S. Moreira et al. (2021), Christiansen et al. (2021), Tang et al. (2022)
Lapachol 	*Alternaria and Penicillium*	Anticancer, antimicrobial, antiviral, anti-inflammatory, and antiparasitic	Hussain and Green (2017), Ortiz-Pérez et al. (2021)
Lawsone 	*Gibberella moniliformis*	Cytotoxic and antiprotozoal	Ortiz-Pérez et al. (2021)
Plumbagin 	*Cladosporium delicatulum*	Anticancer, cytotoxic	Yin et al. (2020), Singh et al. (2021), Sun et al. (2022)
Shikonin 	*Fusarium tricinctum*	Anticancer, cytotoxic	Yin et al. (2020), Singh et al. (2021), Sun et al. (2022)

and *Penicillium* but also in the plant genus *Tabebuia (T. avellanedae* and *T. argentea)*. Lapachol is, as juglone, one of the most studied quinones in terms of biological activity. This compound presents anticancer, antimicrobial, antiviral, anti-inflammatory, and antiparasitic activity. Lawsona is a cytotoxic naphthoquinone isolated from the plant source *Lawsonia inermis* and the endophytic fungus *Gibberella moniliformis.* Many studies reveal strong antiprotozoal activity against *Leishmania* spp., *Trypanosoma cruzi, Plasmodium falciparum*, *Toxoplasma gondii*, and *Toxocara canis* for both compounds (Ortiz-Pérez et al. 2021). Another structure in relation with these compounds is β-lapachone, that can be obtained through chemical synthesis from lapachol; β-lapachone is also quite interesting because the activity of this compound has been also evaluated in clinical trials for the treatment of pancreatic cancer revealing potential applications in that field (Gomes et al. 2021).

Naphthgeranins, **nanaomycin A** and **thysanone** have not been widely studied as the previous mentioned quinones, but while the first reveal antibiotic activity (Wessels et al. 1991), nanaomycin A and thysanone may act as topoisomerase inhibitors leading to apoptotic cell death (Sperry et al. 2009).

Plumbagin and **shikonin** are also cytotoxic naphthoquinones isolated from plants of the genus *Plumbago* and *Lithospermum* but also in endophytic fungi; plumbagin was found in *Cladosporium delicatulum* and shikonin in *Fusarium tricinctum* (Singh et al. 2021). The potential antitumor activity of these molecules is in relation with ROS-mediated apoptosis and DNA double-strand break by oxidative DNA base damage but also with regulation of NF-kB, STAT3, and AKT pathways (Yin et al. 2020; Sun et al. 2022). Both compounds, but particularly shikonin, are drug candidates as clinical trials have also been developed in the field of cancer; although the pharmacodynamic and pharmacokinetic properties of shikonin have been validated through clinical trials, nephrotoxicity and skin allergy have also been recorded.

According to dimeric structures, **xanthomegnins** and **xanthoviridicatins** have been found in *Aspergillus*, *Penicillium,* and *Talaromyces.* Although xanthomegnin did not show teratogenic effects in mice, it has been considered as a mycotoxin with relevance in terms of food safety. These mycotoxins seem to have an ecological role in protecting the filamentous fungi from predators (Xu et al. 2019). Finally, xylindein, a dimeric naphthoquinone from *Chlorociboria,* is a green pigment that can be used in fabrics without using other additional substances (Venil et al. 2020). However, the main problem about using these pigments is their extraction from natural fungi using hazardous solvents.

Naphthoquinones exhibit a wide range of biological activities, some of them are summarized in Table 18.1.

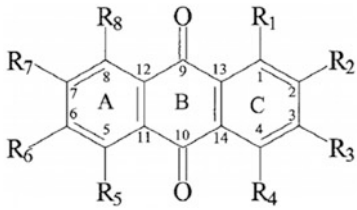


Fig. 18.2 Anthraquinone structure

18.4 Anthraquinones

Anthraquinones are the largest group of natural quinones, with more than 700 compounds. These are present in plants, fungi, and lichens. They are pigments that give color to the fungus, usually yellow, orange, or brown (Gessler et al. 2013). Endophytic fungi that coexist with plants are also a source of anthraquinones. These compounds are present in the endophytes themselves, or are generated by the formation of secondary metabolites from host plants (Huang et al. 2007; Aly et al. 2010). Anthraquinones are widespread in the kingdom of fungi present in organisms like *Aspergillus* spp., *Culvularia lunata*, *Dreschlera* spp., *Emericella purpurea*, *Eurotium* spp., *Fusarium* spp., *Microsporum* sp., *Mycosphaerella rubella*, *Penicillium* spp., etc.) (Gessler et al. 2013).

Marine ecosystems are also home to a wide biodiversity of fungi present free in water, inert organic or inorganic matter. They can also be included as endophytes or pathogens in marine plants, plankton, vertebrates, and invertebrates (Panno et al. 2013).

There is a great diversity of fungal molecules of marine origin still little explored today. These could produce exclusive molecules due to their adaptation to the unfavorable environment: high osmotic pressure, low oxygen, temperature, limited light, high pressure, or tidal water flows (Saleem and Nazir 2015).

Anthraquinones may be of interest due to their various biological properties such as antibacterial, antiparasitic, insecticide, fungicide and antiviral, anticancer, laxative, diuretic, immunomodulatory or other effects (Nemeikaite-Čeniene et al. 2002; Chrysayi-Tokousbalides and Kastanias 2003; Huang et al. 2007; Srinivas et al. 2007; Malik and Müller 2016).

However, many anthraquinones have properties, which can cause mutagenic and carcinogenic effects (Nemeikaite-Čeniene et al. 2002; Masi and Evidente 2020). For this reason, they are usually used in small doses or in combination with other bioactive compounds present in mushrooms or vegetable mixtures.

Anthraquinone molecules are aromatic organic compound based on a structure formed by the fusion of three benzene rings; IUPAC: 9,10-dioxoanthracene (Fig. 18.2). The variety of natural anthraquinones is ensured by the presence of different substituents (R1–8), such as –OH, –CH3, –OCH3, –CH2OH, –CHO, –COOH, etc., as well as by the reduction of carbonyl groups and double bonds in the benzene ring (Malik and Müller 2016; Masi and Evidente 2020). There are usually several lateral substituents on the benzene ring of fungal anthraquinones. The most

widespread in fungi are the 1,8 dihydroxy and 1,5,8 or 1,6,8 trihydroxy anthraquinone derivatives. This biosynthesis of the anthraquinone in fungi is through the acetate-malonate pathway, unlike plants that have shikimate and acetate-malonate pathways.

Some of the main fungal anthraquinones are altersolanol A, averufin, catenarin, chrysophanol, cynodontin, emodin, pachybasin, physcion, questin, and versicolorin.

Altersolanol A is a tetrahydroanthraquinone present in species of the genus *Alternaria*, and *Ampelomyces*, and the species *Stemphylium botryosum*. This compound has shown various biological effects. Altersolanol A exhibited phytotoxicity on lettuce leaves and inhibited the growth of cell cultures of *Nicotiana rustica* (Assante and Nasini 1987; Haraguchi et al. 1996). This anthraquinone is a potent stimulator of mitochondrial NADH oxidation, and an electron acceptor. In addition, it also showed cytotoxicity when tested in vitro against L5178Y cells and protein kinases inhibitory properties (Debbab et al. 2009).

Averufin is a compound present in *Aspergillus versicolor*, *A. parasiticus, Dothistroma pini* (Danks and Hodges 1974; Lee et al. 2010). Averufin has been shown to uncouple oxidative phosphorylation of isolated rat liver mitochondria, and averufin dimethyl ether inhibits mitochondrial respiration, which may explain the toxicity of this anthraquinone (Kawai et al. 1984). It has also shown antiproliferative potential against five human tumor cell lines (A-549, SK-OV-3, SK-MEL-2, XF-498, and HCT-15) (Lee et al. 2010).

Catenarin is presented in species from genus *Aspergillus*, *Curvularia*, *Drechslera, Eurotium*, *Helmintosporium*, *Pyrenophora* (Engström et al. 1993; Hobson et al. 1997; Bouras and Strelkov 2008). This anthraquinone exhibits antibacterial activity in *Bacillus brevis*. At low concentrations, it inhibits the incorporation of uracil and leucine, and at higher concentrations, also that of thymidine. It exhibited antiproliferative activity by inhibiting the incorporation of uridine and thymidine in Ehrlich's ascites carcinoma cells. In addition, it is able to inhibit the DNA-dependent RNA polymerase of *E. coli* (Anke et al. 1980). The fungus *Pyrenophora tritici-repentis*, with high presence of catenarin and emodin phytotoxins, causes spots, foliar and seed disease of wheat (Bouras and Strelkov 2008).

Chrysophanol is an anthraquinone from fungal origin, present in genus *Aspergillus*, *Curvularia*, *Drechslera*, *Phoma*, and *Trichoderma* (Bick and Rhee 1966; Hobson et al. 1997; Liu et al. 2009; Rai et al. 2009; Qian et al. 2011). Chrysophanol has shown various biological activities. One study showed protective effects against ethanol-induced toxicity *in vitro* on HepG2/CYP2E1 cells. It decreased gamma-glutamyl transpeptidase (GGT) activity and increased glutathione (GSH) (Qian et al. 2011). Chrysophanol showed antifungal and antibacterial activity on the plant species it colonizes (Liu et al. 2009). This anthraquinone exhibited anti-inflammatory effects on dextran sodium sulfate-induced colitis and lipopolysaccharide-induced inflammatory responses in mouse peritoneal macrophages. At a molecular level, this compound reduced the production of tumor necrosis factor alpha, interleukin 6 and the expression of cyclooxygenase 2 levels. It also reduced the activation of NF-kappaB and caspase-1 in macrophages

stimulated by LPS which revealing potential uses as anti-inflammatory agent (Kim et al. 2010).

The anthraquinone **cynodontin** is presented in *Drechslera avenae, Curvularia lunata* (Hobson et al. 1997; Chrysayi-Tokousbalides and Kastanias 2003). This compound has been assessed *in vitro* for fungitoxic properties, inhibiting the growth of *Sclerotinia minor*, *Sclerotinia sclerotiorum*, *Botrytis cinerea* and, *Verticillium dahlia*, with similar ED_{50} values to the reference fungicides dichloran and carbendazim (Chrysayi-Tokousbalides and Kastanias 2003).

Emodin is one of the major anthraquinones in nature, as well as being an intermediary for other fungal compounds. It is present in fungi, lichens, or plants. This anthraquinone is highly characteristic of the genus *Aspergillus, Curvularia, Dermocybe, Phoma, Pyrenochaeta, Pyrenophora*, *Trichoderma* (Bick and Rhee 1966; Anke et al. 1980; Calhoun et al. 1992; Hobson et al. 1997; Ren et al. 2006; Frisvad et al. 2009; Liu et al. 2009; Qian et al. 2011; Dong et al. 2016; Stompor-Gorący 2021; Semwal et al. 2021). Emodin possesses a broad spectrum of biological properties, including anticancer, hepatoprotective, anti-inflammatory, antioxidant, and antimicrobial activities (Dong et al. 2016). However, at high and chronic doses, emodin could also cause liver, kidney, and reproductive toxicity. Like catenarin, emodin exhibited antibacterial and antiproliferative activity, and inhibition of *E. coli* DNA-dependent RNA polymerase (Anke et al. 1980). Emodin has been shown to be a potential candidate for attenuating ethanol-induced liver damage, with better effects seen than with chrysophanol (Qian et al. 2011).

Pachybasin is an anthraquinone derived from genus *Ascochyta, Coniothyrium, Phoma,* and *Trichoderma* (Bick and Rhee 1966; Liu et al. 2009; Sun et al. 2013; Masi et al. 2018). This compound from *Coniothyrium* showed weak activity against the Gram-positive bacterium *B. megaterium*., but not others antibacterial or antifungal properties. However, coniothyrinones A–D, derived from pachybasin and others anthraquinones, shown antibacterial and antifungal properties (Sun et al. 2013). In other study, pachybasin from *Trichoderma* also shown antibacterial and antifungal activity against *S. aureus* and *R. solani*, respectively (Liu et al. 2009).

Physcion has been detected in the genus *Aspergillus, Eurotium,* and *Microsporum* (Butinar et al. 2005; Frisvad et al. 2009; Wijesekara et al. 2014). Physcion demonstrated antiproliferative properties on HeLa cells. Physcion-induced apoptosis by downregulating Bcl-2 expression, upregulating Bax expression, and activation of the caspase-3 pathway. In addition, an increase in reactive oxygen species (ROS) was observed in the cells (Wijesekara et al. 2014). Moreover, other properties have been demonstrated by this anthraquinone, but with those of plant origin. These are anti-inflammatory, antimicrobial, and hepatoprotective properties (Zhao et al. 2009; Tamokou et al. 2009; Ghosh et al. 2010).

Questin is presented in genus *Aspergillus, Eurotium,* and *Penicillium.* This anthraquinone has shown allelopathic effects, preventing the germination and growth of wheat (*Triticum aestivum*) and radish (*Raphanus sativus*) seeds. This computed presented a similar activity to glyphosate, a broad-spectrum herbicide (Gui et al. 2015). This phytotoxicity could be due to its antimicrobial properties since it destroys the microbiota present in the soil and plant substrate.

Versicolorin A and B, obtained from *A. versicolor* and *A. parasiticus*, may be furofuran precursors of aflatoxins and sterigmatocystin, hepatotoxic and carcinogenic mycotoxins (Hsieh et al. 1978). This fact is related with the versicolorin cytotoxicity in A549 human adenocarcinoma lung cells. It also caused a statistically significant increase in the number of formed micronuclei and a decrease in the formation of nuclear buds and nucleoplasmic bridges, related to irreversible DNA damage (Jakšić et al. 2012).

Therefore, anthraquinones exhibit a wide range of biological activities, some of them summarized with the main anthraquinone structures in Table 18.2.

Many of them exert phytotoxic activity as previously described with altersolanol A, catenarin, cynodontin, or emodin, but many others have been studied due to it phytotoxicity. Lentiquinones A-C, from *Ascochyta lentis*, showed interesting activities, caused severe necrosis of leaves host plants (Hsieh et al. 1978). **Macrosporin** from *Stemphyfium botryosum* and *Alternaria* also shown phytotoxicity as previously described with altersolanol A (Assante and Nasini 1987).

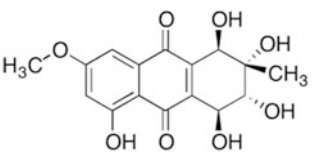
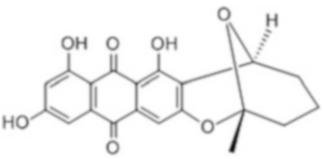
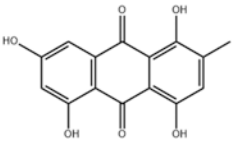
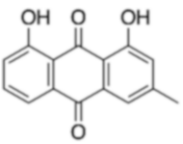
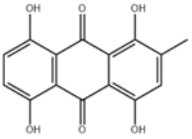
Other common biological activity is the antiproliferative and cytotoxic properties of tumoral cells. Altersolanol, averufin, catenarin, emodin, and physcion, which are described before, have the capacity to inhibit the cell survival of different types of cancer cells. In this line, other anthraquinones such as **stemphylin** and **dactylariol** from *S. botryosum* have shown also anticancer properties like altersolanol A (Assante and Nasini 1987). This could be due to the ability to inhibit protein kinases, responsible for cell proliferation and transformation, as seen previously with emodin, or altersolanol (Debbab et al. 2009). There are other fungal anthraquinones such as tetrahydroxyanthraquinones from *Microsphaeropsis*, which are capable of inhibiting various kinases and the epidermal growth factor (Brauers et al. 2000). Another mechanism that could explain the anticancer effects of fungal anthraquinones is the activation of the caspase cascade and the induction of apoptosis, together with the uncoupling of mitochondrial respiration (Chiang et al. 2011).

These antiproliferative effects can be harmful to the body's own tissues, cells, and DNA, being the cause of anthraquinone toxicity, with potential nephrotoxic, hepatotoxic and carcinogenic effects.

A group of anthraquinones derived from the fungus *Ramularia collo-cygni*, the **rubellins**, cause a leaf spot disease of barley, since they are phytotoxic as others before described. In addition, they have cytotoxicity in human cervix carcinoma (HeLa) and green monkey kidney (GMK) cells, and antiproliferative activity in human umbilical vein endothelium (HUVEC) and human chronic myeloid leukemia (K-562) cells (Miethbauer et al. 2009). This group of anthraquinones has also shown antibacterial activity against *Bacillus subtilis*, *Staphylococcus aureus*, and *Mycobacterium vaccae.*

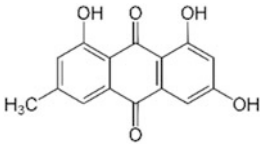
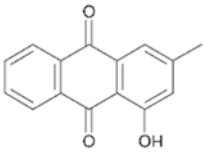
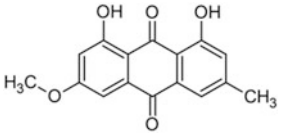
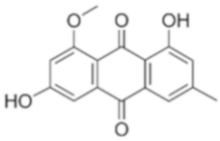
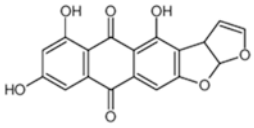
The antibacterial activity is also shared with numerous anthraquinones such as catenarin, chrysophanol, emodin or pachybasin. Others such as **averantin, nidurufin, viocristin,** and **isoviocristin** from the genus *Aspergillus* have also shown antibiotic properties (Anke et al. 1980; Lee et al. 2010). **Engyodontochones** from *Engyodontium album*, exhibited inhibitory activity against MRSA (Wu et al. 2016). **Conyothyrinone B-D** from the culture of *Coniothyrium* inhibited the bacteria

Table 18.2 Chemical structure, source, and biological effects of the interesting anthraquinones from fungal origin

Structures	Origin	Biological effect	References
Altersolanol A 	*Alternaria, Ampelomyces, Stemphylium botryosum*	Phytotoxic, cytotoxic, protein kinase inhibitor.	Assante and Nasini (1987), Haraguchi et al. (1996), Aly et al. (2008), Debbab et al. (2009)
Averufin 	*Aspergillus versicolor*, *A. parasiticus, Dothistroma pini*	Mycotoxin, uncoupling mitochondria oxidative phosphorylation, antiproliferative potential	Kawai et al. (1984), Lee et al. (2010)
Catenarin 	*Aspergillus cristatus, A. glaucus*, *Curvularia lunata, Drechslera, Eurotium*, *Helmintosporium catenarium*, *Pyrenophora tritici-repentis*	Antibiotic and antiproliferative properties, inhibition DNA-dependent RNA polymerase, phytotoxic	Anke et al. (1980), Engström et al. (1993), Hobson et al. (1997), Bouras and Strelkov (2008)
Chrysophanol 	*Aspergillus*, *Drechslera teres*, *Curvularia lunata, D. holmii*, *D. ravenelii*, *Phoma* sp., *P. islandicum*, *P. pachybasium*, *Trichoderma*	Antifungal, bactericidal, anti-inflammatory, hepatoprotective	Bick and Rhee (1966), Hobson et al. (1997), Liu et al. (2009), Rai et al. (2009), Kim et al. (2010), Qian et al. (2011)
Cynodontin 	*Drechslera avenae, Curvularia lunata, Pyrenochaeta terrestris*	Fungicidal, phytotoxic	Hobson et al. (1997), Chrysayi-Tokousbalides and Kastanias (2003)
	Aspergillus glaucus, A. fumigatus, Curvularia lunata, Dermocybe	Hepatoprotective, antibiotic and antiproliferative properties, inhibition DNA-dependent	Bick and Rhee (1966), Anke et al. (1980), Calhoun et al. (1992), Hobson et al. (1997),

(continued)

Table 18.2 (continued)

Structures	Origin	Biological effect	References
Emodin 	*sanguínea, Phoma foevata, Pyrenochaeta terrestres, Pyrenophora tritici-repentis, Trichoderma*	RNA polymerase. Phytotoxic, mycotoxic	Ren et al. (2006), Frisvad et al. (2009), Liu et al. (2009), Qian et al. (2011), Dong et al. (2016), Stompor-Gorący (2021), Semwal et al. (2021)
Pachybasin 	*Ascochyta lentis, Coniothyrium, P. foevata, Trichoderma*	Antibiotic and antifungal properties	Bick and Rhee (1966), Liu et al. (2009), Sun et al. (2013), Masi et al. (2018)
Physcion 	*Aspergillus fumigatus, Eurotium, Microsporum*	Anticancer	Butinar et al. (2005), Frisvad et al. (2009), Wijesekara et al. (2014)
Questin 	*Aspergillus, Eurotium, Penicillium frequentans*	Negative allelopathy	Butinar et al. (2005), Gui et al. (2015)
Versicolorin 	*A. versicolor, A. parasiticus*	Cytotoxicity, mycotoxin	Hsieh et al. (1978), Jakšić et al. (2012)

E. coli and *B. megaterium.* They also showed inhibitory effects against the fungus *M. violaceum*, and the alga *Chlorella fusca* (Sun et al. 2013). These antifungal and antibiotic activity are common in the anthraquinones, as previously described in chrysophanol, cynodontin, or pachybasin.

The presence of a quinoid structure allows anthraquinones to have antioxidant and pro-oxidant properties, which could be beneficial or harmful. Some

anthraquinones such as **alaternin** and emodin are capable of preventing the oxidation of linoleic acid (Choi et al. 2000). The pro-oxidant and toxic effects of many anthraquinones have also been demonstrated and may be due to the formation of ROS (Ueno et al. 1995).

Therefore, anthraquinones of fungal origin have been popularly used for their various activities and biologically active compounds. All this broad spectrum of activity makes them potential compounds for the development of antifungal, anticancer, antibacterial and other drugs. However, its activity must be elucidated with more clinical trials and its toxic potential must be closely monitored.

18.5 Concluding Remarks

Fungal quinones, mainly represented by benzo-, naphtho-, and anthraquinones, comprise a group of natural metabolites with a great chemical diversity and a wide range of biological properties. From the most basic structures to the more complex compounds, they have been found in terrestrial fungi of the Ascomycota and Basidiomycota divisions but also in marine fungi. The most representative genera in which these compounds are found are *Alternaria*, *Aspergillus*, *Fusarium*, *Penicillium*, and *Talaromyces* generating a vast number of molecules with different biological, industrial, and pharmacological properties. Some of them, particularly anthraquinoid derivatives, are used as pigments and colorant agents. In terms of biological activity, certain benzoquinones, such as coenzyme Q10 has demonstrated antioxidant properties at a clinical level, while many naphthoquinones and anthraquinones seem to exert pro-oxidant activities. Many of the naphtho and anthraquinones are here presented as antimicrobial and anticancer agents with potential applications in the pharmaceutical industry; nevertheless, more studies are needed since these compounds might exert toxic effects inducing ROS release and DNA damage in cells and human tissues.

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Chapter 19
Non-Alkaloid Nitrogen-Containing Compounds from Fungi

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Abstract Fungi represent an ancient ubiquitous and interesting kingdom that can be classified into Phycomycota, Oomycota, Microsporidiomycota, Zygomycota, Ascomycota, Basidiomycota, and Deuteromycota divisions. These microorganisms are considered as a reservoir of bioactive compounds that can be exploited in crucial fields: food, pharmaceutical, and agricultural industries. Non-alkaloid nitrogenated compounds are considered a prolific bouquet composed of proteins such as enzymes playing an essential role in the synthesis of compounds that are involved in vital life processes, lytic enzymes possessing depleting effects, antibiotics, toxins applied as antimicrobials and antifungals, immunosuppressive molecules, etc. Moreover, a number of species found in phyla like Oomycota and Microsporidiomycota have been and are still used as biological control agents for crops as a counterpart to chemical products.

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Nevertheless, as potent these secondary metabolites can be, some of them exhibit a pathogenic profile against crops, beneficial insects, humans, mammals, fish, and crustacea.

This chapter is an effort to outlay the nitrogenated secondary metabolites produced by fungal species in terms of functions and applications.

19.1 Introduction

Fungi represent one of the most diverse and ancient evolutionary branches of the life tree (Li et al. 2021). Fungi are found in the kingdom Mycetae (also called kingdom fungi) and the kingdom Straminipila (Raghukumar 2017). Organisms included in these kingdoms play a key role in the nutrient and carbon cycle in terrestrial ecosystems as mutualists, pathogens, and free-living saprotrophs. They are characterized by being one of the largest eukaryotic kingdoms (Tedersoo et al. 2018). The fungal kingdom comprises heterotrophic organisms characterized by the presence of a chitinous cell wall, the lack of phagotrophic capabilities, and cell organizations ranging from completely unicellular monopolar organisms to extraordinarily complex syncytial filaments that can form macroscopic structures (Naranjo-Ortiz and Gabaldón 2019).

Kingdom mycota can be classified into Phycomycota, Oomycota, Zygomycota, Ascomycota, Basidiomycota, and Deuteromycota divisions (Fig. 19.1). This classification is based on the organization of the vegetative thallus, the morphology of reproductive structures, the way of spores' production and the particular life cycle involved (Patterson and Sutton 2018).

The exploitation of these microorganisms for extracting bioactive compounds can be interesting since many fungi are a rich source of amino acids and other digestible nitrogen compounds (Wallis et al. 2012). These organisms are the only ones capable

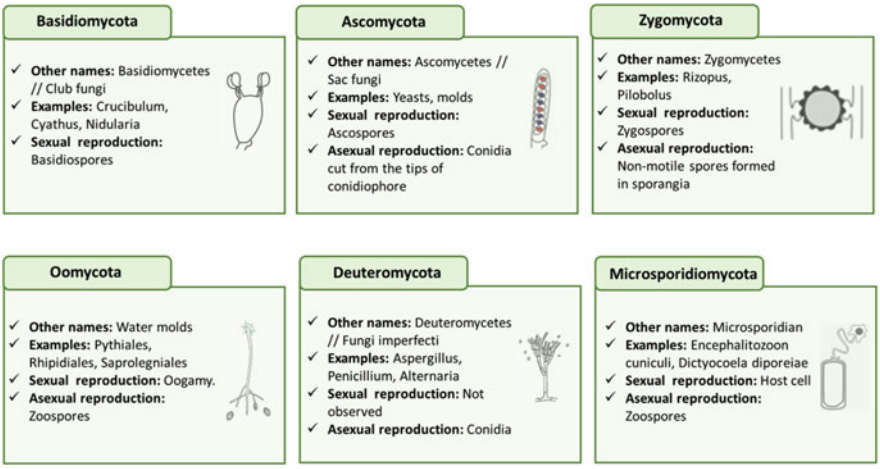

Fig. 19.1 Classification of main commercial fungi

of degrading complex nitrogen molecules (proteins, amino acids, nucleic acids and nucleotides) to simple compounds (ammonia or ammonium ion) (Gupta et al. 2017). However, the content of nitrogen compounds and the rest of the elemental compounds of fungal biomass significantly varies between species (Wallis et al. 2012). For example, carbon levels range from 38 to 57% dry matter, nitrogen values range between 0.23 and 15% dry matter, and phosphor ranges from 0.040 to 5.5% dry matter (Zhang and Elser 2017). In edible mushrooms, nitrogenous compounds represent the second largest fraction after carbohydrates. Their protein content varies from 4% to 53.7% dry matter depending on the species (Barros et al. 2007, 2008; Bauer-Petrovska 2001). However, more studies are needed to fully understand changes produced in the protein content during the fruiting body development, as well as the digestibility of the protein fraction. A study carried out with *Pleurotus ostreatus* showed that during the development phase of the fruiting body, 92% of the protein content (36.4–11.8% of proteins in cap and stem) was digestible. In addition, mushrooms possess large amounts of nitrogen in the form of free amino acids and nucleic acids, present mainly in the cell walls' chitin (Chakraborty et al. 2021; Fujihara et al. 1995; Mattila et al. 2002). The amount of non-protein nitrogen in some species reaches 33.4% of the total nitrogen (Bauer-Petrovska 2001). This nitrogen concentration is also due to the presence of toxins, siderophores, beta-lactams, cyclopeptides, non-protein amino acids, cyanogenic glycosides and glucosinolates. In this chapter, the main non-alkaloid nitrogenous compounds present in common edible mushroom families are reviewed. In addition, the biological properties of these compounds and their potential applications are deeply discussed.

19.2 Non-alkaloid N-biomolecules in Fungi

19.2.1 Basidiomycota

Basidiomycota comprises the most morphologically complex family of fungi. They include mushrooms, toadstools, rust, and smut parasites of plants (Watkinson 2008). The nitrogen content of basidiomycota biomass varies from 2 to 4% dry matter (Zhang and Elser 2017). Nitrogen sources in basidiomycota are from proteins and other compounds such as toxins, siderophores, beta-lactams, cyclopeptides, non-protein amino acids, cyanogenic glycosides and glucosinolates. Generally, fungi produce hydroxamate and carboxylate types of siderophores (Khan et al. 2018). Basidiomycota is reported to have siderophore synthetases type I, II, and VI that primarily produce hydroxamate siderophores with N-hydroxylated and N-acylated ornithine residues (Gressler et al. 2021). These compounds are interesting as they have a high affinity for ferric iron, which is necessary for many vital life processes (Schneider et al. 2020). Moreover, siderophores have antibacterial activity and can act as chelating agents forming soluble complexes (Chowdappa et al. 2020). These properties make siderophores interesting in biotechnology, medicine, bioremediation of heavy metal-polluted environments, biocontrol of plant pathogens, and

plant growth enhancement (Pecoraro et al. 2022). Ferricrocin was mainly found in *Hebeloma crustuliniforme*, potassium nutrition is a significant regulatory factor for ferricrocin production (Van Hees et al. 2006). Ferrichrome and ferrichrome A have been reported in *Ustilago maydis*, being both compounds obtained by hydroxylation of L-ornithine (Budde and Leong 1989; Winterberg et al. 2010), while hydroxamate and catecholate siderophore were found in Wolfiporia cocos (Arantes and Milagres 2008). In *Laccaria laccata* and *Laccaria bicolor,* the main siderophore is linear fusigen (hydroxamate siderophore). Ferricrocin, coprogen, and triacetylfusarinine C were also reported in small quantities in these organisms. The production of large amounts of linear fusigen may explain the observed suppression of plant pathogenic Fusarium species (Haselwandter et al. 2013). Rhodotorulic acid is present in *Microbotryum violaceum* and its concentration in the culture medium can increase in response to iron stress (Birch and Ruddat 2005).

Basidiomycota is a rich source of cyclopeptides, mainly present in *Amanita*, *Conocybe*, *Lepiota*, *Galerina* and *Omphalotus* species. Cyclopeptides have shown certain toxicity in mammals, but also bioactivities of interest such as cytotoxic, insecticidal, antimalarial, estrogenic, sedative, nematicidal, antimicrobial, immunosuppressive and enzyme-inhibiting activities (Pomilio et al. 2006). This group of compounds is formed by toxins such as amatoxins and phallotoxins characteristic of *Amanita* spp. (Hallen et al. 2003). Amatoxins (*e.g*., α-amanitin, amatoxin, related bicyclic heptapeptide phallacidin, phallotoxin) are the most common lethal toxins of poisonous mushrooms in the genus *Amanita*. They are potent and selective inhibitors of RNA polymerase II (Hallen et al. 2007). α-amanitin can be found in certain species of the genera *Amanita*, *Galerina*, *Lepiota*, and *Conocybe* (*e.g*., *A. bisporigera, G. marginata, C. apala*) (Luo et al. 2012). Fomannoxin and sesquiterpene phytotoxin fomannosin are produced by *Fomes annosus* (Heslin et al. 1983). Clitidine is produced by *P. amoenolens* (Khovpachev et al. 2021). Alloviroidin and virotoxins are produced by *A. suballiacea* (Little et al. 1986). Cylindrocyclin A was isolated from *Cylindrocarpon sp*. (Weber et al. 2006). Omphalotins B, C and D are nematicidal cyclopeptides from *Omphalotus olearius* (Büchel et al. 1998).

Fungi have several non-protein amino acids, for example, α-aminoadipic acid, ornithine, citrulline, β-alanine, γ-aminobutanoic acid, cysteic acid and ethanolamine (Hatanaka 1992). Gamma-aminobutyric acid (GABA), a non-protein amino acid abundant in food matrices, is found in several species of basidiomycota such as *Agaricus* spp., *Auricularia* spp., *Boletus edulis, Clitocybe maxima, Lentinus edodes, Ophiocordyceps sinensis* and *Pleurotus ostreatus* (Ramos-Ruiz et al. 2018) and it is the main non-protein amino acid of *A. bisporus* (Oka et al. 1981). GABA acts as a potent neural signal transmitter. It can be used to regulate blood pressure, treat brain and psychiatric diseases, enhance the immunity system, protect against cancer, exhibit anti-inflammatory activity, or hormonal regulation (Diana et al. 2014). Se-methyl-seleno-l-cysteine, a seleno non-protein amino acid, is produced by *Lentinula edodes*. This molecule is characterized by having strong anticancer activity and the ability to remove excess selenium (Klimaszewska et al. 2016). Ornithine and N-(gamma-L-glutamyl)-4-hydroxyaniline are the main non-protein amino acids found in *A. bisporus* (Oka et al. 1981).

Two cyanogenic glucosides (linamarin and lotaustralin) were identified in psychrophilic basidiomycota (Stevens and Strobel 1968; Zuk et al. 2020). Despite the psychrophilic basidiomycota called snow mold does not produce linamarin or lotaustralin in detectable amounts, it can synthesize other unstable cyanogens (*e.g.*, glyoxylic acid cyanohydrin and pyruvic acid cyanohydrin) in the range of 0.8–1.4 mg/mL (Tapper and Macdonald 1974).

19.2.2 Ascomycota

Ascomycetes are, together with basidiomycetes, very well-documented prolific producers of secondary metabolites. Among the variable non-alkaloid structures synthesized by ascomycetes, the most abundant structures can be classified as toxins, peptides, or amines with both cyclic and linear structures.

The toxin synthesis of non-alkaloid structures by ascomycetes is considered to create negative impacts on agriculture production and public health. Currently, more efficient detection techniques to improve food safety are being developed and even some of them are regarded to exploit their potential in alternative applications.

Regarding food safety issues, fumonisins are a relevant group of mycotoxins mainly involved in the contamination of *Zea mais* (maize) and many other grains and derived products. Fumonisins are principally synthesized by *Fusarium* sp., although *Aspergillus nigri* has been also described to produce them (Kamle et al. 2019). The analysis of contaminated maize samples has shown that B-series fumonisins (FB_1, FB_2, FB_3, and FB_4) are the most relevant detected mycotoxins, even though two other groups, A and C, have been described (Proctor et al. 2003). Fumonisins are constituted by a linear 20-carbon backbone of polyketide origin that presents several substitutions: 1 amine ($–NH_2$) or amide (NH_2COOCH_3), 1–4 hydroxyls ($–CH_3$), 2 methyls ($–CH_3$), and 2 tricarboxylic acid groups. Fumonisins A possess an amide substitution, while the B group presents an amine. At the same time, fumonisins B present a methyl group at the C1-terminal, whereas fumonisins C do not have it. This group of toxins was described to induce diseases such as kidney, liver and esophageal cancers (Krska et al. 2007; Proctor et al. 2003). Similarly, ochratoxins, especially ochratoxin A, have been described to cause severe health damage mostly associated with their main organ target, the kidneys, where it can induce nephrotoxicity or tumors. Ochratoxin A is mainly synthesized by *Aspergillus ochraceus, A. carbonarius, A. niger,* and *Penicillium verrucosum.* The chemical structure of ochratoxin A includes the amino acid L-phenylalanine bound by an amide linkage to a dihydroisocoumaric acid with a chlorine substitution which seems to be related to its toxic activity. Other analogs are ochratoxin B (a non-chlorinated form), ochratoxin C (an ethyl ester derived), the methyl esters of the ochratoxin A and B, ochratoxin α (free dihydroisocoumaric acid) and its amides bound to serine, hydroxyproline and lysine (EFSA 2020). Another important group of toxins involved in food safety concerns is the enniatins and beauvericin, a group of cyclic hexadepsipeptides initially found to be synthesized by *Fusarium oxysporum*,

recently described in the genera *Beauveria*, *Halosarpheia*, *Paecilomyces*, *Polyporus* and *Verticillium*. Both groups share a very similar chemical structure. Enniatins consist of a cyclic hexadepsipeptide bond to 3 methyl-amino acid residues, mainly valine and/or isoleucine, and the most relevant analogues are A, A1, B, and B1. Whereas the hexadepsipeptide in beauvericin is bonded to 3 hydroxy-isovaleryl, 3 N-methyl-phenylalanyl, or a combination of both. Both beauvericin and enniatins create weak ion-dipole interactions with cations and act as ionophores so they can increase the membrane permeability of alkali cations. However, there is limited information about the toxicological risk assessment for long-term exposure to these compounds (Caloni et al. 2020). Up to date, enniatins have been used for the development of a nasal/oromucosal spray for the treatment of upper respiratory infections due to their antibiotic and anti-inflammatory properties (EFSA 2014). Beauvericin was also demonstrated to have a wide variety of biological activities as an insecticide (against *Aedes aegypti* or *Calliphora erythrocephala*, among others), antibiotic (against *Bacillus* spp., *Listeria monocytogenes*, *Pseudomonas aeruginosa*, *Salmonella enterica*, *Shigella dysenteriae*, and *Escherichia coli*, among others), antiviral (against HIV-1), antiplasmodial, antifungal, anticarcinogenic and phytotoxic agent (Caloni et al. 2020). Finally, another interesting molecule that has been described both as mycotoxin and cytochalasan is the chaetoglobosin A which was found to be produced by *Chaetomium globosum*, *Chaetomium. subbafine,* and *Penicillium aquamarinium*. Chaetoglobosin A has been demonstrated to have very strong cytotoxic activity toward chronic lymphocytic leukemia by attacking the cytoskeleton and inducing apoptosis (Knudsen et al. 2014).

Other secondary metabolites from ascomycota are the polyketide-non-ribosomal peptide (PKS-NRPS) cytochalasins. This group is represented by numerous and diverse analogs. For instance, *Aspergillus sclerotioniger* was identified to produce sclerotionigrin A and B and proxiphomin. This latter showed a strong cytotoxic effect against chronic lymphocytic leukemia although it was less effective than chaetoglobosin A (Petersen et al. 2014). Isolated from *Alternaria alternata* the alternariasin A was demonstrated to possess antibacterial activity against *S. aureus*, *B. subtilis* and *E. coli* (Guo et al. 2021). Species belonging to *Xylaria* have been targeted for their content in cytochalasins. From *Xylaria* cf. *curta*, curtachalasins C and E were capable of inducing a reversion of the fluconazole-resistance in *Candida albicans*, and xylarichalasin A which showed strong antiproliferative activity against human breast and hepatic cancer cell lines. Similarly, other cytochalasins such as xylochalasin or daldinin (from *Daldinia concentrica*) showed cytotoxic effects against several human cell lines but showed much weak activity. Besides, other activities were described for this group of cytochalasins. For instance, nodulisporic acid was obtained from *Hypoxylon pulicicidum* and showed insecticidal and antiparasitic capacity and the cyclooctadepsipeptide "PF-1022A," which can be produced by several strains of *Rosellinia* and *Astrocystis* has shown veterinary applications as anthelmintic. Besides, phytotoxic effects were reported for cytochalasin K (or epoxyrosellichalasin) which displayed stronger inhibition power against wheat shoot and turnip root elongation than glyphosate. Cytochalasin C and D from

Xylaria cubensis also exhibited stronger growth inhibition of wheat coleoptiles than the oxyfluorfen-based commercial herbicide (Becker and Stadler 2021).

Other fungal peptides include chlorofusin, a nonacyclopeptide isolated from *Microdochium caespitosum* that was disclosed to act as an antagonist of the MDM2 (mouse double minute 2 homolog) protein. MDM2 protein is a negative regulator of the p53 tumor suppressor to which chlorofusin gets directly bound through the *N*-terminal domain (Qiu et al. 2014). Another fungal peptide is the group of cyclic structures named after pseudoxylallemycins and isolated from *Pseudoxylaria* sp., which contains hydrophobic and aromatic amino acids and an alternating pattern of N-methylation. Pseudoxylallemycins A–D have been described to have antimicrobial activity against the *Pseudomonas aeruginosa* and *Mycobacterium vaccae* and antiproliferative activity against human cell lines used to evaluate cardiovascular diseases (HUVEC) and leukemia (K-562). Besides, they reported cytotoxic activity against cervical carcinoma cells (HeLa) (Guo et al. 2016). Other fungal cyclic structures are the family of caprolactams named pestalactams A–C and extracted from the endophytic fungus *Pestalotiopsis* sp. The pestalactams A and B showed modest activity against two different strains of the malaria parasite *Plasmodium falciparum* evaluated through *in vitro* assays (Davis et al. 2010).

Finally, several siderophores were described to be synthesized by Ascomycota fungi. Siderophores can be mainly classified into three categories: coprogens, fusarinines, and ferrichromes. Regarding the family of coprogens *Fusarium dimerum* produces dimerum acid and coprogen B, *Penicillium sp.* coprogen and *Alternaria longipes* hydroxycoprogen, hydroxyneocoprogen I, hydroxyisoneocoprogen-I, N-dimethyl coprogen, and few derivatives*:* The group of the fusarinines is represented by cis- and trans-fusarinines produced by *Fusarium roseum* and *Fusarium dimerum,* fusarinine A and B by *Gliocladium virens* or neurosporin by *Neurospora crassa.* As representatives of ferrichromes different asperchrome analogs, ferrichrome C and ferrirhodin are synthesized by *Aspergillus ochraceous* (Shukkoor and Khalivulla 2021).

19.2.3 Zygomycota

The study of the secondary metabolites of zygomycetes has been repeatedly hindered by the study of higher fungi and because this type of metabolites has been rarely observed in zygomycetes (apart from the terpenoid trisporic acid from *Blakeslea trispora* and *Mucor mucedo*). Even though information regarding Zygomycota is scarce, a brief review of some interesting secondary metabolites is presented below.

Some mycotoxins have been identified in Zygomycota fungi. For instance, rhizoxins (16-membered macrocyclic lactones) isolated from *Rhizopus microsporus* are considered potent anticancer drugs since they possess a similar structure to vinca derivatives (vincristine and vinblastine) extensively described as inhibitors of microtubule polymerization and thus, they can inhibit mitosis (Garcia-Oliveira et al. 2021;

Jennessen et al. 2005) Besides, rhizonin is another class of cyclic peptides extracted from *Rhizopus microspores*. Rhizonin A is a cyclic heptapeptide containing 2 valines, 1 *allo*-isoleucine, 1 leucine, 1 *N*-methylalanine, ND 2 *N*-methyl-3-(3-furyl)alanine (Steyn et al. 1983). Both rhizonin A and B are also considered mycotoxins with strong hepatotoxic activity (Jennessen et al. 2005).

Regarding *Mortierella alpina,* different oligopeptides have been isolated. Calpinactam, a chemical structure characterized by being a hexapeptide with a caprolactam ring at the C-terminal, possesses antimycobacterial activity against *Mycobacterium smegmatis* and *Mycobacterium tuberculosis* (Koyama et al. 2010). Another family of linear peptides includes the hexapeptides malpinins A-D which can act as natural emulsifiers since they can reduce the surface tension. Concerning their biological activity very modest antibacterial properties and antiproliferative effects against a few cancer cell lines were observed (Baldeweg et al. 2019). Regarding cyclic peptides, two classes of cyclopentapeptides, malpibaldins A and B and malpicyclins A to F were also isolated from *Mortierella alpina* and showed moderate activity against Gram-positive bacteria (Baldeweg et al. 2019; Wurlitzer et al. 2021). Malpicyclins are composed of leucine, valine, d-phenylalanine, d-tryptophane, d-tyrosine, and d-arginine (Wurlitzer et al. 2021). Zygosporamide, a cyclic pentadepsipeptide in which structure is present the α-hydroxyleucic acid and a few d- and l-amino acids, was purified from *Zygosporium masonii*, a marine Zygomycota fungus. Zygosporamide displayed significant cytotoxicity in the global panel of 60 types of cancer cell lines (NCI's 60 panel) and showed high selectivity against the specific cancer glioblastoma (SF-268 cell line) that affects the central nervous system and renal cancer (RXF 393 cell line) (Oh et al. 2006). Another cyclic molecule, named Ro 09–1679, consisted of fumaryl-L-arginyl-L-leucyl-arginal and it was shown to have thrombin inhibition activity (Kamiyama et al. 1992). The cyclic compound MBJ-0174 displayed a similar structure to plactins which has previously been shown to act as a stimulator of cellular fibrinolytic activity (Kawahara et al. 2017).

Different siderophores have been isolated from Zygomycota. Among them, rhizoferrin, initially isolated from *Rhizopus microsporus*, represents the most common siderophore. Many siderophores show the structural presence of citrate, an intracellular primary metabolite capable of binding iron and thus, they can be defined as the simplest siderophores (Winkelmann 2001). Indeed, the polycarboxylate or complexone structure of the rhizoferrins consists of a diaminobutane backbone linked to 2 citric acid moieties and presents a *R,R*-configuration around the chiral center. Rhizoferrins have been identified by analytical analyses in several families of Mucorales such as in Mucoraceae (*Mucor mucedo* and *Phycomyces nitens*), Thamnidiaceae (*Chaetostylum fresenii* and *Cokeromyces recurvatus*), Choanephoraceae (*Cunninghamella elegans* and *Mycotypha africana*), Mortierellaceae (*Mortierella vinacea*) and in Entomophthorales (*Basidiobolus microsporus*) (Carroll et al. 2017; Thieken and Winkelmann 1992). Besides, specific classes of rhizoferrins have been isolated such as glomuferrin, which consists of a putrescine (1,4 butanediamine) bond by amides to 2 dehydrated citrate residues. This

compound was obtained from *Glomus etunicatum*, *G. mossae,* and *Glomus* sp. (Winkelmann 2017).

19.2.4 Oomycota

The Oomycota (water molds) are fungus-like filamentous eukaryotic microorganisms associated to the kingdom Straminipila. However, they are similar to fungi due to their filamentous growth and their mode of nutrition that occurs via absorption (Kaczmarek 2021). This phylum is divided into two classes: *Saprolegniomycetes* and *Peronosporomycetes* and consists of more than 1500 species in 100 genera (Silva 2017; Kaczmarek 2021). Among the Oomycota, *Phytophthora infestans* has notably impacted the history by causing late blight of potatoes which was considered as the origin of the Irish potato famine that led to the emigration of 1.5 million people and the death of approximately one million people in the 1840s (Rossman 2006). The phylum Oomycota, which includes saprobes and parasites (Rossman 2006), is known to be one of the most ubiquitous eukaryotic life forms as they can be found in terrestrial and aquatic environments (Thines 2018). Saprobic species participate in the cycling of nutrients by the degradation of organic material becoming as well available for secondary colonizers (Silva 2017; Thines 2018). Oomycetes are also capable of secreting effectors that promote pathogenicity, which may stay outside the host cells to stop non-specific defense mechanisms or may be delivered into host cells to attack different organelles (Fry 2010; Thines 2018). Oomycota is best known as plant pathogens, but numerous species attack other organisms, such as the case of Saprolegnia species which are pathogens of amphibians, crustaceans, fish, and insects. *Pythium oligandrum* and *Pythium periplocum* are pathogens of fungi and oomycetes. *Pythium guiyangense* is an extremely virulent pathogen of mosquitos. In addition, some species like *Pythium insidiosum*, can cause fatal infections in humans and other mammals that can lead to amputations or even death (Silva 2017; Shen 2019).

Oomycetes have long been classified as fungi, however numerous differences are identified that involve the morphology and the physiology (Latijnhouwers 2003). One of the most considerable distinctions is that Oomycetes' cell walls are composed of cellulose derivatives and other β-glucans with little to no chitin, contrary to true Fungi that have a chitinous cell wall (Hassett 2019; Kaczmarek 2021). Besides, during a major part of fungi's life cycle, they are considered as haploid or dikaryotic, while oomycetes are diploid (Latijnhouwers 2003). In addition, during the sexual reproduction of Oomycetes, the oospores are formed, whereas none of the true Fungi produce oospores (Rossman 2006). Oomycetes are known to have a limited capacity to produce secondary metabolites. However, they secrete different enzymes to attack their hosts or to survive (Bělonožníková 2022; Judelson 2009). Indeed, phytopathogenic oomycetes secrete degradative enzymes that are required for host cell degradation, such is the case of carbohydrate-active enzymes (CAZymes) including cutinases to degrade cutin, pectinases to degrade pectin, endoglucanases and

β-glucosidases to degrade xyloglucan, α-glucosidases, α-amylases, α-glucoamylases, and starch-binding modules to metabolize starch (McGowan and Fitzpatrick 2020; Wang 2018). Among the CAZymes and glycoside hydrolases, oomycetes secrete chitinases which play a crucial role in mycoparasitism (Bělonožníková 2022). In addition, according to Rodenburg et al. the study of the phylum oomycota shows that the pathways related to lipids like sphingolipid, glycerolipid, glycerophospholipid, and ether lipid are the highest which confirm that both lipids and fatty acids have an important role in oomycetes. Besides, the same study mentioned that *Phytophthora* spp. is able to reduce the naphthalene due to the action of salicylate hydroxylase that degrades salicylic acid which confers a defensive reaction to the plants (Rodenburg et al. 2020). Moreover, some oomycetes produce toxic molecules in order to suppress immune responses such is the case of *Saprolegnia parasitica* which attacks its fish hosts by producing prostaglandin E2 (Judelson 2009). As for *Plasmopara halstedii*, it uses campesterol to produce a plant hormone called brassinolide by being able to acquire this latter phytosterol and converts it to brassinolide that will be absorbed by the plant host which can lead to the deregulation of its immune system (Judelson 2009). Oomycetes are barely used by the industry due to their pathogenicity. However, some genera are used as bio-control agents like *Pythium oligandrum* (Berger et al. 2016), which presents different advantages according to several studies. *P. oligandrum* acts like a colonizer to the rhizosphere of many crop plants and a competitor for space and nutrients. It also induces plant growth via the production of an auxin precursor which is the tryptamine (TNH2). This oomycete activates the plant immune system and as a result promotes defense response and resistance against soilborne fungal pathogens, such is the case of ascomycetes, basidiomycetes and bacteria. *P. oligandrum* has the capacity to promote resistance against pathogenic oomycetes like *Phytophthora* species by the production of extracellular proteins termed Oligandrins (Bělonožníková 2022; Benhamou et al. 2012).

19.2.5 Deuteromycota

The deuteromycota phylum includes species that do not yet have a well-defined classification, and, for this reason, its representatives are often called imperfect fungi. This phylum receives various denominations ranging from deuteromycota, deuteromycetes, conodial fungi, imperfect fungi, mitosporic fungi, and anamorphic fungi. The deuteromycetes are an artificial group of fungi, of which there are approximately 15,000 species, commonly called molds, and are called imperfect fungi since they are second-class fungi and do not have a known sexual state in their life cycle. This undefined asexual state is called anamorphic state, able to occur through the production of conidia or chlamydospores, which are structures that resemble spores but formed only by mitosis (asexual spores) or by budding leading to the formation of the pseudomycelium, or even differentiation of the somatic hyphae, therefore they are placed in a separate phylum (Alexopoulos et al. 1996;

Grandi 1998; Kirk et al. 2008). Although their conidial states are similar to the phyla of the ascomycota, some species show affinity to lower (primitive) fungi and the basidiomycota phyla. The characteristic reproductive structures of these fungi are conidiophores and conidia and can occur in different forms (Grandi 1998). These fungi colonize, survive, and multiply on various substrates, and are subject to extreme climates, such as those that characterize the Arctic and Antarctic, deserts, as well as milder environments such as tropics and zones with a temperate climate (Cannon and Sutton 2004). They perform several ecologically important functions, such as decomposition of organic matter, release of minerals, production of humic substances, promotion, and alteration of niche development, among others. They also play important roles in enzyme production and industrial production (antibiotics, immunoregulators, etc.), in case genetic manipulations, biological control, and cause profound mycoses, allergies, and diseases in plants and animals. These Fungi have great economic value for industries worldwide, because through secondary metabolism they can produce antibiotics, vitamins and their precursors, enzymes, and organic acids, besides fermenting beverages and maturing foods (Bononi and Grandi 1999; Kendrick 2000; Mercado-Sierra 1984). However, they can cause serious harm to humans by producing serious pathologies or mycotoxins. The use of deuteromycete fungi is very important for industry in the fermentation process. Many new and innovative chemical compounds are obtained from these fungi on an industrial level. Some members are used in food processing and flavoring such as *Penicillium camemberti* and *Penicillium roqueforti* in the cheese industry, such as brie and blue cheese, these, after going through processing are immersed in a spore solution and remain there until they age before going to the market. In addition, many species produce mycotoxins, for example, *Aspergillus flavus*. Deuteromycetes, on the other hand, are very important in their negative roles. Many of them bio-deteriorate our materials, while many more produce a variety of toxins in food, feed, and grain in storage. Some of these toxins are carcinogenic to humans and animals. They can act as plant parasite species of higher plants, causing severe leaf and fruit diseases. Special mention can be made of *Alternaria solani*, which causes early blasting of potato, *Helminthosporium oryzae* causes brown spot of rice, *Heliminthosporium gramineum* caused Stripe disease of barley, *Pyricularia oryzae* causes rice blast, *Septoria tritici* causes leaf spot of wheat, *Colletotrichum gloeosporioides* causes anthracnose of mango, *Colletotrichum falcatum* causes red rot of sugarcane, *Fusarium oxysporum* var. cubense causes Panama wilt of banana, Fusarium udum causes wilt of pigeon pea, and *Cercospora personata* causes leaf spot or tikka diseases of peanut. One example is aflatoxin, which is produced by the fungus *Aspergillus flavus*, found in peanuts. A general screening of the fungus can be done using a black light, under which the fungus fluoresces a greenish-yellow color. Fungi use their ability to produce enzymes to enter growing plant tissue and then destroy the tissue. Annual crop losses caused by fungi in the United States can be measured in billions of dollars. In addition, the small spores of deuteromycetes can directly affect animals and humans (Chahal 2021). Most human mycoses are developed by deuteromycete fungi. The diseases causing fungi of the skin of humans, animals or both are known as dermatophytes or ring fungi and the diseases

are dermatomycosis. These fungi cause ringworm of the scalp (found mainly in children) or other hairy parts of the smooth skin of the nails and hands and feet. Ringworm of the feet is popularly known as "athlete's foot." *Trichophyton gypseum* and *Thalassoma purpurium* cause athlete's foot. Another disease, called moniliasis, that mainly affects the toes, is caused by *Candida albicans*. Candida species also cause a disease of the throat and mouth called thrush, lung infections, infection of the mucous membranes of the genital organs, and various diseases. Spores are released in air currents and are blown from one place to another. As humans breathe this air, the spores enter the nasal passages and lungs and react with the immune system, causing allergies.

Some deuteromycete fungi are medicinally very important. For convenience, the miracle drug penicillin is obtained commercially from *Penicillium chrysogenum*, while *Penicillium griseofulvum* is the source of the production of the antibiotic griseofulvin. In addition, these fungi are used for their capacity for biochemical synthesis and conversions, such as that of steroids, which are of great importance in pharmaceuticals. Commonly the anamorph fungi, Aspergillus, Penicillium, Fusarium and Trichoderma, produce most of the widely used antibiotics, rarely are such compounds produced by species of the phylum Zygomycota. New compounds have been investigated on an industrial pharmacological screening scale, such as antitumor and anticancer agents, enzyme inhibitors, immunomodulators, and cardiovascular agents (Brizuela et al. 1998; Okanade et al. 2004).

19.2.6 Microsporidiomycota

Microsporidiomycota are obligate intracellular, eukaryotic, unicellular, and spore-forming parasites that have a rather reduced form and that belong to the fungal kingdom (Goyal et al. 2016). They can invade multiple organisms through their impeccable way of infecting almost all animals (vertebrates and invertebrates), humans, as well as some protists (Didier et al. 2004; Han and Weiss 2017). More than 1400 species are reported and described, which are distributed into about 200 genera able to cause both benign and lethal infections (Cali et al. 2017; Han and Weiss 2017). From arthropods, insects are considered as type hosts of almost half of the nearly 200 described genera (Solter et al. 2012).

Microsporidian parasites are omnipresent in the environment in various ecosystems (terrestrial, marine, and freshwater), and they can cause devastating economic damages as they infect important organisms in agriculture and in fishery such as honeybees, silkworms, crustacea, and fish (Han et al. 2020). Initially, Microsporidiomycota were described as "yeast-like fungi" (Capella-Gutiérrez et al. 2012), especially in 1857 when the Swiss botanist Carl Nägeli discovered the first known microsporidium that has eventually led to the historical collapse of the silk industry in Europe, *Nosema bombycis*, which is famous for causing the pébrine disease in silkworms, from the domesticated silkworm *Bombyx mori* considered as the major source for sericulture all around the world for thousands of years (Pan et al.

2013; Solter et al. 2012). However, later, the Microsporidiomycota phylum was placed as Protozoa by Bilbani in 1882, and it wasn't until 1971 that Tutzet et al. proposed the first modern classification for the phylum, which has generated a controversy that lasted for years (Solter et al. 2012). The perplexations regarding the classification of Microsporidiomycota persisted long enough until only in the past 25 years that molecular biology techniques have been introduced and used to help the scientific committee reclassify Microsporidiomycota from protozoa to fungi (Mhaissen and Flynn 2018; Solter et al. 2012), and that was through the sequencing of a number of genes such as the building blocks for microtubules α- and β-Tubulin, the largest subunit of ribonucleic acid (RNA) polymerase II, TATA-box-binding protein, translation elongation factors EF-1α and EF-2, glutamyl transfer RNA (tRNA) synthase, and mitochondrial heat shock proteins 70 (HSP70) (Didier et al. 2004). Moreover, the sequencing of the pathogenic fungus *Encephalitozoon cuniculi* and the discovery of microsporidian mitosomes, as well as the presence of fungal traits such as closed mitosis, chitinous spores, and the production of trehalose have also contributed to recently reclassify microsporidomycota as fungi instead of protozoa (Capella-Gutiérrez et al. 2012). Microsporidiomycota is considered to be a very interesting microorganism that has peculiar characteristics allowing its species to survive outside a host cell as metabolically inactive spores. These features include a unique metabolism and, mainly, the absence of elements commonly found in eukaryotic cells such as mitochondria, peroxisomes, and the Golgi apparatus in its classic form (Buczek et al. 2020). The remarkably compact eukaryotic content is due to the intracellular parasitic life cycle of Microsporidiomycota which led to colossal depletions in the totality of genes throughout time (Huang et al. 2021). These elements are substituted by other structurally similar elements; for instance, the Golgi apparatus is replaced by a mass of vesicular tubes (Buczek et al. 2020), and the mitochondria is replaced by microsporidian mitosomes, which are degenerated mitochondria that lack a genome and the ability to produce adenosine triphosphate (ATP) (Han and Weiss 2017) making them the reason behind the Microsporidiomycota total energetic dependence on its hosts due to a restricted energetic functioning (Huang et al. 2021). To compensate for the lack of energy and metabolites, Microsporidiomycota utilizes its transport systems to profit from the host cell's nutrients and ATP in exchange for injecting cytotoxic compounds that cause oxidative stress in host cells eventually, inactivating and killing the host organism (Jena et al. 2016). Though microsporidiomycota species are considered a danger to a myriad of beneficial species such as honeybees and silkworm, they are of a remarkable agricultural importance seen the role some microsporidian species play as biological control agents, namely in biological control of pests, in managing insect diseases, and in insect population dynamics (Solter et al. 2012). Amongst the several *Nosema* species that are used as biological control agents seen their ability to infect different insects, *Nosema pyrausta, a microsporidian species that spreads both vertically and horizontally, is usually used as a biological control agent for insect pests of maize* (Gassmann and Clifton 2017), while *Nosema locustae*, later named *Paranosema locustae*, which is another microsporidium that spreads both vertically and horizontally and has a wide host range, is considered as a natural

control agent for European corn borer (*Ostrinia nubilalis*). Until the early to mid-nineties, it was the only commercially available species of microsporidium used to control grasshoppers and crickets following a long-term management strategy of rangeland pests due to its slow mode of action (Hoffmann and Frodsham 1993). On the other hand, *Vairimorpha necatrix*, a microsporidium that spreads horizontally, presents a high commercial potential in the field of biological control seen its ability to infect various caterpillar pests including European corn borer, cabbage looper, fall webworm, etc., and its high virulence leading to a quick death in its hosts in a matter of six days post invasion (Hoffmann and Frodsham 1993). Although microsporidiomycota presents interesting biocontrol efficacy, it is still not widely used due to the slow mode of action that takes many weeks, and more importantly due to its ability to also infect beneficial insects and natural enemies, which impairs the ecological balance on the long-term run (Bjørnson and Oi 2014).

19.3 Conclusions

Fungi are sources of various nitrogenated secondary metabolites that have applications in several industries. Although some are very important for the food and pharmaceutical industries, many compounds are still poorly studied due to having toxic effects. Still, further research is needed to understand potential effects of these nitrogenated molecules.

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Chapter 20
Sulfur-Containing Compounds from Fungi

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Abstract Sulfur contributes greatly to the chemical richness of nature and, due to its unique properties, enables essential biological interactions that no other element can. Sulfur-containing compounds are bioactive/nutraceuticals substances from different

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sources, including fungi. They comprise sulfur atoms that are cyclically or noncyclically bonded to a cyanate group or a carbon atom. Sulfur-containing compounds, already identified in various mushrooms species, include compounds such as ergothioneine (EGT), glutathione (GSH), and lenthionine (LT). These compounds exhibit important therapeutic properties such as antioxidant, anti-inflammatory, anti-neurodegenerative, and antiplatelet characteristics potentially useful in the treatment of a variety of disorders and diseases, including neurodegenerative and cardiovascular diseases and even diabetes. The purpose of this chapter is to explain the generally recognized sulfur-containing compounds from fungi covering the distribution in species, and genera, as well as the extraction and identification by contemporary analytical techniques. The biosynthetic elements of fungal sulfur compounds, as well as the state of art concerning their biological activities, are also discussed.

Abbreviations

ALD	alcoholic liver disease
DM	diabetes mellitus
EGT	ergothioneine
HBV	hepatitis B virus
HCV	hepatitis C virus
HIV-1	immunodeficiency virus-1
HPLC-PDA	High-Performance Liquid Chromatography - Photodiode Array Detector
GSH	glutathione
GTT	γ-glutamyl transpeptidase
IGT	impaired glucose tolerance
LC/ESI-MS/MS	liquid chromatography/electrospray ionization-tandem mass spectrometry
LT	lenthionine
SPME	solid-phase microextraction

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NAFLD	non-alcoholic fatty liver disease
OCTN1	organic cation transporter 1
ROS	reactive oxygen species

20.1 Introduction

Sulfur is one of the most basic elements in living beings and the seventh most prevalent mineral in the human body. Sulfur is classified as chalcogen and nonmetal, belongings to the group of elements in the periodic table that presents a wide range of properties in redox states. The multiplicity of chemical structures of sulfur-containing compounds, influenced in part by the element's several oxidation states, directly results in diverse modes of action for sulfur-containing natural products synthesized as secondary metabolites. Natural sulfur compounds are used by all living organisms and are presented in several kingdoms. The kingdom Fungi is an important and valuable source of these compounds (Francioso et al. 2020). Fungi are found and distributed ubiquitously in all ecosystems, performing a variety of functions that includes biomass degradation and participation in mutually beneficial symbiotics with other organisms (Naranjo-Ortiz and Gabaldón 2019). Macrofungi or mushrooms are well-known for their significant nutritional value due to their high protein, essential amino acids, and fiber content, as well as their low-fat level (Kalač 2013). Mushrooms are an important matrix due to their functional benefits, which are correlated to the occurrence of diverse bioactive compounds such as antioxidants, anticancers, and antimicrobials (Garcia et al. 2021, 2022). The bioactive compounds include phenolic compounds (caffeic acid, gallic acid, cinnamic acid, melatonin, *p*-hydroxybenzoic acid, *p*-coumaric acid, and protocatechuic acid), carbohydrates (chitosans, glucans/lentinan, trehalose), fatty acids (linoleic, oleic, and palmitic acid), proteins (lectins), and sulfur compounds (lenthionine, ergothioneine, and glutathione). From those, some of the unique bioactive sulfur molecules found in mushrooms are ergothioneine (EGT), glutathione (GSH), and lenthionine (LT) (Borodina et al. 2020; Gaucher et al. 2018; Wang et al. 2021b). EGT is derived mostly from edible mushrooms including *Agaricus bisporus*, *Lentinula edodes*, *Pleurotus ostreatus*, and *Grifola frondosa* (Kalač 2013) and it has been authorized for supplementation. Because of the discovery of its transporter (OCTN) in the body, as well as its antioxidant, anti-inflammatory, and cytoprotective actions in injured tissues, EGT has gotten a lot of attention (Halliwell et al. 2018). Supplementing EGT with other pharmacological drugs has also been linked to better treatment results in several illness models (Dare et al. 2022) GSH is the major sulfur-containing compound with widespread distribution in eukaryotic cells (plants, animals, fungi) and Gram-negative bacteria. Mushrooms are likely to be a significant source of GSH in the diet, especially in areas where mushroom consumption is high (Richie et al. 2015). However, the oral bioavailability of GSH is still a topic of controversy, with research yielding conflicting findings.

LT a cyclic sulfur molecule discovered in *L. edodes* is the source of the mushroom's distinct odor and one of the most important olfactory markers (Hiraide et al. 2004). Scarce studies have been conducted concerning to biological activities of LT,

however, the current chapter is intended to promote more study into this intriguing chemical, with research focusing on the LT's biological activities.

The present chapter aimed to clarify the main emerging techniques of extraction and the analytical methods for EGT, GSH, and LT analysis, as well as the recent findings concerning biosynthesis and biological activities, emphasizing their potential therapeutic action.

20.2 Commonly Known Sulfur-Containing Compounds

Several sulfur compounds have been identified in fungi, however undoubtedly many other compounds are yet to be discovered. Sulfur compounds such as dimethyl sulfide, dimethyl disulfide, dimethyl trisulfide, and S-methyl methanethiosulfonate are found in low content in fungus-like *Fusarium* sp. and *Penicillium* sp. (Dickschat et al. 2011; Larsen 1998). Mercaptoacetone is a rare sulfur compound volatile found solely in fungi, such in the saprophyte *Schizophyllium commune* (Schalchli et al. 2011). Besides this compound, in *S. commune* cultivated vials, other volatile sulfur compounds such as dimethyl disulfide, methyl ethyl disulfide, diethyl disulfide, dimethyl trisulfide, and dimethyl tetrasulfide were identified (Toyotome et al. 2021). *Tuber melanosporum* produces 3-methylsulfanylpropan-1-ol, which is likely produced from methionine and may play a role in truffle flavor (Splivallo et al. 2007; Toyotome et al. 2021; Dickschat 2017). Sulfur-containing chemicals such as 3-thiophenecarboxaldehyde, 2-acetylthiazole, S-methyl methanethiosulfonate, and benzothiazole were also discovered in the edible mushroom *Boletopsis leucomelas* and are identified as the key odor components (Nosaka and Miyazawa 2014). From the sponge-associated fungus strains *Aspergillus violaceus*, five novel methylsuccinimide-based sulfur-bearing compounds, violaceimides A–E, were found with potential anticancer activity (Yin et al. 2018). Other sulfur-containing compounds have been identified as toxic and constitute the most lethal toxins. Among them, alpha-amanitin has been extensively studied due to its chemical nature and it is known as the most lethal of the toxins from fungi (Garcia et al. 2015a, b, 2019). Indeed, several poisonings and most fatalities caused by mushroom consumption are attributed to alpha-amanitin (Garcia et al. 2015a, c). Although other sulfur chemicals may be found in fungi, the present chapter will focus on the most relevant molecules with several therapeutic actions. In this context, EGT, GSH, and LT will be scrutinized and described in detail in the next sections.

EGT (Fig. 20.1a) is a colorless and odorless molecule presented a molecular mass of 229.30 g mol^{-1} and high solubility in water. EGT occurs in solution as a tautomer between thiol and thione forms, thereby conferring greater stability on this biomolecule since it does not suffer auto-oxidation compared to other "acclaimed" antioxidants (Hartman 1990). Another distinguishing feature of EGT is the conventional redox potential of the thiol-disulfide pair, which is 0.06 V, as opposed to 0.2–0.32 V for other naturally occurring thiols (Cheah and Halliwell 2012). Its formal name is (2S)-3-(2-thioxo-2,3-dihydro-1H-imidazol-4-yl)-2-(trimethylammonio)-propanoate,

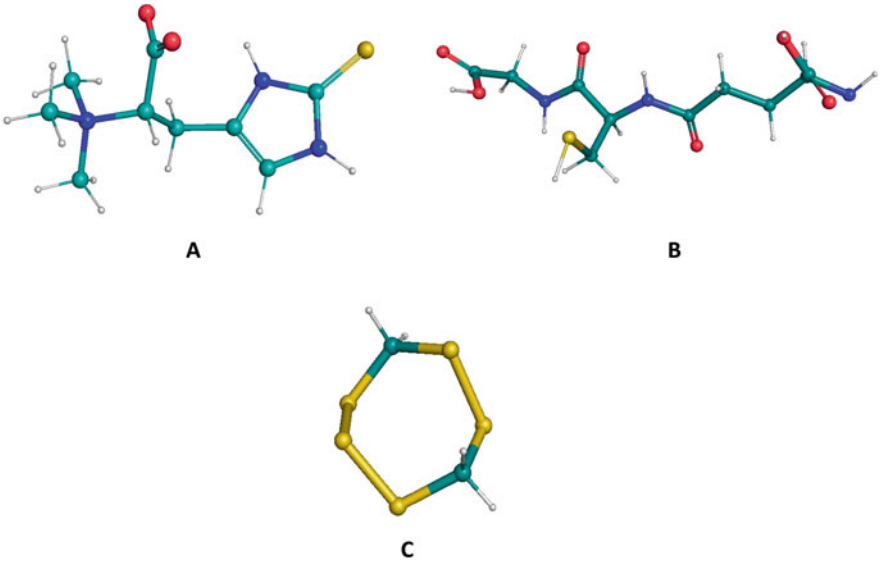

Fig. 20.1 3D Structure of (**a**) L-Ergothioneine, (**b**) Glutathione, and (**c**) Lenthionine

but it is also known as 2-mercaptohistidine trimethylbetaine. It is an N, N, N-trimethyl-l-histidine derivative with a mercapto group in lieu of the hydrogen at position 2 on the imidazole ring (Borodina et al. 2020).

The GSH (Fig. 20.1b) is a low molecular weight compound with a molecular mass of 307.32 g/mol. GSH is composed of three important amino acids, L-cysteine, L-glutamic acid, and glycine (γ-Glu-Cys-Gly) with a gamma peptide linkage between the carboxyl group of the glutamate side-chain and the α-amino-group of cysteine.

LT (Fig. 20.1c), a volatile cyclic sulfur compound with 188.4 g mol^{-1} of molecular mass is found in both fresh and dried *L. edodes* (shiitake) mushrooms. It is the source of the species' distinctive aroma and is one of the most important odor indicators. LT is a polysulfides polymer and is classified as the most significant nonaromatic sulfur derivative (Francioso et al. 2020).

20.3 Edible Mushrooms as Source of Sulfur-Containing Compounds

Data on EGT, GSH, and LT contents in mushrooms are presented in Table 20.1. Kalaras et al. (2017) performed a comparative study on the determination of EGT and GSH levels in several species of mushrooms. EGT levels ranged from 0.2 to 7.3 mg g^{-1} dry weight (dw) with the highest concentrations being observed for *Boletus edulis* (porcini) and *Pleurotus citrinopileatus* (yellow oyster). The GSH

Table 20.1 Content of ergothioneine, glutathione, and lenthionine of mushrooms

Mushroom species	Glutathione (mg/g d.w)	Ergothioneine (mg/g d.w)	Lenthionine (mg/g d.w)	References
Agaricus bisporus	0.63 ± 0.57	0.41 ± 0.18	–	Kalaras et al. (2017)
Agrocybe aegerita	1.92	2.56	–	
Boletus edulis	1.38	7.27	–	
Cantharellus cibarius	0.11	0.20	–	
Ganoderma lucidum	0.41	0.56	–	
Grifola frondosa	2.41 ± 2.00	1.11 ± 0.49	–	
Hericium erinaceus	1.50 ± 0.56	1.12 ± 0.12	–	
Morchella esculenta	0.75	0.47	–	
Pleutotus citrinopileatus	1.39	3.94	–	
Pleutotus ostreatus	1.32 ± 0.45	1.21 ± 0.25	–	
Boletus auripes	–	2.40 ± 0.05	–	Nachimuthu et al. (2019)
Fomitopsis pinicola	–	0.07 ± 0.01	–	
Ganoderma applanatum	–	0.06 ± 0.02	–	
Ganoderma neo-japonicum	–	0.07 ± 0.00	–	
Grifola gargal	–	2.04 ± 0.20	–	
Hydnum repandum	–	0.78 ± 0.02	–	
Hygrophorus russula	–	4.98 ± 0.31	–	
Lactarius torminosus	–	0.82 ± 0.15	–	
Lampteromyces japonicus	–	0.43 ± 0.16	–	
Lepista nuda	–	5.54 ± 0.26	–	
Pleurotus eryngii	–	1.41 ± 0.12	–	
Neolentinus lepideus	–	2.41 ± 0.09	–	
Ramaria botrytis	–	0.29 ± 0.03	–	
Russula virescens	–	0.68 ± 0.04	–	
Sarcodon aspratus	–	1.79 ± 0.02	–	
Suillus bovines	–	1.09 ± 0.07	–	
Suillus granulates	–	0.09 ± 0.03	–	
Suillus luteus	–	2.27 ± 0.24	–	
Sparassis crispa	–	2.37 ± 0.42	–	
Tremella foliacea	–	0.61 ± 0.03	–	

(continued)

Table 20.1 (continued)

Mushroom species	Glutathione (mg/g d.w)	Ergothioneine (mg/g d.w)	Lenthionine (mg/g d.w)	References
Tricholoma matsutake	–	0.74 ± 0.08	–	
Tricholomopsis rutilans	–	2.50 ± 0.30	–	
Lentinula edodes	1.14 ± 0.46	0.92 ± 0.29	0.0039 ± 0.04	Wang et al. (2021a)

content varied from 0.11 mg/g dw and 2.41 mg g^{-1} dw with *G. frondosa* (maitake) and *Agrocybe aegerita* (pioppini) as the most GSH-enriched species. Considering these two important antioxidants, the EGT content was equivalent or higher than GSH, with *P. citrinopileatus* and *B. edulis* presenting fourfold to sevenfold higher levels of EGT than GSH. A correlational analysis was also performed, which revealed that the levels of EGT and GSH seem to be directly proportional (Kalaras et al. 2017).

Chilanti et al. (2022) determined the contents of EGT in broth (submerged culture), mycelium (submerged culture and Petri dish), and basidiome of several *Pleurotus* strains, finding that the 122H.5 strain had the highest concentration (2.93 mg g^{-1}) followed by the 189H.5 (1.99 mg g^{-1}) (Chilanti et al. 2022). Corroborating these results Lin et al. (2015) found in submerged culture of *P. citrinopileatus* EGT value of 10.65 mg g^{-1} dw (Lin et al. 2015). Given these findings and the inherent heterogeneity in fungal metabolism, it is reasonable to conclude that the cultivation procedure has an impact on EGT synthesis. Therefore, to improve the EGT content, the cultivation conditions for edible mushrooms must be optimized. The advantages of cultivating edible mushroom mycelia under submerged circumstances are ease of EGT production, high yield, and unexpensive production. The greatest level of EGT generation under fermentation conditions has been recorded to reach 200 mg L^{-1} (Jiang et al. 2014). The addition of various additives such as aspartic acid, lysine, and methionine resulted in increased EGT biosynthesis. Additionally, it has been demonstrated that EGT production increases with rising pH and alkaline conditions (Martinez-Medina et al. 2021). According to a study published by Dubost et al. (2006), the concentration of EGT increased with the harvest cycle, with 0.6 mg g^{-1}, 1.0 mg g^{-1}, and 1.32 mg g^{-1} dw of EGT observed in the first, second, and third harvest cycles, respectively (Dubost et al. 2006).

Concerning LT mushrooms content, few studies have been conducted and the amount found in mushrooms shiitake can be found in Table 20.1. From the literature, it is known that drought stress enhanced the accumulation of LT, and the essential γ-glutamyl transpeptidase (GTT) genes and cysteine (Cys) sulfoxide lyase of *L. edodes* are crucial for its biosynthesis (Wang et al. 2021b).

20.4 Biosynthesis of Sulfur-Containing Compounds

The biosynthesis of EGT involves the methylation of histidine to hercynine and the addition of sulfur obtained from Cys (Melville et al. 1957). Considering the bacterium *Mycobacterium tuberculosis*, Richard-Greenblatt et al. (2015) defined the later stage in biosynthesis as the introduction of glutamylcysteine as a GSH precursor in hercynine (Richard-Greenblatt et al. 2015). Additionally, in EGT-producing species, GSH and its precursors appear to be essential for EGT production. EGT can promote GSH synthesis by induction of the Nrf2/ARE-mediated signaling pathway (Hseu et al. 2015).

GSH biosynthesis implies a two-step enzymatically catalyzed reaction that is shown in Fig. 20.2. The cysteine sulfhydryl group is involved in the reduction and conjugation reactions, which are the most significant functions of GSH.

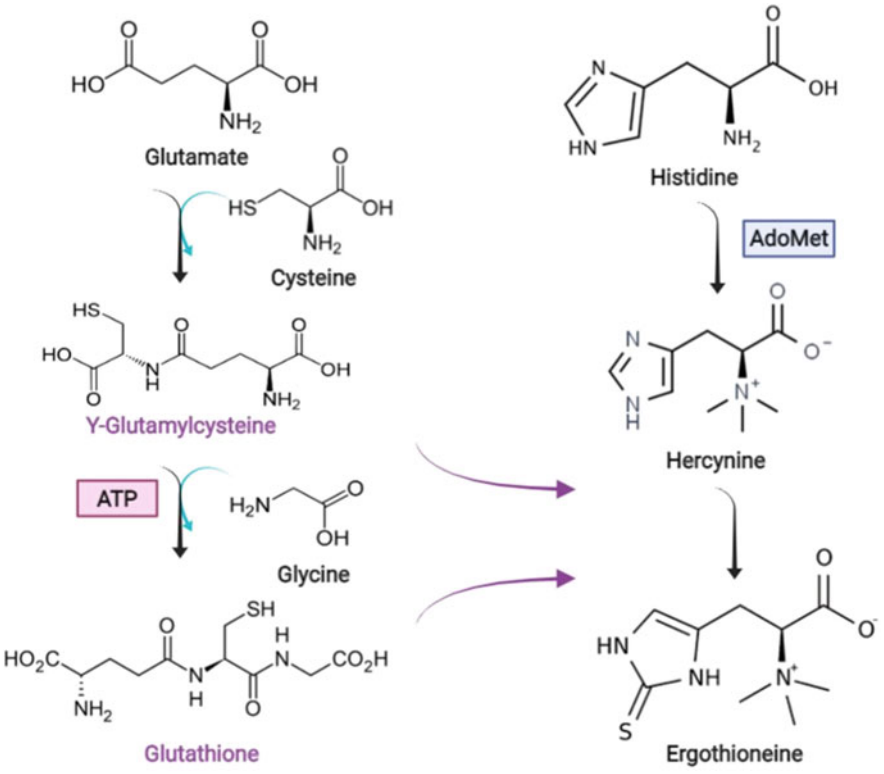

Fig. 20.2 Biosynthesis of glutathione and ergothioneine adapted from Kalaras et al. (2017) (Created with BioRender.com)

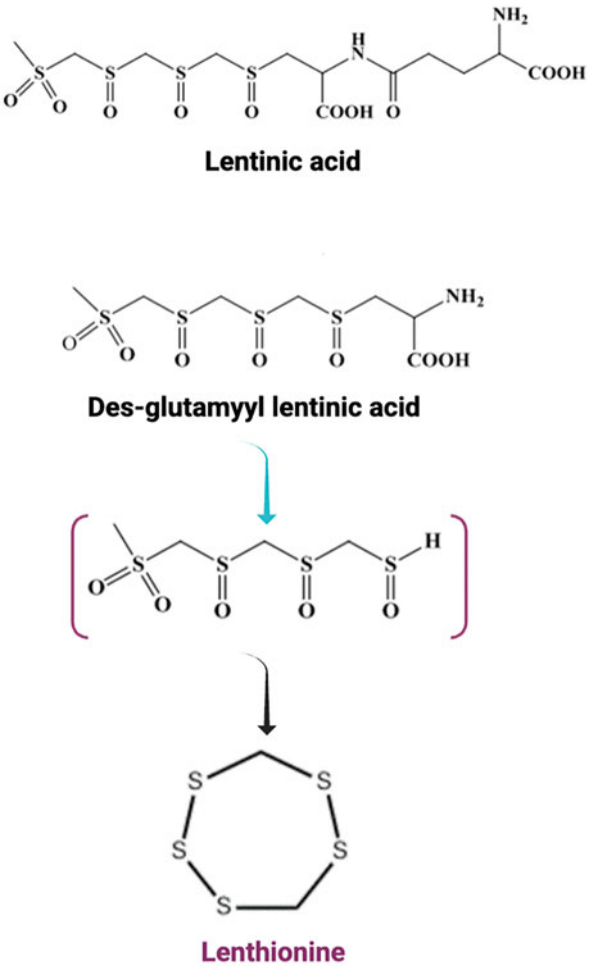

Fig. 20.3 Biosynthesis of lenthionine adapted from Wang et al. (2021b) (Created with BioRender.com)

LT biosynthesis was not completely explained, but it seems that catalytic cleavage of the C-S lyase enzyme is the crucial step for the final product formation (Francioso et al. 2020). LT is produced from lentinic acid by a two-step enzyme-catalyzed reaction and through a complex nonenzymatic reaction (Fig. 20.3). The γ-glutamyl peptide which binds lentinic acid is hydrolyzed by GTT and releases γ-glutamylpeptides. These peptides are hydrolyzed by cysteine sulfoxide lyase and produce thiosulfate and, consequently, LT is produced by a complex nonenzymatic reaction with thiosulfate as a substrate.

20.5 Extraction and Analytical Methods for Sulfur-Containing Compounds Analysis

For the extraction of sulfur-containing compounds from mushrooms, several methods have been improved, including solid-phase microextraction (SPME) and supercritical CO_2 fluid extraction. In 2014, Bhattacharya et al. used a Response Surface Methodology to optimize the extraction of EGT from *P. ostreatus*, namely by the study of three independent variables: extraction pressure, extraction temperature, and amount of co-solvent. After an extraction by supercritical carbon dioxide using a Spe-ed, the optimal conditions to obtain the highest EGT concentration in the mushroom extract with reduced IC50 value were the pressure of 21 MPa, temperature of 48 °C, and co-solvent amount of 133 mL. Furthermore, this research demonstrated that EGT concentration is strongly dependent on the delicate balance between the supercritical fluid's density and vapor pressure, which are controlled by the extraction pressure and temperature, respectively. In reality, the vapor pressure and the amount of co-solvent used influenced EGT extraction (Bhattacharya et al. 2014).

In 2017, Kalaras et al. determined and compared levels of EGT by High-Performance Liquid Chromatography - Photodiode Array Detector (HPLC-PDA), in different species of mushrooms as stated in Sect. 20.6.3. Recently, Horie et al. (2020) developed and validated a novel liquid chromatography/electrospray ionization-tandem mass spectrometry (LC/ESI-MS/MS) method for the determination of EGT in the *Aspergillus oryzae*-fermented rice bran and rice. The derivatization using 2-iodo-N-(8′-quinolinyl)acetamide improved the reversed-phase LC retention and ESI-MS/MS sensitivity of EGT, consequently, enabling its precise and accurate quantification in the fermented product samples (Horie et al. 2020).

Kalaras et al. (2017) performed the first comprehensive analysis of GSH levels in different edible mushrooms and the authors reported that levels varied by more than 20-fold (0.11–2.41 mg g^{-1} dw).

The methodology for quantifying GSH is crucial, not only for achieving the required specificity for distinguishing the various forms of GSH in biological matrices, but also for distinguishing between reduced and oxidized GSH forms.

There are numerous ways for determining GSH in biological materials nowadays, ranging from the classic enzyme-colorimetric method (Tietze 1969) to spectrophotometric (Chen et al. 2012) or spectrofluorimetric (Xu and Hepel 2011) approaches. Due to the GSH family's lack of chromophores and fluorophores, numerous derivatization methods, such as O-phthalaldehyde or N-pyrenemaleimide have been developed (Piccoli et al. 1994), as well as the use of electrochemical methods (Rezaei et al. 2015), chemiluminescence (Han et al. 2006), mass spectrometry (Burford et al. 2003), and nuclear magnetic resonance (Mandal et al. 2012). Chromatographic techniques (Schneider 2013; Vallverdú-Queralt et al. 2015) mass spectrometry (Burford et al. 2003), and nuclear magnetic resonance (Mandal et al. 2012). Chromatographic techniques (Schneider 2013; Vallverdú-Queralt et al. 2015)

and gas chromatography (Neuschwander-Tetri and Roll 1989) or electrophoretic techniques (Serru et al. 2001) were also highly used.

More recently, the use of nanotechnologies appears to be an interesting solution for quantifying glutathiolated species, however, the development of such nanosystems can lead to many problems of interference, particularly when chemical structures close to GSH in large amounts in the body, such as homocysteine or cysteine, are present (Gaucher et al. 2018).

LT may be found in the fruiting body of *L. edodes* as well as in mycelium biomass generated in submerged culture (Enman et al. 2008). This compound may be extracted from the shiitake mushroom with an organic solvent or a mixture of an organic solvent with water or also by buffer solutions, followed by identification through High-Performance Liquid Chromatography (Wang et al. 2021b). Hiraide et al. (2010) investigated the amount of LT produced by rehydrating dry shiitake mushrooms under various pH values using several types of buffers at 25 °C after 3 h of incubation. The ideal pH was 8.0, and the LT concentration was 0.54 mol g^{-1} (Hiraide et al. 2004).

Morales et al. (2019), on the other hand, demonstrated that LT, which was present in the shiitake powder before heating, was not detected in any of the produced extracts (Morales et al. 2019), corroborating Shiga et al. (2004) findings that LT is thermally degraded above 80 °C. Wada et al. (1967) also found that LT was unstable when heated for 1 h at 100 °C in a 10% alcohol solution at a pH greater than 5.0. In reality, heat treatment has a significant impact on LT concentration.

However, Shiga et al. (2004) referred that the heat treatment of the extracted shiitake liquid at 80 °C resulted in a 5.3-fold increase in the concentration of LT in the feed solution (Shiga et al. 2004).

Regarding the volatile LT compound, it is thought to be responsible for shiitake mushrooms' scent (Wu and Wang 2000). This compound could be also effectively extracted in *L. edodes* with the solid-phase microextraction (SPME) (Wu and Wang 2000) method after supercritical CO_2 fluid extraction, compared to the absence of SFE extraction (Li et al. 2020).

20.6 Bioactive Properties and Therapeutic Potential of Sulfur-Containing Compounds

20.6.1 Ergothioneine (EGT)

EGT has a significant impact on human health, since a specific carrier was discovered in numerous tissues namely in lungs, liver, kidneys, heart, red blood cells, and sperm. Notwithstanding the EGT with extremely hydrophilic and membrane-impermeable properties is actively carried into mammalian cells by the organic cation transporter 1 (OCTN1), firstly described by Gründemann et al. (2005).

In vitro studies have shown that EGT has high antioxidant and cytoprotective properties, and that increasing OCTN1 levels can improve the body's concentration at damaged sites. EGT deficit may play a role at the beginning of the course of several diseases since its levels in the blood and/or plasma are low in numerous pathologies (Halliwell et al. 2018). Furthermore, various studies on the efficacy of EGT in the treatment of a wide range of clinical disorders, including neurodegenerative, cardiovascular, and COVID-19 diseases, imply that EGT might be an essential nutraceutical for general health (Cheah and Halliwell 2020; Nakamichi et al. 2016; Smith et al. 2020). As a result of these findings, the European Food Safety Authority has certified EGT as a supplement, and the US Food and Drug Administration has designated it a safe supplement (Turck et al. 2017).

20.6.1.1 Antioxidant Activity

Since its discovery in 1909, the function of EGT has been a source of debate and the functions of EGT are still unknown. The antioxidant effect of EGT is due to its sulfhydryl group, which confers a powerful radical scavenging activity, especially for hydroxyl radicals, hypochlorite, peroxynitrite, and also chelates redox-active cations like Cu^{2+} (Fig. 20.4) (Akanmu et al. 1991). Noteworthy in vitro studies revealed that EGT exhibits high intrinsic antioxidant activity when compared to GSH, trolox, and uric acid, which are some of the most well-known antioxidant compounds (Franzoni et al. 2006). In human cultured fibroblasts, EGT was more effective than coenzyme Q10 or idebenone at preventing lipid peroxidation due to its superior ability to scavenge free radicals (Dong et al. 2007). Because of its capacity

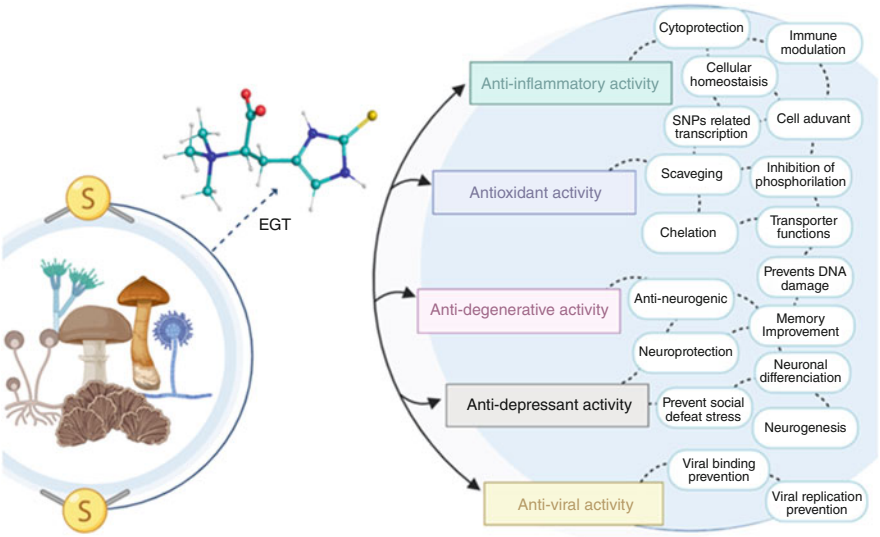

Fig. 20.4 Summary of possible mechanisms of action of EGT (Created with BioRender.com)

to be irreversibly oxidized by relevant oxidants prevalent in cells, EGT is a remarkable cell adjuvant (Servillo et al. 2015). It has also been demonstrated that EGT be an oxidation inhibitor of oxyhemoglobin mediated by copper ions, as well as, prevent the peroxidation of arachidonic acid induced by combinations of myoglobin or hemoglobin and hydrogen peroxide (H_2O_2) (Akanmu et al. 1991). Other antioxidant mechanisms have been proposed by Song et al. (2017) namely blocking the NF-kB transcription pathway. Indeed, EGT appears to protect PC12 cells from hyperglycemia by preventing cytotoxicity, the increase of protein carbonyl and reactive oxygen species (ROS) levels (Song et al. 2017).

Myeloperoxidase is a heme-containing peroxidase expressed mainly in neutrophils, which catalyzes the formation of reactive oxygen intermediates, including hypochlorous (HOCl), hypobromous, and hypothiocyanous acids, as a result of its reaction with H_2O_2 and chloride ions (Aratani 2018). As so, Ito et al. (2011) reported that extract from an edible mushroom *Grifola gargal* containing EGT, effectively inhibited the protein chlorination by HOCl. EGT also inhibited TNF-induced IL-6 release in adipocytes, as a result, it would regulate IL-6, a cytokine that has been linked to insulin resistance in adipocytes (Ito et al. 2011).

EGT has also been shown to chelate divalent metal ions, producing complexes with metal cations that prevent divalent metal ions from participating in redox cycling (Zhu et al. 2011). Cheah et al. (2017) reported that the oral administration of EGT to humans leads to a decrease in biomarkers associated with oxidative damage and inflammation, including allantoin, 8-hydroxy-2′-deoxyguanosine, 8-iso-PGF2a, protein carbonylation, and C-reactive protein, suggesting an important physiological function (Cheah et al. 2017). Animal studies suggest that EGT may act as a significant antioxidant and the decreasing trend of oxidative damage indicators corroborating the previous findings.

20.6.1.2 Anti-inflammatory Activity

Owing to its prominent role in counteracting inflammatory responses, EGT has received special attention in recent years in a variety of inflammation-related disorders (Cao et al. 2020; Cheah and Halliwell 2012). Indeed, the plasmatic EGT concentration in patients with inflammatory bowel disease has been reported to be lower when compared to healthy controls, suggesting that EGT may play a role in the regulation of inflammation (Cao et al. 2020). From a pharmacogenomic point of view, functional polymorphisms on the organic cation transporter genes, SLC22A4 and SLC22A5, have been implicated in chronic inflammatory conditions Crohn's disease. These variations have been shown to affect the transcription of organic cation transporters and interact with Crohn's disease-related genes (Cheah and Halliwell 2012; Leung et al. 2006).

An in vivo analysis performed by Repine and Elkins (2012), showed that EGT could reduce the oxidative stress and the activation of TNFα/NF-κB, as well as the release of important cytokines, IL-8 and IL-6, in alveolar epithelial cells, limiting acute lung injuries and inflammation (Repine and Elkins 2012). Overstimulation and

dysfunction of IL-8 recruited neutrophils within the airways results in the release of several pro-inflammatory molecules and proteases resulting in further damage of lung tissue. Additionally, Asahi et al. (2016) found that EGT-extracted metabolite from *Coprinus comatus* (shaggy ink cap), an edible mushroom described to contain a high amount of EGT, reduced the DNA halogenation and prevented the UV radiation-related inflammation (Asahi et al. 2016). This action may result in neutralizing radiation-induced ROS and possibly also by the activation of Nrf2 and, ultimately, downstream antioxidant genes (Obayashi et al. 2005).

Despite the great promise of EGT, research into its anti-inflammatory effects is still missing, and the specific and detailed mechanisms underlying the EGT in human disease need to be clarified.

20.6.1.3 Antiviral Activity

This fascinating topic was recently reviewed by Cheah and Halliwell (2020), focusing on the potential application of EGT in the treatment of the underlying pathology of COVID-19. The authors suggest that EGT might be employed as therapy to minimize the severity and mortality of COVID-19, particularly in the elderly and those with underlying health problems (Cheah and Halliwell 2020). This review illustrates the capacity of EGT to control inflammation, scavenge free radicals, protect against acute respiratory distress syndrome, and prevent endothelial dysfunction as well as other COVID-19 complications (Cheah and Halliwell 2020).

Additionally, the antioxidant and anti-inflammatory purposes of EGT administration could be a step ahead in chronic illnesses, such as immunodeficiency virus-1 (HIV-1) related disease. Xiao et al. (2006) demonstrated that EGT reduced both TNF- and protease frameshift-mediated increases in -galactosidase activity in a dose-dependent manner (IC50 of roughly 6 mM), suggesting that EGT directly inhibits HIV transcription (Xiao et al. 2006). A study performed by Gallego et al. (2019) using aqueous extracts obtained from *Agaricus bisporus* was found to inhibit NS3/4A protease (involved in viral replication) activity in 85–90%, and hepatitis C viral genome replication (Fig. 20.4), in 93% (Gallego et al. 2019).

20.6.1.4 Anti-neurodegenerative Activity

The pathogenesis of neurodegenerative diseases, including Alzheimer's, Parkinson's, and Huntington's, has been linked to an increase in free radical production. Thiols, as EGT, are hydrophilic molecules capable of radical-scavenging antioxidants in neural cells (Nachimuthu et al. 2019; Phan et al. 2017). Among other functions, EGT protects neurons from cytotoxicity induced by a variety of neurotoxins (N-methyl-D-aspartate, β-amyloid, and cisplatin) (Jang et al. 2004; Moncaster et al. 2002; Song et al. 2010). In addition, EGT is involved in the cellular homeostasis of neuronal cells, restoring the cellular equilibrium (Fig. 20.4) (Phan et al. 2017). As a result, edible mushroom extracts have been shown to exhibit

anti-neurogenic action, indicating that bioactive chemicals are responsible for neuroprotection. According to Ishimoto et al. (2014), EGT suppressed the proliferation of mice-cultured cortical neural progenitor cells and enhanced the differentiation of neural progenitor cells into neurons at a concentration of 500 M. (Ishimoto et al. 2014). EGT (0.5 mg/kg/body weight of mice) substantially reduced -amyloid protein deposition in the hippocampus of the D-galactose-treated animal model, according to Song et al. (2014). In addition, it dramatically reduced lipid peroxidation and preserved the GST/GST disulfide ratio and superoxide dismutase activity in brain tissues, resulting in reduced oxidative damage and improved learning and memory (Fig. 20.4) (Song et al. 2014). A study performed by Nakamichi et al. (2016) the daily oral ingestion of *Pleurotus cornucopiae*-derived EGT (1.2%, w/w, for 2 weeks) is transported across the blood-brain barrier, where it may promote neuronal differentiation (Nakamichi et al. 2016).

20.6.1.5 Antidepressant Activity

Oxidative stress is a hallmark of several diseases and disorders, including depression, and it tends to increase when antioxidant function decreases (Ng et al. 2008). A pre-clinical study performed by Nakamichi et al. (2016) using EGT extracted from *P. cornucopiae* mushroom indicated that this compound alleviates the symptoms of depression in mice (Nakamichi et al. 2016). In mice, oral administration of EGT (1.2% w/w, for 2 weeks) reduced immobility time, a measure of depression-like behavior. Increased EGT concentrations in plasma and brain were linked to antidepression effects evaluated by forced swimming and tail suspension tests (Nakamichi et al. 2016). The intake of EGT may promote neuronal differentiation and exert antidepressant-like activity, potentially through neurogenesis enhancement (Nakamichi et al. 2016). Interestingly, Matsuda et al. (2020) also reported that oral administration of EGT prevented depressive behavior and depression-like sleep abnormalities in mice (Matsuda et al. 2020). The prophylactic administration of EGT (0.25 mg mL^{-1}) corresponded to approximately 30 mg kg^{-1} *per* day based on water intake and body weight, which avoided social and depression-like sleep abnormalities, such as rapid eye movement sleep (Fig. 20.4).

20.6.2 Glutathione (GSH)

In nearly all organisms, this ubiquitous tripeptide (γ-L-glutamyl-L-cysteinyl-glycine) is considered the major intracellular antioxidant. GSH also has other activities including detoxification of a variety of toxins and carcinogens, post-translational modulation of protein activity, and immune function maintenance. (Giustarini et al. 2004). Depletion of GSH can impair immunological function, increase sensitivity to a wide range of xenobiotics and oxidative damage. Therefore maintaining appropriate tissue levels of this compound is crucial for preserving health (Kalaras et al.

2017). The usefulness of oral GSH in boosting body GSH storage has been suggested by the results of a randomized controlled trial of oral GSH supplementation (Richie et al. 2015). As a result, it is necessary to identify the key sources of GSH and its inducers in the diet. Previous research on the GSH content of popular foods has identified mushrooms as especially high on this compound (Kalaras et al. 2017). Even partial GSH depletion increases vulnerability to xenobiotics and oxidative stress, low GSH levels have been linked to an increased risk of oxidative-related diseases such as cancer, neurodegenerative diseases, liver dysfunctions, and diabetes (Teskey et al. 2018).

20.6.2.1 Antioxidant and Anti-neurodegenerative Activities

GSH reduces electrophilic and oxidant species either directly or through enzymatic catalysis: i) it directly reduces reactive hydroxyl free radicals, other oxygen-centered free radicals, and radical centers on DNA as well as other biomolecules such as methylglyoxal and 4-hydroxynonenal; and ii) it is the co-substrate of GSH peroxidase, allowing peroxide reduction. NADPH reducing equivalents and GSH disulfide reductase catalysis are then used to convert glutathione disulfide (GSSG) to 2 GSH. Through the activation of glutathione-S-transferases, electrophilic endogenous molecules and xenobiotics (drugs, pollutants, and their phase I metabolites) are conjugated with GSH (Gaucher et al. 2018). The overall objective of cellular redox homeostasis is to reduce harmful ROS and nitrogen species, as well as GSSG, to a minimum while maintaining high levels of GSH.

The pathogeneses that mediate neurodegenerative disorders are yet unknown; however, there is strong evidence that ROS may be a critical event since an enhanced level of oxidative stress has been identified in the brains of neurodegenerative diseases patients. Herein, GSH's involvement in fighting the development of neurodegenerative disorders is crucial and relevant (Fig. 20.5). Indeed, GSH depletion is a typical hallmark of neurodegenerative disorders, and it can be caused by a variety of factors, including disruptions in GSH homeostasis and changes in GSH-related enzymes. Multiple cellular issues linked to GSH and GSH-dependent enzyme dysregulation result in mitochondrial dysfunction, increased oxidative damage, alteration of intracellular signal transduction pathways, protein aggregation, and ultimately cell death (Bagatini et al. 2020).

20.6.2.2 Liver Diseases

Numerous studies have found a relationship between GSH levels and the pathogenesis of liver diseases such as non-alcoholic fatty liver disease (NAFLD), alcoholic liver disease (ALD) and hepatitis C virus (HCV), hepatitis B virus (HBV) (Fig. 20.5) (Vairetti et al. 2021).

NAFLD is a multifactorial illness, with insulin resistance, lipotoxicity, gut/nutrient-derived signals, adipocytokines, oxidative stress, and genetic factors, all

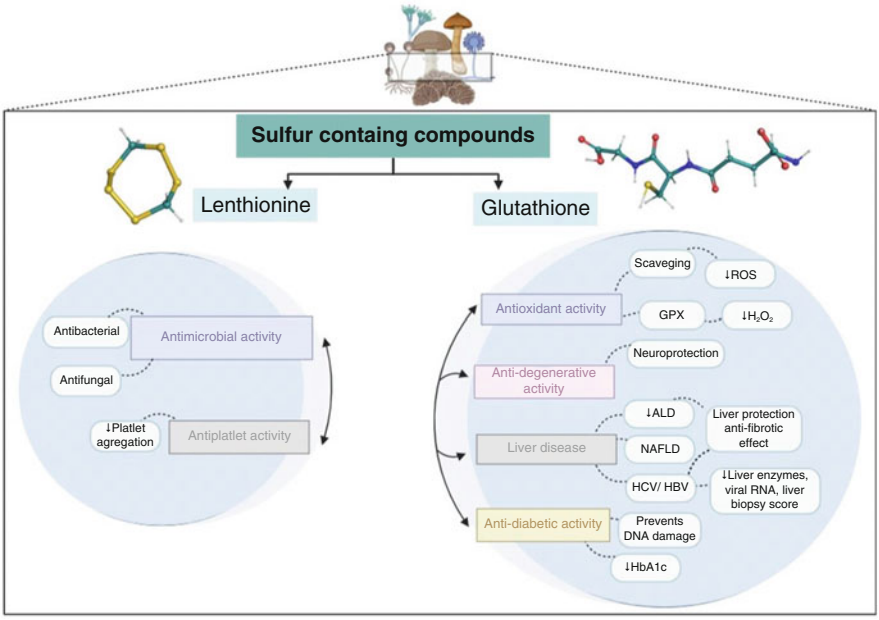

Fig. 20.5 Summary of possible mechanisms of action of LT and GSH (Created with BioRender.com)

considered to play a role in its etiology (Bovolini et al. 2020). In an open-label, multicenter pilot research, the therapeutic potential of oral GSH administration in NAFLD illness was revealed. In fact, after 4 months of therapy with GSH, serum alanine aminotransferase levels, a marker of NAFLD development, reduced considerably (Honda et al. 2017).

A deficient host antiviral immune response and oxidative stress are linked to the pathophysiology of chronic HCV infection (Millman et al. 2017). The increased liver steatosis, inflammation, and hepatic cells necrosis are influenced by oxidative stress and lipid peroxidation. Therefore, patients with persistent HCV infection may benefit from antioxidative treatment, which includes GSH. In a phase I clinical trial, a combination of antioxidants that contained GSH resulted in a favorable response in one of the examined criteria in 48% of patients (e.g., liver enzymes, HCV RNA levels, or liver biopsy score) (Melhem et al. 2005). Similar findings were achieved for HBV therapy. Compared to a control group, an intravenous 1200 mg of GSH was effective in lowering serum levels of transaminases, and inflammation parameters suggesting that GSH treatment can improve liver function, suppress inflammation and hepatic fibrosis in chronic hepatitis B patients (Qian et al. 2017).

20.6.2.3 Anti-diabetic Activity

Diabetes mellitus (DM) has become one of the most common chronic diseases among many people throughout the world. Type 1 is a hereditary condition, whereas type 2 is mostly a lifestyle disease. Glycation, a process that increases the formation of free radicals, is linked to diabetes and hyperglycemia (Olokoba et al. 2012). In this context, diabetes has been associated with low levels of GSH. Indeed, Hakki Kalkan and Suher (2013) showed that new-onset DM patients presented lower GSH levels than impaired glucose tolerance (IGT) and healthy patients. Furthermore, the discovery of reduced GSH levels in IGT patients compared to controls has revealed that the antioxidative defense system is compromised at each stage of glucose metabolism impairment (Hakki Kalkan and Suher 2013). Case-control research found that individuals with T2DM, especially those with microvascular issues, exhibit GSH deficiency when compared to non-diabetic controls. This is most likely due to increased irreversible consumption and reduced synthesis in non-glycemic environments (Lutchmansingh et al. 2018). Moreover, there is evidence that abnormal GSH status is implicated in beta-cell dysfunction and in the etiology of long-term diabetic complications (Livingstone and Davis 2007). Corroborating these findings, a randomized clinical trial demonstrated that oral GSH supplementation restores the body's GSH reserves and dramatically lowers oxidative DNA damage (Fig. 20.5) in diabetes patients (Kalamkar et al. 2021). Additionally, it decreases HbA1c within 3 months and then stabilizes it (Fig. 20.5). Over 6 months, the patients above 55 years appear to benefit the most, as demonstrated by a considerable drop in HbA1c and an increase in insulin release by beta-cells (Kalamkar et al. 2021). These findings suggest that oral GSH can be employed as an adjunct therapy to anti-diabetic drugs, to help patients achieve better glycemic control, especially in elderly people.

20.6.3 Lenthionine (LT)

Surprisingly, little research has been undertaken to assess LT's diverse bioactivities. However, it is anticipated that this chapter will expand new perspectives and inspire more investigation into this fascinating molecule. It is expected that the information available in the literature on the few explored biological activities would be gathered in the following sections.

20.6.3.1 Antiplatelet Activity

Blood platelet activation has a role in the pathophysiology of a variety of disorders such as cardiovascular diseases. Atherosclerosis and other cardiovascular dysfunctions, such as blood clots and blockages, are connected to dysregulation of blood

platelet activity (Olas 2018). The inhibition of platelet aggregation can prevent serious clinical complications such as thrombosis, myocardial infarction, and stroke, thus the development of novel platelet inhibition treatment options is critical for the prevention of cardiovascular complications. Here, sulfuric chemicals, such as LT, appear to be a promising antiplatelet approach (Fig. 20.5). Indeed, the essential oil from shiitake mushrooms, which contains LT as a primary sulfuric component, reduced platelet aggregation generated by arachidonic acid and U-46619, a thromboxane A(2) analog (Shimada et al. 2004). Additionally, the antiplatelet aggregation activity of shiitake mushroom extract was investigated in an in vitro study by assessing collagen-induced platelet aggregation and DPPH radical scavenging (Kim et al. 2013). The findings of this study reveal that shiitake mushroom can reduce platelet aggregation (which may be linked to LT) and can be utilized to treat clinical conditions such as cardiovascular diseases (Kim et al. 2013).

20.6.3.2 Antibacterial and Antifungal Activities

The antibacterial activity of *L. edodes* mycelium culture fluid was investigated against various common bacterial species as well as the fungus *Candida albicans*. The species *Streptococcus pyogenes*, *Staphylococcus aureus*, and *Bacillus megaterium* were all inhibited by the mycelium-free culture fluid. The activity-causing compound was heat-stable, chloroform extractable, and had a molecular weight of less than 10,000 which suggests that the antimicrobial compound was LT (Hatvani 2001).

More recently the potential LT antifungal activity was proved against several microorganisms such as *Glomerella cingulate*, Pyricularia *oryzae*, *C. albicans*, *Trichophyton mentagrophytes*, *Saccharomyces cerevisiae*, *Cryptococcus neoformans,* and *Trichophyton rubrum* with minimum inhibitory concentration ranging from 3.12 μg/mL to 12.50 μg/mL (Raimundo et al. 2018). Concerning LT antimicrobial activities (Fig. 20.5), scarce studies are available in the literature, as a result, there is still a lot of work to be done in this area.

20.7 Conclusion

Sulfur has distinct properties which contribute greatly to the chemical richness of nature, and its unique activities enable essential biological interactions. Indeed, in this chapter, the biochemistry of sulfur-containing compounds found in fungi was reviewed, as well as the enormous pool of naturally occurring sulfur-containing compounds in fungi. We focused primarily on the chemistry and biochemistry of sulfur-containing compounds EGT, GSH, and LT, emphasizing their importance as sulfur natural products, whose biological role is still unknown and requires further investigation. Additionally, sulfur-containing compounds have an important role in human health and well-being, and their therapeutic potential has been documented.

In recent years, significant progress in the understanding of EGT has been performed, including the identification of a highly specialized transporter in higher species and humans, numerous antioxidants and cytoprotective effects in vitro. Few in vivo studies including free radical scavenging activity, anti-inflammatory actions, and protection against neuronal injury have also been recognized. The molecular mechanisms that underpin these cytoprotective effects are still largely unknown.

Disturbances in GSH content contribute to the etiology of many diseases, making it one of the most studied redox-active molecules. GSH is more than just a ROS and RNS scavenger. GSH plays several roles in cell physiology, including chemical metabolization, cell signaling via protein S-glutathionylation, and cell survival. It is implicated in fibrotic development in NAFLD and ALD and it is also a hallmark of viral hepatitis. As a result, supplementing with GSH or GSH precursors has been shown to be an effective in addition to the treatment of several hepatic diseases. However, large-scale clinical trials are needed to clarify the oral efficacy of GSH.

Shiitake mushrooms have a unique flavor that comes from LT, a unique sulfur chemical found in nature. Surprisingly, few studies on LT bioactivities have been focused on antiplatelet activity and antimicrobial activities. Considering the encouraging results, further work is needed to better clarify these biological activities. Despite the therapeutic properties, the LT antibacterial and antifungal action as well as aromas, and flavors may play a significant role in the production innovative nutritionally enriched functional food. Moreover, this kind of compound might be employed instead of chemical agents used to clean surfaces or as preservation agents in food packaging technologies.

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Chapter 21
Mycosterols

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Abstract Sterols are amphipathic lipids that play essential roles in the physiology of eukaryotic organisms in general. The fungal sterols are collectively known as mycosterols and they exert numerous physiological functions. For humans, the interest on this class of compounds relies heavily on the fact that they can promote health benefits. For this reason, fungal extracts rich in sterols of various forms are valuable and promising ingredients. One of the best-known benefits of mycosterols is their inhibitory actions on cholesterol absorption and biosynthesis, but there are several interesting regulatory and modulatory phenomena that mycosterols can affect and that might eventually be of therapeutic interest. Within this domain, the practical application of mycosterols or mycosterol-enriched fungal extracts presents several challenges. The latter include isolation of novel bioactive mycosterols from still underexploited fungi species, the optimization of existing methodologies for production and recovery, extensive study of their applications and, finally, substantial clinical trials for attesting their health benefits and safety.

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21.1 Introduction

Sterols are amphipathic lipids that play essential roles in the physiology of eukaryotic organisms in general. In animals, for example, the most common form cholesterol is an integral part of the cellular membrane, where it exerts a key role in determining its fluidity and plasticity. Sterols are also important in plants and fungi. In the latter they are collectively designated mycosterols and are also involved in many physiological functions. The by far most abundant form of this class of compounds in fungi is ergosterol, but there are many other derivates exerting a great number of physiological functions. Besides the importance for the fungi themselves, on the other hand, many mycosterols have been described to possess pharmacological and therapeutic effects in mammals, a feature that makes investigations on their respect a highly interesting field. These properties will be analyzed in the present chapter after a short overview on their biosynthetic routes.

21.2 Sterol Biosynthesis

A key intermediate of the biosynthetic pathway of sterols in general is squalene as this compound already contains the carbon backbone from which all other compounds of the class are derived. The carbon backbone of squalene comes ultimately from acetyl-CoA. In a long series of reactions 3 acetyl-CoA molecules are firstly condensed into β-hydroxy-β-methylglutaryl CoA (HMGCoA). In the sequence, the six carbons of the β-hydroxy-β-methylglutaryl moiety are deprived of one carbon unit giving origin to the more proximal precursor of squalene synthesis, which is the 5-carbon isoprenoid isopentenyl pyrophosphate. The latter, through condensation reactions, gives origin to the 15-carbon molecule farnesyl-pyrophosphate. Condensation of two farnesyl-pyrophosphate molecules, finally, forms the 30-carbon molecule squalene. To arrive at this point, thus, 12 acetyl-CoA molecules are required. Figure 21.1 provides a summary of these transformations. The condensation reaction that forms squalene is catalyzed by squalene synthase and the subsequent epoxidation that produces 2,3-oxidosqualene is catalyzed by an epoxidase. Lanosterol is the first cyclic intermediate in the production of mycosterols and contains 30 carbon atoms. Singularly, this is also the precursor in the cholesterol biosynthesis in animals (Weete et al. 2010). The fungal sterol pathway, that involves at least 20 enzymes, is analogous to that one operating in animal cholesterol biosynthesis, usually known as the acetate mevalonate pathway (Dhingra and Cramer 2017). The formation of mycosterols initiates by the methylation of lanosterol at C-24, which is followed by a series of demethylations at C-4 and C-14 and double bond rearrangements that (in most cases) produce the C28 sterols that are frequently found in most fungi (Fig. 21.2; Weete et al. 2010). The diverse pathways culminating with the formation of ergosterol vary in accordance with the sequence by which the double bonds are transformed (Song and Nes 2007). In some taxa, a second methylation that originates

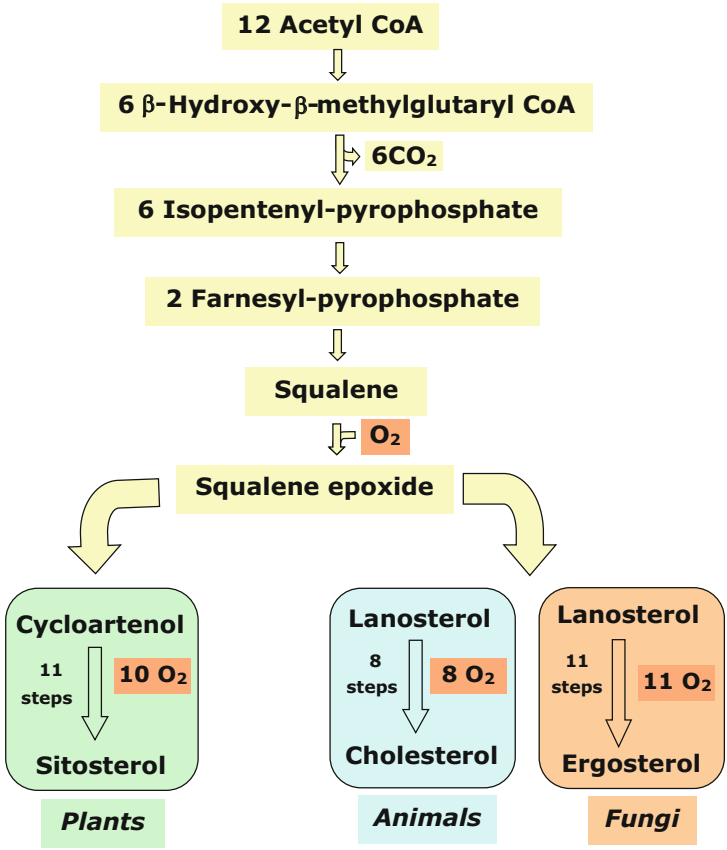

Fig. 21.1 Simplified biosynthetic pathways of sterols

a 24-ethylidene, which is later reduced to 24-ethyl, produces C29 sterols (Fig. 21.2; Weete et al. 2010). For additional information on ergosterol biosynthesis see these references (Alcazar-Fuoli et al. 2008; Abe and Hiraki 2009; Weete et al. 2010; Dupont et al. 2012).

21.3 Main Mycosterols

Ergosterol (ergosta-5,7,22-trien-3β-ol), a type of natural steroid alcohol, has been considered the main sterol of hyphal membranes, followed by derivatives such as ergosta-5,8,22-trien-3-ol, ergosta-7,22-dien-3-ol, ergosta-5,7-dien-3-ol, and ergosta-7-en-3-ol (fungisterol) (Gil-Ramirez et al. 2013). Other products of sterol biosynthesis, such as cholesterol, 24-methyl cholesterol, 24-ethyl cholesterol and brassicasterol, are some examples of the main sterols present in fungi (Weete et al.

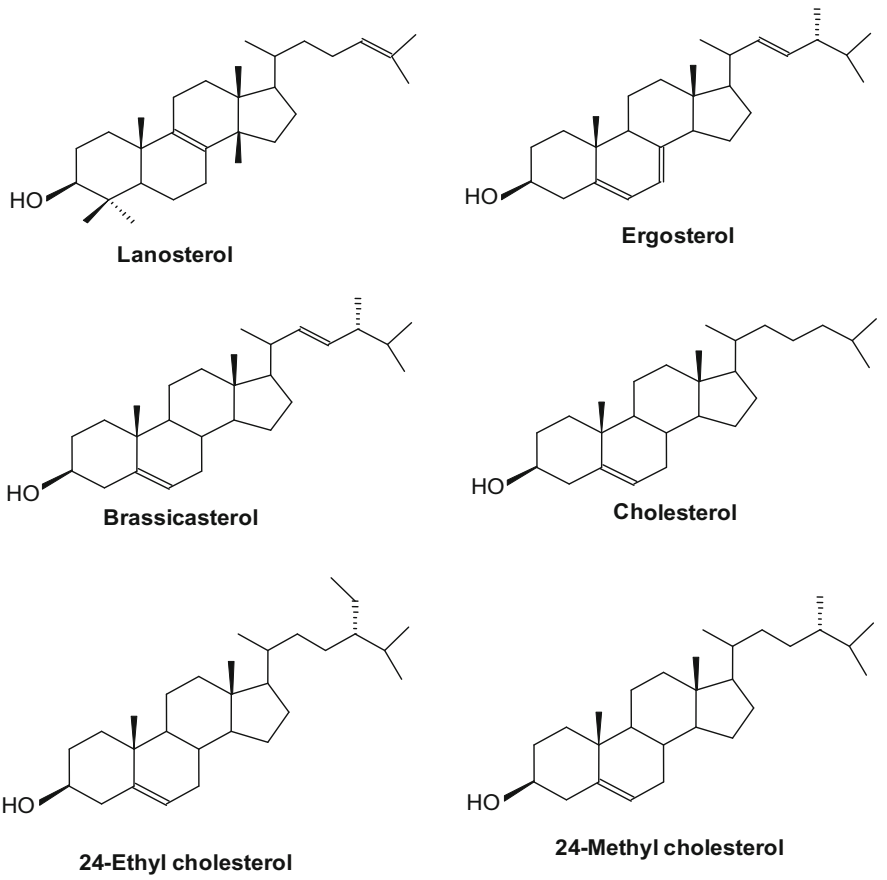

Fig. 21.2 Structure of sterols found in fungi

2010; Fig. 21.2). For most fungi, ergosterol may represent up to 80% of their sterol content (w/w), and it plays an essential role in membrane function, regulating its fluidity, plasma membrane biogenesis and permeability (Abe and Hiraki 2009). Proper cellular ergosterol levels are important for maintaining normal cellular functions that include environmental stress response, cellular detoxification, nutrient transport, and host-pathogen interactions (Bhattacharya 2021). It is completely or almost absent in animal, plant, and bacterial cells (Gomez-Lopez et al. 2011). The ergosterol content is amply related to structural and growing fungal features (Barreira et al. 2014), for example, maturation, hyphal formation, and sporulation (Villares et al. 2014), and can vary according to the fungal species (Phillips et al. 2011). For all the above-cited reasons, ergosterol and its biosynthetic pathways are crucial for fungal growth, so much that both are under consideration for the development of azole antifungals (Alcazar-Fuoli et al. 2008).

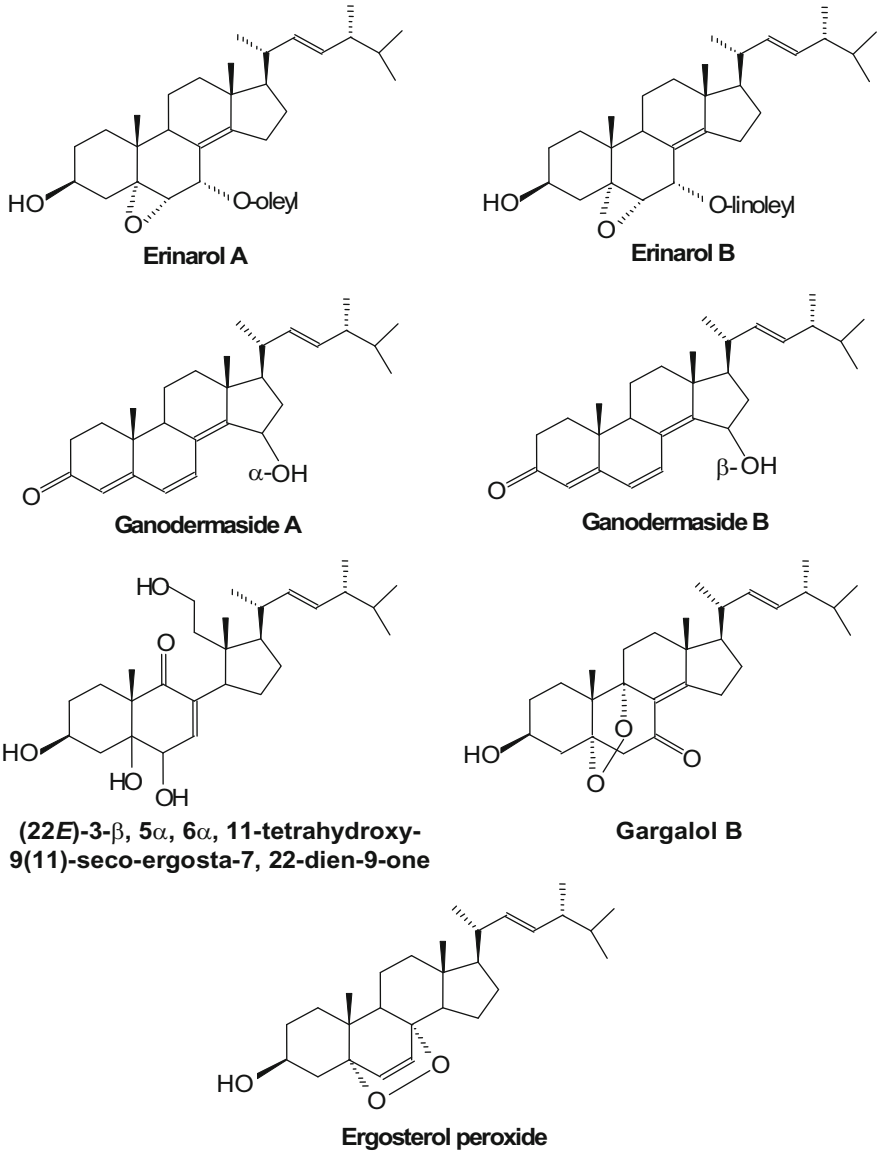

Fig. 21.3 Structure of some ergosterol derivatives from common edible/medicinal mushrooms (Chen et al. 2017, modified)

In the last few years, various new mycosterols have been isolated in both basidiomata (fruiting bodies) and mycelial masses of different fungi and their structures elucidated (Fig. 21.3). Several of them are ergosterol derivatives and present important biological properties (Chen et al. 2012; Shimizu et al. 2016). One of the most studied is ergosterol peroxide, which is an ergostanoid, namely

ergosta-6,22-dien-3-ol, with a peroxyl group between positions 5 and 8 (the 3beta, 5alpha, 8alpha, 22E stereoisomer; see Fig. 21.3). Isolated from *Ganoderma lucidum* and *Cordyceps* sp. (Chen et al. 2017) it exhibits antimycobacterial, trypanocidal, and antineoplastic activities. It has also a role as metabolite, antineoplastic agent, antimycobacterial drug, and trypanocidal drug.

Depending on the mushroom species, the ergosterol concentration may vary from 0.2 to 10 mg/g. In general, the most cultured and consumed species worldwide are precisely those ones containing the highest amounts. Probably the main reason for this phenomenon is that mushroom farmers cultivate them under strict observation of the ideal nutritive requirements (using specific and enriched substrates), and harvest the basidiomata at their optimal developmental stage, while the mushrooms that grow in nature rarely benefit from optimal environmental conditions (Gil-Ramírez et al. 2014; Gil-Ramirez and Soler-Rivas 2014). Hence, the genera *Agaricus* and *Pleurotus* not only concentrate the largest number of studies on bioactive mycoesterols, but also on sterol composition and recovery methodologies (Barreira et al. 2014; Gil-Ramirez et al. 2013; Phillips et al. 2011; Villares et al. 2014).

21.4 Bioactive Properties of Mycosterols

Numerous studies have indicated that fungal extracts rich in ergosterol and ergosterol derivatives can reduce cholesterol absorption (Corrêa et al. 2017; Gil-Ramírez et al. 2014; Yeh et al. 2014) as well as inhibit its biosynthesis in the human body (Chen et al. 2012; Caz et al. 2016). Other health benefits described for this type of extracts are antitumoral and antiproliferative (Kang et al. 2015; Nowak et al. 2016; Torres et al. 2017), anti-inflammatory (Li et al. 2015a, b) and anti-microbial (Sinanoglou et al. 2015) effects. Moreover, being a precursor of vitamin D2, ergosterol (and its derivatives) might enhance bone metabolism, immunity, and mood (Feeney et al. 2014; Xu et al. 2020).

21.4.1 Agaricus bisporus Mycosterols

Agaricus bisporus L., the most consumed mushroom in the world, contains a fraction of mycosterols composed mainly by ergosterol (∼90%) (Barreira et al. 2014). Ergosterol can be easily converted into vitamin D via irradiation, a useful property in industrial applications such as dietary supplements and food fortifiers (Heleno et al. 2016a). Gil-Ramirez et al. (2013) efficiently recovered sterol-enriched fractions (ergosterol and other minor sterols) from *A. bisporus* basidiomata and corresponding by-products via pressurized liquid extraction and supercritical fluid extraction techniques.

Heleno et al. (2016a) studied the use of ultrasound-assisted extraction to recover mycosterols from *A. bisporus* basidiomata applying response surface methodology,

and found that this methodology is powerfully efficient in terms of ergosterol extraction yield and extract purity, also allowing a dramatic reduction of extraction time in comparison with the traditional Soxhlet method. In a later work, Heleno et al. (2016b) proved the feasibility of using *A. bisporus* by-products as a valuable source of ergosterol and microwave-assisted extraction as a suitable technique for its extraction. The authors successfully optimized the extraction process by applying response surface methodology and reported a recovery yield of more than 550 mg of ergosterol/100 g of mushroom (on dry basis) at the best-optimized conditions, using ethanol as extractor solvent. However, other species of the genus *Agaricus* still remain as underutilized sources of ergosterol and could be exploited to obtain this molecule of interest. Mokochinski et al. (2015) studied the production of ergosterol by *A. brasiliensis* in mycelium phase cultures using different agro-industrial by-products as substrates. According to the authors both solid-state fermentation and submerged fermentation were efficient in generating mycelia biomass. The combination of malt substrate and submerged fermentation was the one that generated the highest yields in terms of biomass and ergosterol. Ergosterol and β-sitosterol were the major sterol compounds identified in their samples.

21.4.2 *Lentinula edodes* Mycosterols

Extracts of the second most extensively cultivated mushroom worldwide, *Letinula edodes* (Berk.) Pegler, displayed plasma cholesterol-lowering activity in hypercholesterolemic mice fed with lard (Caz et al. 2016). The ongoing expansion of mushroom industries has generated enormous spent mushroom substrates with the potential for the production of valuable chemicals. To valorize this waste, spent shiitake substrate was submitted to ultrasound-assisted extraction (Wang et al. 2018). Under optimized conditions, the extract contained ergosterol, ergosta-7,22-dienol, and β-sitosterol as the main sterols. The extract showed a comparable antitumor effect against three cancer cell lines.

21.4.3 *Pleurotus spp.* Mycosterols

Schneider et al. (2011) investigated the cholesterol-lowering properties of *Pleurotus ostreatus* in a randomized placebo-controlled intervention. The authors suggested that the significant improvements in human blood parameters, including triglyceride levels, total cholesterol concentration, and oxidized low-density lipoprotein levels, are related to the presence of linoleic acid, ergosterol, and ergosterol derivatives of *P. ostreatus* fruiting bodies.

Numerous studies have attributed functional properties to both basidiomata and mycelia of *Pleurotus* spp., some of them precisely defining the compounds, among them sterols, involved in these bioactivities (Corrêa et al. 2016). Some examples are:

(1) an ergosterol peroxide isolated from the dried fruiting bodies of *Pleurotus ostreatus* (Jacq.) P. Kumm showed potent amoebicidal activity against the intestinal parasite *Entamoeba histolytica*, but no toxicity against human colon cells (Meza-Menchaca et al. 2015); (2) a novel 5,6-seco-ergostane-type steroid from the basidioma of *Pleurotus eryngii* (DC.) Quél., showed anti-inflammatory potential in lipopoly-saccharide-induced mouse macrophages along with slight cytotoxicity (Kikuchi et al. 2015); (3) six ergostane-type steroids were isolated from *P. eryngii* basidioma, and presented inhibitory effects on nitric oxide production (Kikuchi et al. 2016).

21.4.4 Ganoderma lucidum Mycosterols

Polysaccharides and triterpenoids are considered the most important bioactives of the medicinal mushroom *Ganoderma lucidum* (Lu et al. 2020; Liang et al. 2019. However, the biological properties of their mycosterols have received more attention in the last years (Chen et al. 2017; Xu et al. 2021; Weng et al. 2010; see some chemical structures of *G. lucidum* mycosterols in Fig. 21.3). Recently, ergosterol and ergosterol peroxide from *G. lucidum* were innovatively isolated by means of a single-step procedure in an aqueous two-phase system. Interestingly, ergosterol and ergosterol peroxide were obtained with much higher yields compared to the traditional saponification method, and exhibited promising anti-inflammatory properties (Xu et al. 2021).

21.4.5 Hericium erinaceum Mycosterols

Fruiting bodies of *Hericium erinaceum* (Hericiaceae) are a traditional herbal medicine widely used in China, Korea, and Japan. *H. erinaceum* is also a well-known edible mushroom, known as the Lion's Mane Mushroom (Li et al. 2015a, b). Several ergostane-type sterol fatty acid esters, including erinarol A and erinarol B (see Fig. 21.3), were isolated from the dried fruiting bodies of *H. erinaceum* and presented anti-inflammatory and peroxisome proliferator-activated receptors (PPARs) transactivational effects (Li et al. 2014).

21.5 Potential of Mycosterols in Foods

To the best of our knowledge, until the present moment, no functional food capable of inhibiting cholesterol synthesis has been introduced into the market. In this sense, edible mushroom extracts might be explored as sources of biomolecules that could not only impair cholesterol absorption (such as ergosterol and/or derivatives, soluble

polysaccharides, β-glucans and chitins), but also inhibit its biosynthesis (e.g., natural statins).

In a pioneer study on the cholesterol-lowering activity of mycochemicals added to food matrices, Caz et al. (2016) investigated the hypocholesterolemic effects of lard, functionalized by the addition of a *L. edodes* ergosterol-enriched extract (0.44%) at the proportion of 12%, used as animal feed. The ergosterol-added lard intake significantly reduced plasma cholesterol, LDL-cholesterol, and HDL-cholesterol levels of hypercholesterolemic mice, being the hypocholesterolemic response not related to transcriptional changes (post-transcriptional mechanisms might be involved).

Heleno et al. (2017) studied, for the first time, the incorporation of ergosterol obtained from *A. bisporus* into dairy beverages at concentrations mimicking commercial phytosterol-added yogurts. The ergosterol-enriched yogurt was assessed for nutritional and bioactive properties and compared with controls (no additives or phytosterol-added yogurts), at two storage times (right after product manufacture and after seven days at 4 °C). The ergosterol-enriched yogurt showed similar antioxidant properties as the PS-added yogurt. It had, however, superior cytotoxicity against tumor cells, being that the ergosterol-enriched yogurt sample was the strongest in both bioactivities. Although nutritional parameters were identical for all samples, ergosterol protected ergosterol-enriched yogurt from oxidation throughout the 7-day storage period.

More recently, Corrêa et al. (2018) used commercially discarded *Agaricus blazei* fruiting bodies for obtaining an extract rich in ergosterol as a fortifier ingredient for yogurts. When added to the latter it significantly enhanced their antioxidant properties. Thus, A. *blazei* fruiting bodies that do not conform to the commercial requirements of the market and are normally discarded could be exploited for obtaining a natural high-added value food additive, following the circular bioeconomy concept.

Lovastatin (mevinolin), a secondary metabolite from fungal growth, integrates the class of statin drugs, which diminish cholesterol biosynthesis in the liver by competitively inhibiting 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCoA reductase) (Chen et al. 2012). Thus, lovastatin can reduce LDL-cholesterol and triglyceride levels while it increases HDL-cholesterol, consequently lowering cardiovascular disease risk (Ng and Ng 2014). Atli and Yamac (2012) identified six isolates of Turkey basidiomycete mushrooms as good lovastatin producers. The best results were found for *Omphalotus olearius* (DC.) Sing. (5.8 mg/L) and *Pleurotus ostreatus* (4 mg/L). Chen et al. (2012) investigated the contents of lovastatin in both fruiting bodies and mycelia of 29 fungi species, including edible and medicinal mushrooms. Among the assessed basidiomata, *P. ostreatus* (606 mg/kg) and *A. bisporus* (565 mg/kg) showed the most expressive contents of lovastatin, while among mycelia, *Cordyceps sinensis* (Berk) Sacc. (1365 mg/kg) and *Antrodia salmonea* TT Chang & WN Chou (1032 mg/kg) had the highest concentrations.

Several authors have reported other fungal compounds with HMGCoA reductase inhibitory activity. For instance, Gil-Ramirez et al. (2013) suggested that β-glucans are the compounds involved in the HMGCoA reductase inhibitory effects displayed by *A. bisporus* fractions obtained via pressurized solvent technologies. The tested

A. bisporus extracts, which were also able to lower cholesterol levels in hypercholesterolaemic rats (probably by inhibiting cholesterol absorption), are great examples of promising ingredients for cholesterol-lowering functional foodstuff formulations.

Ergosterol plays also a substantial role in the human body as a precursor of vitamin D_2 (ergocalciferol), which is generated in response to ultraviolet radiation (sunlight) on sterols present in the skin (Mokochinski et al. 2015). Thus, the intake of food products enriched with fungal ergosterol might contribute to address vitamin D deficiency issues, and consequently offer to consumers several vitamin D-related health benefits such as improvements in immunity, bone metabolism, muscle function and cognition, and mood outcomes (Feeney et al. 2014).

21.6 Challenges for Obtaining Mycosterols on Large Scale

Almost all the fungal ergosterol fractions (and other bioactive mycosterols) are obtained from fruiting bodies. Although the by-products of basidioma's commercial production represent a sustainable and quite interesting source of ergosterol (Heleno et al. 2016a, b), producing fruiting bodies for sterol obtainment purposes is not viable. Large-scale mushroom production is an effortful and time-consuming process (can take several months), what demands huge volumes of substrate and consequently wide spaces, high energy costs to ensure the right temperature and humidity for cultivation (especially in tropical countries) and skilled labor (Corrêa et al. 2016). On the other hand, vegetative phase cultivation techniques are dramatically faster and more functional for the obtainment of high value-added molecules like mycosterols, as they require much smaller spaces, and, more important, when automatized, enable the accurate control of cultivation parameters (Inácio et al. 2015). This strict control of temperature, humidity, pH, and aeration provided by bioreactors can be extremely useful in optimizing the production of specific biomolecules.

While submerged fermentation has been considered the most effective technique to produce fungal mycelia and their bioactive compounds after shorter fermentation times (in up to a week), minimum space and superior control of process parameters, solid-state fermentation reproduces the natural environment of fungi development, demands low capital investment and minimizes contamination due to the little amount of water that is used, thus figuring out as the ideal technique for large-scale production (Mokochinski et al. 2015). However, the resemblances of the bioactive compound profiles in basidiomata and mycelia need to be previously confirmed (Corrêa et al. 2016). In an inedited comparative study on the ectomycorrhizal symbiont *Suillus bellinii* (Inzenga) Watling, Souilem et al. (2017) found that its mycelium, independently of the culture conditions, presented higher contents in ergosterol (8.9–12.4 mg/g extract) than its corresponding basidioma sample (6.5 mg/g extract).

21.7 Conclusion and Perspectives

Mycosterol-enriched fungi extracts are potential sources of biomolecules that could not only impair cholesterol absorption but also inhibit its biosynthesis and to promote other significant human health benefits. This makes them valuable and promising ingredients for the development of sterol-enriched food products. The isolation of novel bioactive mycosterols (especially ergosterol derivatives) from still underexploited fungi species, the optimization of the existing methodologies for production and recovery of these target mycochemicals (mainly automatized liquid fermentation technologies), the extensive study of their application in food product formulations (including products' bioaccessibility, efficacy, stability, as well as nutritional and sensorial evaluation), and finally further clinical trials for attesting their health benefits and safety, are challenges that science should seek to overcome in the coming years.

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Chapter 22
Influence of Genetics on the Secondary Metabolism of Fungi

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Abstract Since the discovery of penicillin in 1928, one of the most famous bioactive molecules that changed the world, studies pertaining to secondary metabolites produced by fungi increased to be explored as prototype drugs for use as pharmaceuticals and agrochemicals. Although thousands of natural products are discovered every year, the recurrent discovery of known compounds represents a problem in the field. In the laboratory, fungi are grown under axenic conditions, and many of their genes are not transcribed, remaining silenced. For this reason, researchers should seek strategies to activate these silent gene clusters to express their metabolic potential and then obtain structurally diverse biosynthesized natural products. Several effective approaches have been reported to expose the cryptic natural biogenetic capacity of fungi, such as the One Strain MAny Compounds (OSMAC) approach, co-cultivation and epigenetic modification. These techniques will be discussed in this chapter.

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22.1 Introduction

Secondary metabolites, also known as natural products, are structurally heterogeneous and low molecular weight molecules that, unlike primary metabolites, are not essential to ensure the growth of the organisms that produce them (Brakhage 2013). These metabolites are produced by filamentous fungi and play a key role in the interactions they have with other organisms like plants, animals, bacteria, and other fungi. Among these interactions, protection, defense, virulence, and nutrient acquisition are the most understood (Rokas et al. 2020). Although thousands of natural products are discovered every year, the recurrent rediscovery of known compounds represents one of the most important problems in the field (Pye et al. 2017). From 33.500 biologically active microbial metabolites described so far, approximately 47% (15.600) have fungal origin, being produced predominantly by those fungi belonging to the phyla *Ascomycota* and *Basiodiomycota* (Nett et al. 2009; Bérdy 2012; Krause et al. 2018). Due to the advancement of technologies in the processes of fungal isolation, sorting, separation, and isolation, the number of discovered natural compounds derived from fungi is growing every year (Ma et al. 2016).

Fungal natural products are produced by enzymatic pathways encoded by a group of genes called biosynthetic gene clusters (BGCs), which include expression control, self-resistance and export genes (Walsh and Fischbach 2010; Tenconi and Rigali 2018). Secondary metabolites are usually synthesized by proteins encoded in BCGs, which typically contain a synthase protein belonging to one of the following families: nonribosomal peptide synthetase (NRPS), polyketide synthase (PKS), terpene cyclase, dimethylallyl tryptophan synthetase or cyclodipeptide synthase (CDPS) (Finking and Marahiel 2004; Weissman and Leadlay 2005; Donadio et al. 2007; Gondry et al. 2009). Most secondary metabolites produced by fungi are derived from NRPs or polyketides (Brakhage 2013). Examples include the antibiotics penicillin and cephalosporin, and the immunosuppressant cyclosporine, derived from NRPs, and the cholesterol-lowering drug lovastatin, derived from polyketides (Brakhage 1998; Hoffmeister and Keller 2007).

To ensure that energy and chemical precursors are used only in environments where the production of metabolites is beneficial, fungi use a mechanism to regulate metabolism and control the biosynthesis of metabolites (Hoffmeister and Keller 2007). Filamentous fungi have up to 90 groups of BCGs in their genome, however, many of them are silenced under laboratory conditions, preventing scientists from exploring these gene clusters and collecting their products. In some cases, others have resorted to heterologous expression of the genes of fungal gene clusters in order to determine the identity of the product of the encoded pathway (Yan et al. 2018). This is a laborious undertaking. These limitations have led researchers to develop strategies to activate these pathways, such as co-cultivation techniques, One Strain MAny Compounds (OSMAC) and epigenetic modulation (Clevenger et al. 2017; Pfannenstiel and Keller 2019).

Fungi produce a multitude of compounds with varied biotechnological applicability, for example, in the pharmaceutical industries against neglected tropical

diseases and in the agrochemical industries for the production of pesticides such as herbicides and antifungals (Höller et al. 2000; Sparks and Bryant 2022). The ecological success of fungi in colonizing different habitats on the planet is due their capability, in part, to produce secondary metabolites, together with their substrate-penetrating and absorbing life form for competition for nutrients. The dependence of fungi on their metabolites to prosper in different habitats is evidenced by the fact that most species produce several different types of metabolites, their expression being adapted to the environment. Significant fractions of their genomes are dedicated to coding and regulating the production of these substances (Bills and Gloer 2016).

22.2 Strategies to Activate Silent Gene Clusters

Fungi usually produce secondary metabolites in small amounts, this level being enough for the organism to compete and coexist with other species in nature (Demain 2014). Under standard laboratory conditions, only a part of the secondary product biosynthetic genes is transcribed, while the rest remains silenced (Marmann et al. 2014). It may happen that some silent BGCs are expressed under laboratory conditions, but at very low levels that are not detected in analytical processes. In these cases, even guided chromatographic fractionation of crude metabolite exctracts may not identify these low concentrations of biomolecules, as secondary metabolites produced in abundance would mask the presence of these compounds (Romano et al. 2018). In nature, fungi produce secondary metabolites in sufficient quantities to compete and coexist with other species. Such amounts are not always sufficient to be detected with standard chemical analysis methods. Thus, some methods using biotechnology have been developed to increase the production of the desired metabolites (Adrio and Demain 2003).

Currently, there are some methodologies that reduce this limitation during the fermentation of microorganisms, aiming to activate biosynthetic pathways that result in the production of new secondary metabolites. The simplest strategy to activate the expression of BGCs is to cultivate a fungus in a variety of culture media, which may include co-cultivation of the fungus of interest with another organism to induce the expression of BGCs. Although successful in identifying some secondary metabolites, these two strategies will not activate all clusters (Frisvad 2012; Marmann et al. 2014). Co-cultivation also creates the additional complication of confirming which microbe is producing which secondary metabolite. A more refined strategy is epigenetic modulation, in which the microorganism cultures are treated with epigenetic modifiers such as DNA methyl transferase inhibitors or histone deacetylase inhibitors, with the aim of modulating histones or DNA, promoting the transcription of genes so far silenced, which could lead to the production of new secondary metabolites (Marmann et al. 2014; Zhang et al. 2014; Liu et al. 2019).

22.2.1 One Strain MAny Compounds (OSMAC)

In the One Strain MAny Compounds (OSMAC) approach, strains of interest are grown in different media and under different culture conditions, to increase the diversity of compounds, which would not be produced in conventional fermentation conditions. The OSMAC approach focuses on environmental changes, being able to induce the expression of BGCs and the exploration of the resulting chemical diversity. By changing the culture conditions such as the composition of the medium, temperature, pH, and osmolarity, the production of secondary metabolites of microorganisms can be modulated (Fuchser and Zeeck 1997; Bode et al. 2002). The OSMAC technique was originally conceptualized by the group of Zeeck (2002) and collaborators, when changing the cultivation conditions, they observed that the fungus *Aspergillus ochraceus*, which until then was thought to produce only the metabolite aspinonene, was able to produce 15 additional metabolites (Bode et al. 2002).

Özkaya et al. (2018) applied the OSMAC method, changing the composition of the medium, as a strategy for comparing the metabolic profiles of the fungus *Aspergillus carneus* isolated from marine sponges. The fungus was cultivated in three different media: solid rice medium containing sea salt, modified Czapek medium, and solid rice medium lacking sea salt. The HPLC-UV profiles of the ethyl acetate (EtOAc) extracts obtained from these different cultures revealed prominent variations in their chemical compositions. Three new natural products, isopropylchaetominine, isoterrelumamide A, and 5′-*epi*-averufanin were isolated. Isopropylchaetominine was isolated from all three media, isoterrelumamide A was only found when the fungus was grown in modified Czapek medium, while 5′-*epi*-averufanin was isolated from solid rice media with or without sea salt. In addition, the cytotoxic and antimicrobial activities of all isolated compounds were reported. Isopropylchaetominine was strongly cytotoxic against the mouse lymphoma cell line L5178Y with IC_{50} values of 0.4 μM, and 5′-*epi*-averufanin was active against the gram-positive bacteria *Staphylococcus aureus* ATCC 700699 and *Enterococcus faecium* ATCC 35667 with MIC values of 12.42 mM and 25.11 mM, respectively. Interestingly, 5′-*epi*-averufanin lacked cytotoxicity against the L5178Y cell line, indicating a high degree of selectivity for the antibacterial versus cytotoxic activity, which makes these two compounds interesting antibiotic candidates for further studies.

Endophytic fungi, those that are found inside plants throughout or at least part of their life cycle without causing apparent damage, also produce structurally diverse secondary metabolites that exhibit a variety of biological activities (Schulz et al. 2002). In the study of Wang et al. (2018), the endophytic fungus *Aspergillus aculeatus*, isolated from leaves of the papaya tree (*Carica papaya*), was cultivated on rice solid medium and produced a new conjugate of L-tryptophan-L-phenyllactic acid. Due to the potential of this fungus, an OSMAC approach was employed by adding eight different sodium or ammonium salts (3.5% NaCl, 3.5% NaBr, 3.5% NaI, 1% NaF, 3.5% $NaNO_3$, 3.5% NH_4Cl, 3.5% $(NH_4)_2SO_4$ or 3.5% NH_4OAc) to

the rice medium. The selection of these salts for the OSMAC study was based on previous experiments with other fungi that indicated the usefulness of these chemical stimuli for the accumulation of cryptic metabolites (Hammerschmidt et al. 2015; Wang et al. 2016, 2017). The addition of 3.5% of $NaNO_3$ caused a significant change in the fungal metabolite profile as indicated by High-Performance Liquid Chromatography (HPLC) analysis. Subsequent isolation of the extract yielded ten new substituted conjugates of L-tryptophan-L-phenyllactic acid, among which nine compounds were not detected in the rice control medium.

Variations in the type and intensity of light during the incubation period can also induce or suppress the biosynthesis of secondary metabolites by fungi. Bayram et al. (2008) observed that the mycotoxin sterigmatocystin produced by the fungus *Aspergillus* sp. was repressed by white light. Pruß et al. (2014) observed that the same type of light induced the biosynthesis of two mycotoxins, alternariol and altertoxin, in *Alternaria alternata*. Culture volume is also a factor that can influence the production of secondary metabolites. Daletos et al. (2017) observed that cultivation of fungi in microtiter plates (1 mL of medium per well) led to the production of several metabolites with antifungal activity that were not detected when the same strains were cultured in 1-L culture flasks.

Other culture parameters that influence the biosynthesis of fungal secondary metabolites include incubation time, level of aeration, cultures grown statically or in shake flasks and oxygen concentration (Daletos et al. 2017; Hautbergue et al. 2018; Pan et al. 2019). Even the type of water used for the preparation of the medium can result in a remarkable change in the biosynthetic profile of the fungi due the fact that tap water has certain trace ions, while distilled water does not (Paranagama et al. 2007). In addition, different culture media brands can affect the metabolite production. For this reason, researchers have to be aware about the origin of the culture medium used in fungal fermentation processes. Even if all culture conditions are the same, a different medium brand may contain a few differences in nutrient composition and affect the expression of secondary metabolites. Hewage et al. (2014) reported that the cultivation of the fungus *Dothideomycetes* sp. in Czapek malt medium prepared from commercial malt extracts from different countries completely altered its metabolic profile. Westphal et al. (2021) investigated the use of four brands of Potato Dextrose Agar (PDA), namely Fluka, Oxoid, Sigma and VWR, and evaluated their effect on the metabolic profile of four *Fusarium* species (*F. fujikuroi*, *F. graminearum*, *F. pseudograminearum,* and *F. avenaceum*). Secondary metabolites were analyzed using HPLC-HRMS, from which statistically significant differences in intensities were observed for 9 out of 10 metabolites. Even with all these potential variables, the OSMAC approach has become popular among natural product chemists because it does not involve sequencing and genetic manipulations, at the same time, it may reduce the chances of rediscovering known compounds frequently produced by laboratory-grown fungi (Gakuubi et al. 2021). The sensitivity of fungal secondary product production (the compound and its amount) to culture media composition and culture conditions has been a problem in the industrial production of some specific fungal compound products.

22.2.2 Microbial Co-cultivation

The co-cultivation technique, where two or more different organisms are grown in the same culture medium, aims to simulate on a laboratory scale the natural environmental ecosystem situation where these microorganisms would be coexisting in complex communities. The first report of co-culture of microorganisms was published by Sonnenbichler et al. (1989). They observed an increase in the chemical diversity of secondary metabolites when the fungus *Heterobasidion annosum* was cultivated together with *Gloeophyllum abietinum* in solid medium. In the following years, different studies were published showing the structural diversity and different bioactivities of compounds obtained using this strategy (Peng et al. 2021).

In nature, competition for limited resources triggers defense mechanisms that depend on the production of bioactive secondary metabolites. When coexisting in the same environment, microorganisms can interact in three ways to activate the silenced gene clusters: (1) one strain produces molecules that diffuse in the medium and act as precursors or substrates so that the other strain can produce new secondary metabolites; (2) exogenous molecules are produced as a form of chemical defense such as antibiotics or signaling molecules for competition; and (3) direct cell-cell contact trigger clusters of silenced genes in the challenged strains, resulting in the regulation biosynthesis of enzymes (Okada and Seyedsayamdost 2017). Under laboratory conditions, competition between these organisms is instigated so that biosynthetic genes silenced in monoculture are activated and transcribed under stress conditions (Ola et al. 2013; Marmann et al. 2014).

Different published studies show that co-cultivation is a viable experimental strategy to increase the diversity of chemical compounds produced by fungi when cultivated *in vitro*. Wang et al. (2022) showed that the co-cultivation of the *Aspergillus niger* and *Pleurotus ostreatus* resulted in the production of extracellular detoxifying enzymes that improved the degradation of aflatoxin B_1 (AFB_1), one of the most harmful mycotoxins for humans and other animals, commonly found in maize, cottonseed, legumes, and peanuts. The maximum degradation of AFB_1 with the co-culture reached 93.4%, which increased by 65.9% and 37.6%, respectively, in relation to the monoculture of *P. ostreatus* and *A. niger*. After purification, these enzymes had a high detoxification effect, with a degradation rate of 94.7%, proving to be very useful to be used in AFB_1 detoxification in food products, which could increase the safety of some food products.

Another example of successful co-cultivation is the substance paclitaxel, an anticancer drug derived from the plant *Taxus* sp. Due to its limited supply, the clinical use of this drug has been a major obstacle, causing scientists to seek new ways to improve its production. According to Li et al. (2009), the co-culture of cells in suspension of the plant *Taxus chinensis* var. *mairei* together with its endophytic fungus *Fusarium mairei* in a bioreactor under ideal culture conditions, not only increased the paclitaxel yield but also shortened the culture time. *Taxus* sp. cell cultures by co-culture produced 25.63 mg L^{-1} of paclitaxel in 15 days, equivalent to

a productivity of 1.71 mg L^{-1} per day and 38 times higher than the uncoupled culture (0.68 mg L^{-1} in 15 days).

According to Lade et al. (2012), the co-cultivation of the fungus *Aspergillus ochraceus* and the bacterium *Pseudomonas* sp. was evaluated for its potential to improve the discoloration and detoxification of the azo dye Rubine GFL from textile effluent. Azo dyes are widely used as raw material in the textile industry, and because they are resistant to degradation, they remain in the environment for a long time, resulting in serious ecological damage when they reach water resources, changing their pH, increasing the Chemical Oxygen Demand (COD) and giving an intense coloration to the site (Levine 1991; Xu et al. 2006). The efficiency of pollutant removal (100 mg L^{-1}) from liquid matrix in a period of 30 h reached 95% using the co-culture of microorganisms, contrasting with 46% and 63% for fungal and bacterial monoculture, respectively. The results obtained suggest that co-cultivation can be effectively used for complete detoxification of dyes and effluents and has potential environmental implications in the cleaning of azo dye-containing effluents. In addition to being simple and efficient, the co-cultivation technique has been widely and successfully applied in the field of discovery of new bioactive natural products. Its biggest advantage is that it does not need complex operations at the genetic level and does not use expensive reagents (Hoshino et al. 2019).

22.2.3 Epigenetic Modulation

Another option to activate silenced biosynthetic pathways involves epigenetic approaches, the science that studies hereditary changes in gene expression that occur without modifying the DNA sequence (Byrum et al. 2012). There are two main mechanisms involved in epigenetics: changes in histones and DNA methylation patterns (D'Alessio and Szyf 2006). In addition to these main epigenetic mechanisms, there are also non-coding RNAs, which can act by interfering with gene transcription (Tang and Ho 2007).

Histones are structural proteins associated with DNA that have the function of organizing chromatin, and changes in their structure directly influence gene expression. When chromatin is condensed, genes are inactive, when chromatin is relaxed, genes are expressed (Rodenhiser and Mann 2006). These chromatin states can be controlled by reversible epigenetic patterns of DNA methylation and histone modifications (Feinberg and Tycko 2004). When DNA is hypomethylated, chromatin is active, allowing transcription of genes, and when DNA is hypermethylated, chromatin is inactive, preventing gene expression (D'Alessio and Szyf 2006). DNA methylation and chromatin structure modulation impact gene expression, resulting in qualitative or quantitative changes in secondary metabolites produced, enabling the use of chemical agents that interfere with epigenetic mechanisms to induce transcription of silent gene clusters (Williams et al. 2008; Yang et al. 2013).

For epigenetic modulation to occur, microorganisms are treated with epigenetic modifiers, such as DNA methyl transferase inhibitors and histone deacetylase inhibitors, to modulate DNA or histones, to initiate the transcription of silenced genes (Cichewicz 2010; Vervoort et al. 2011). Several epigenetic modifying enzymes have been identified to date, such as DNA methyltransferases (DNMT), histone acetyltransferases and histone deacetyltransferases (HDAC) (Williams et al. 2008; Li et al. 2017). Examples of DNMT inhibitors include 5-azacytidine, 5-aza-2′-deoxycytidine, methylthioadenosine and S-adenosylhomocysteine, while HDAC inhibitors are trichostatin A, suberoylanilide hydroxamic acid (SAHA), trapoxin B, apicidine, HC-toxin, sodium butyrate and valproic acid (Cichewicz 2010), as well as romedepsin (Owens et al. 2020) and 2-amino-3*H*-phenoxazin-3-one (Venturelli et al. 2015).

In the last two decades, the discovery of several new fungal compounds as a result of treatment with HDAC and DNMT inhibitors has been reported. Several published studies show different effects after treating fungi with these inhibitors, often resulting in an increased diversity of metabolites produced by them (Toghueo et al. 2020). Wang et al. (2010) reported that epigenetic modulation of *Penicillium citreonigrum* profoundly changed its metabolite profile. The culture was treated with 50 μM of the DNMT inhibitor 5-azacytidine, resulting in the production of six azaphylones (sclerotiorin, sclerotioramine, ochrephilone, dechloroisochromophilone III, dechloroisochromophilone IV, 6-((3E,5E)-5,7-dimethyl-2-methylenenonone-3,5-dienyl)-2,4-dihydroxy-3-methylbenzaldehyde), pencolide, and two new meroterpenes (atlantinones A and B). Only pencolide, inactive against a panel of bacteria and fungi, was detected in the control. On the other hand, sclerothiorin and sclerothioramine, produced in the 5-azacytidine-induced culture, exhibited moderate inhibition of *Staphylococcus epidermidis*. Sclerthioramine was very active against several strains of *Candida* spp. Furthermore, none of the induced metabolites were structurally similar to pencolide. Fritz and Papavasiliou (2010) and Hagemann et al. (2011) reported that the use of 5-azacytidine led to increased levels of hypomethylated DNA, that is, chromatin was active allowing the transcription of genes.

Cultures of the fungi *Alternaria alternata* and *Penicillium expansum* were treated with the HDAC inhibitor trichostatin. As a result, Shwab et al. (2007) observed a statistically significant increase in several previously unidentified metabolites in both species. After treatment with the histone deacetylase inhibitor SAHA (suberanylhydroxamic acid), the fungus *Cladosporium sphaerospermum*, derived from sediment from the Mariana Trench (depth 6.562 m), produced four new tetramic acids, cladosins H − K, and a related known compound, cladodionen. The cytotoxicity of these compounds was evaluated against multiple cell lines, and the compound clasosin I showed promising cytotoxicity against the HL-60 cell line with an $IC_{50} = 2.8$ μM (Zhang et al. 2018). Fungi have been the main targets of epigenetic modifications in order to gain access to cryptic natural product genes. With several works published in the area of epigenetics, histone deacetylase (HDAC) and DNA methyltransferase (DNMT) inhibitors have proven their efficacy in inducing the synthesis of new secondary metabolites in fungi.

22.3 Natural Products from Fungi Against Neglected Diseases

According to the World Health Organization (WHO), neglected tropical diseases (NTDs) are a group of 20 conditions prevalent in tropical areas, where they mostly affect impoverished communities and disproportionately affect women and children (WHO 2022). Among these diseases are included Chagas disease (also known as American trypanosomiasis) and leishmaniasis (WHO 2022). NTDs can cause devastating health, social, and economic consequences to more than one billion people in tropical and subtropical countries, affirming the need for and importance of drug discovery and development for NTDs (Cheuka et al. 2017; WHO 2022). In this scenario, the natural products produced by fungi are investigated by many research groups aiming to discover novel and effective treatments for these diseases.

22.3.1 Chagas Disease

Chagas disease is an anthropozoonosis occurring in the South American continent. It is caused by the protozoan *Trypanosoma cruzi* and remains a major social and public health problem in Latin America (Pérez-Molina and Molina 2018). Benznidazole and nifurtimox are the only two drugs licensed for the treatment of Chagas disease, and both have been the mainstay of parasiticidal treatment for almost 50 years, although their safety and efficacy profile is far from ideal (Pérez-Molina and Molina 2018), necessitating the urgent need for the discovery of new classes of trypanocidal drugs with better efficacy and safety.

Rateb et al. (2013) demonstrated that the co-culture of *Aspergillus fumigatus* MBC-F1-10 with the type strain of *Streptomyces bullii* led to the isolation of ergosterol, seven metabolites belonging to the diketopiperazine (DKP) class of alkaloids, along with brevianamide F, spirotryprostatin A, 6-methoxy spirotryprostatin B, fumitremorgin C and its 12,13-dihydroxy derivative, fumitremorgin B, verruculogen, 11-O-methylpseurotin A and 11-O-methylpseurotin A2. In addition, an independent experiment was conducted in which N-butyryl-DL-homoserine lactone was added to the culture medium, resulting in the production of emestrins A and B. Neither microbe produced these compounds when cultured alone. The compounds were tested for their potential antiprotozoal activity and fumitremorgin B and verruculogen were the most potent growth inhibitors exhibiting EC_{50} values of 0.2 μM, followed by 12,13-dihydroxyfumitremorgin C which exhibited an EC_{50} value of 7.4 μM on *T. brucei* (Rateb et al. 2013).

Do Nascimento et al. (2020) cultivated the endophytic fungi *Phanerochaete* sp. H2 and *Talaromyces purpurogenus* H4, isolated from *Handroanthus impetiginosus* leaves, in mixed (co-culture) and single (axenic) cultures, in solid and liquid media. From the mixed co-culture extracts the authors reported the detection of the meroterpenoid austin. The compound possessed trypanocidal

activity, with an IC_{50} of 36.6 ± 1.2 μg mL^{-1} against the epimastigote form of *T. cruzi* (Do Nascimento et al. 2020). These results highlight the importance of the co-culturing of endophytic fungi to obtain natural, bioactive products.

22.3.2 Leishmaniasis

Leishmaniasis is a parasitic disease caused by protozoans belonging to the genus *Leishmania.* The parasite is transmited when infected, female phlebotomine sandflies (*Phlebotomus ariasi*) bloodfeed on humans (Ross 1903; Cheuka et al. 2017). A wide variety of clinical manifestations, ranging from cutaneous to the visceral forms, can be manifested after infection. The currently available treatments remain mostly ineffective, some requiring hospitalization and causing severe side effects and leading to the development of resistance (Gervazoni et al. 2020).

In the study conducted by Rateb et al. (2013), the compounds obtained from the co-culture of *Aspergillus fumigatus* MBC-F1–10 with *Streptomyces bullii* were tested against *Leishmania donovani*. All tested compounds proved active except brevianamide F and 11-O-methylpseurotin A. Fumitremorgin B and verruculogen were the most potent, exhibiting EC_{50} values of 3.1 and 3.9 μM, respectively (Rateb et al. 2013).

Fill et al. (2016) used the OSMAC approach and demonstrated that *Penicillium brasilianum* LaBioMMi 136, an endophytic fungus isolated from the root bark of *Melia azedarach*, was able to produce an active compound when cultivated in a medium supplemented with $CuSO_4$ and $MnSO_4$. The compound was isolated and identified as the cyclodepsipeptide JBIR 113. The compound was submitted to biological assays and evaluated against promastigote forms of *L. amazonensis*, and epimastigote and trypomastigote forms of *T. cruzi*, with an IC_{50} value of 63.2 ± 2.5 μM (Fill et al. 2016).

Demers et al. (2018) reported that 530 fungal isolates obtained from mangrove plant tissues (roots, stems, leaves, and flowers) were cultivated with and without the modulators of epigenetic regulation: sodium butyrate (HDACi) and 5-azacytidine (DNMTi). Of the total of extracts tested, 40 with DNMTi and 35 with HDACi showed IC_{50} value of <1 μg mL^{-1} for *L. donovani*. Although few studies using the techniques described in this chapter have been found in the literature, they are promising approaches for the discovery and development of new antiparasitic drugs.

22.4 Application of Secondary Fungal Metabolites in Agriculture

Agriculture contributes to the economic development of most countries around the world and guarantees food security in these places and others. Currently, with the rapid growth of the world population, the agricultural sector has been greatly

overburdened (Alston and Pardey 2014). In addition, this sector has been facing a major challenge, which is to control the pests that affect farming and can cause billions of dollars in losses per year (Ricroch et al. 2016). These pests include insects, nematodes, fungi, and weeds, which are responsible for most of the losses in crop productivity, with herbicides representing 60% of the pesticides used worldwide (Dayan 2019).

Weeds are plants that have no commercial value and end up competing successfully for nutrients from the soil, water, sunlight, thus harming the growth of crops of interest. In addition, they have high phenotypic plasticity and spread easily within a farm, which makes their control even more difficult (Vigueira et al. 2013; Singh and Pandey 2019). Due to this major problem, agriculture has become dependent on herbicides. However, with the continuous and often indiscriminate use of herbicides that have the same mechanism of action, weeds have evolved resistance to most of them (Dayan 2019; Gaines et al. 2020). So far, 509 cases of herbicide-resistant weeds have been recorded globally, with 266 species (153 dicots and 113 monocots). Weeds have evolved resistance to 21 of the 31 known herbicide sites of action and to 164 different herbicides (Heap 2022). Research shows that secondary metabolites produced by fungi are very promising in the search for new substances, as they have unique mechanisms of action, with low toxicity to humans, in addition to not being persistent in the environment, standing out as an alternative to minimize the negative impacts caused to the environment and human health by synthetic herbicides (Dayan et al. 2009; Duke et al. 2010).

After a refined search in the literature on research that used techniques such as OSMAC, co-cultivation, and epigenetics to induce the production of new secondary metabolites in fungi that had the objective of producing new herbicides, only one article was found. The endophytic fungus *Clonostachys rosea* isolated from mangrove plants had its biosynthetic profile of secondary metabolites altered by the OSMAC technique. Supratman et al. (2021) observed that the addition of apple juice to the solid rice medium induced the production of a known compound, (−)-vertinolide, in addition to four new compounds, (−)-dihydrovertinolide and the clonostach acids A, B and C. The compound (−)-dihydrovertinolide had moderate phytotoxic activity against lettuce seedlings above a concentration of 50 mg L^{-1}.

Phytopathogenic fungi affect crops around the world causing high losses in productivity (Shafique et al. 2016). The resistance acquired over the years by these phytopathogens to fungicides used in agriculture and the emergence of new pathogenic fungal species reinforces the need to discover new agricultural fungicides with new mechanisms of action (Strobel 2006; Farhat et al. 2019). Strategies such as OSMAC, co-culture and epigenetic modulation can help in the discovery of new secondary metabolites of fungi with antifungal activity to be studied as prototypes of new products.

Few studies have been published using the three strategies to activate silent gene clusters addressed in this chapter aiming at the production of antifungal metabolites with a focus on agriculture. We highlight here the most recent of them. Pang et al. (2018) used the OSMAC approach with the endophytic fungus *Emericella* sp., which resulted in the production of four new polyketides (emericelactones A–D).

In their previous work, seven prenylxanthones and two alkylated furan derivatives were isolated from solid fermentation culture medium composed of rice plus glucose medium. Continuing the search for structurally diverse compounds generated by this fungus, diverse secondary metabolites were produced when cultured in a solid medium of rice broth plus malt extract broth (MEB). The four compounds showed moderate antimicrobial activities against three agricultural pathogenic fungi (*Verticillium dahliae*, *Rhizoctonia solani* and *Gibberella saubinetii*) with MIC values of 25–50 μg mL^{-1}.

A new alkaloid, (±)-preisomide and five known polyketides were isolated from the endophytic fungus *Preussia isomera* obtained from *Panax notoginseng* using the OSMAC (Chen et al. 2020). In a previously published work, the research group investigated the chemical constituents of *P. isomera* using rice broth plus malt extract (MEB) medium (Xu et al. 2019). To explore its biosynthetic potential, they applied the OSMAC approach and observed that this fungus can produce several secondary metabolites when grown in rice plus wheat bran medium. Five of the isolated polyketides exhibited moderate antifungal activity against *Gibberella saubinetii*, a wheat phytopathogen, with a MIC value of 50 μg mL^{-1}.

Costa et al. (2019) used the co-cultivation technique to activate cryptic biosynthetic pathways and discover novel antimicrobial metabolites. They cultivated *Penicillium citrinum* and *Penicillium digitatum*, pathogens responsible for 90% of citrus fruit losses worldwide. Mass spectrometry imaging (MSI) was used to track potential antifungal metabolites involved in the interaction between these microorganisms, revealing a chemical warfare between fungi, where two tetrapeptides, deoxycitrinidine A, citrinadine A, chrysogenamide A and tryptochialanines were produced in the zone of fungal confrontation. Antimicrobial assays confirmed the antifungal activity of the investigated metabolites. In addition, tryptochialanines inhibited *P. citrinum* sporulation. The fungal metabolites reported by those authors demonstrate that exploration of co-cultures of phytopathogens that compete for the same host is a sensible strategy to discover new antifungal agents.

A new nonadhydride derivative, (−)-bisoclamic acid imide, was isolated from the co-culture broth of two fungi occurring in mangroves. The bioassays showed that this compound, when compared to the positive control carbendazim, a broad-spectrum fungicide, has moderate *in vitro* antifungal activity against the crop pathogens *Fusarium graminearum* and *Fusarium oxysporum* (Ding et al. 2017). Zhang et al. (2019) used three chemical epigenetic modifiers, 5-azacytidine, nicotinamide, and suberoylanilide hydroxamic acid (SAHA), to induce the metabolites of *Penicillium mallochii*, a fungus isolated from the gut of the lepidopteran insect *Ectropis obliqua*. The metabolic profiles of *P. mallochii* were altered by treatment with SAHA, resulting in the production of four metabolites, including two new sclerothioramine derivatives, isochromophyllone XIV and isochromophyllone XV, and two known compounds, sclerothioramine and (+)-sclerothiorine. The anti-phytopathogenic activities of the isolated compounds were evaluated, and (+)-sclerothiorine showed broad and important inhibition activities against *Curvularia lunata* ($IC_{50} = 2.1$ μg mL^{-1}), *Curvularia clavata* ($IC_{50} = 21.0$ μg mL^{-1}), *Fusarium oxysporum* ($IC_{50} = 40.4$ μg mL^{-1}) and *Botryosphaeria dothidea*

(IC_{50} = 27.8 μg mL^{-1}), which were comparable with IC_{50} values shown by cycloheximide of 0.3, 5, 12.4 and 15.3 μg mL^{-1}, respectively. Isochromophyllone XV and sclerothioramine had selective and potent activities against *Colletotrichum graminicola* with IC_{50} values of 29.9 and 9.7 μg mL^{-1}, respectively. The results obtained suggest that the chemical induction by epigenetic modifiers is a promising approach to obtain antifungal metabolites encoded by cryptic *P. mallochii* biosynthetic genes.

Chemical fungicides have been widely used in agriculture to control phytopathogenic fungi. However, they present increasing risks to the environment and human health, in addition to the problem of fungicide resistance caused by these products. As a consequence, the search of natural products as possible substitutes is growing.

22.5 Conclusions

Fungi represent a highly diverse microbial group, including those recognized as endophytes. Due to their high worldwide diversity, many species of high hierarchical taxonomic levels still remain unknown, which can include taxa that could be prolific factories of novel bioactive compounds. There is a great need for new pharmaceuticals and pest management compounds from natural sources. Discovery of such compounds from known and newly discovered fungi by means of methods such as the OSMAC approach, co-cultivation or epigenetic modification or other techniques that activate cryptic gene clusters encoding biosynthetic pathways for new bioactive compounds is promising.

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Part IV
Food Industry

Chapter 23
Applications of Plant Secondary Metabolites in the Food Industry

Spyridon A. Petropoulos

Abstract The Plant kingdom is abundant with numerous secondary metabolites, which despite not being essential for plant growth and development, they exert significant properties related to the adaptation and survival of the species against exogenous stressors. These compounds are synthesized when plants are subjected to unfavorable conditions, and they represent the armory of plants against abiotic and biotic stress. Moreover, they exert significant bioactive properties and are associated with beneficial effects on human health. Considering the bioactivities of secondary metabolites, current research focuses on the recovery of these compounds and their valorization in various industrial applications, including the food industry. This chapter will present the most common categories and sources of secondary metabolites usually found in plants, focusing on cultivated species. Moreover, it will highlight the most noteworthy applications of secondary metabolites in the food industry, while it will also discuss the current trends and future perspectives related with the recovery and valorization of these compounds.

23.1 Introduction

Secondary metabolites are considered those small molecules that are not regularly composed in plant tissues and are not obligatory for the growth, development, and reproduction of plants (Hassan et al. 2012). Their function is focused on plant defense against abiotic and biotic stressors, which differentiates them from primary metabolites that are involved in vital functions of plants and their presence is necessary for life maintenance (Tiwary and Rana 2015). However, this segregation is equivocal since in some occasions the intermediates in the biosynthetic pathways of both metabolites are common, making the distinction very complex (Pichersky and Gang 2000). Moreover, secondary metabolites can be used by plants in primary

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metabolism functions, while they are associated with multiple functions (Erb and Kliebenstein 2020). Therefore, a new definition was coined and now secondary metabolites are considered those specialized compounds (hence the term "specialized metabolites") that can be found in specific species, which differentiates them from primary metabolites that are omnipresent in all living organisms and plant hormones that have regulatory functions (Erb and Kliebenstein 2020). The recent review of Erb and Kliebenstein (Erb and Kliebenstein 2020) clearly identifies the obstacles in defining and dividing primary and secondary metabolites, while they also highlight that recent advances in analytical chemistry and genetics made this task more complex. Moreover, the same authors suggest the whole perspective for these compounds have to be revised and considering them as components of dynamic metabolic pathways will improve our understanding regarding the functionality of these valuable compounds (Erb and Kliebenstein 2020).

The biosynthesis of secondary metabolites comes to cover specific needs of plants at specific stages of their growth (e.g., the synthesis of volatiles to attract pollinators during the reproductive stage) or when plants have to overcome exogenous restraints (e.g., pest and pathogens attacks, unfavorable environmental conditions) (Hartmann 2007; Ramakrishna and Ravishankar 2011; Wink 2018). The numerous compounds that have been identified in various plant species so far are classified based on their chemical structures and they are categorized into three main groups, namely terpenes, polyphenols, and nitrogen-containing compounds (e.g., alkaloids and glucosinolates) (Hartmann 2007). Despite their structural similarities there are several individual compounds that are species specific and maybe synthesized through the activity of specific enzymes on different or the same substrate (Pichersky and Gang 2000). Therefore, each species composes a different set of secondary metabolites even when the stimuli are the same, e.g. attraction of the same species of pollinators or protection against the same herbivores (Knudsen and Tollsten 1993; Mello and Silva-Filho 2002).

The modern food industry sectors must come up against several contradictory challenges related to food security, on the one hand, and, the health safety, on the other hand. The increasing demands for food production due to increasing population necessitate the production of larger amounts of food products, whereas the consumers' concerns regarding the food safety and their demands for functional and healthy foods call for the redesign of food industry sector (Carpentieri et al. 2022). Functionality of plant-based food products is associated with the presence of secondary metabolites which are normally produced in various plant tissues and organs, while modern food research focuses on eliciting the biosynthesis of such compounds or incorporating them in the food products (Isah et al. 2018; Carpentieri et al. 2022; Ceccanti et al. 2022). Therefore, the recovery of bioactive compounds from natural sources such as plants and agro-industry by-products is gaining interest aiming to design new functional and healthy foods (Galanakis 2021).

The present chapter will discuss the main categories of secondary metabolites, as well as their applications in the food industry, focusing mostly on commercially available solutions and secondary metabolites obtained from cultivated species. Moreover, the future perspectives that must be considered in order to improve the

valorization of these compounds and increase the added value of crops will be discussed.

23.2 Secondary Metabolites Categories

The term "secondary" or "specialized metabolites" refers to various compounds that are responsible for plant responses to environmental conditions and biotic factors. They are classified based on their chemical structure, composition, or biosynthetic pathways and include phenolic compounds, terpenes and steroids, and nitrogen-containing compounds (mainly alkaloids and glucosinolates) (Bourgaud et al. 2001). There is a great diversity in the chemical structures and biological functions of these compounds, which are species or genotypes specific, while they can be synthesized in particular plant organs when plants are subjected to specific conditions (Petropoulos et al. 2018; Chiocchio et al. 2021).

Phenolic compounds are one of the most common groups of secondary metabolites which are widely distributed in the plant kingdom and are synthesized via the shikimic acid and phenylpropanoid biosynthetic pathways (Bennett and Wallsgrove 1994). There is a great diversity of phenolic compounds which can be further classified into two distinct groups, namely flavonoids (e.g., anthocyanins, flavanols, flavanones, flavonols, flavonones, and isoflavones) and non-flavonoids (e.g., phenolic acids, xanthones, stilbenes, lignans, and tannins) (Durazzo et al. 2019). The non-flavonoid group includes simple phenols which contain a single benzenic ring, phenolic acids which also contain one benzenic ring with a substituent of the carboxylic group and are further classified into benzoic acid and hydroxycinnamic acid derivatives, and other polyphenols with diversified structures (Chiocchio et al. 2021).

On the other hand, the flavonoid group includes compounds with fifteen atoms of carbon that consist of two benzenic (aromatic A and B) rings linked with a unit of three atoms of carbon that forms or not an extra (C) ring (Panche et al. 2016). They are further divided into several sub-groups (chalcones, stilbenes, aurones, flavanones, flavones, isoflavones, phlobaphenes, dihydroflavonols, flavonols, leucoanthocyanidins, proanthocyanidins, and anthocyanins) based on the presence or absence of C ring between the aromatic ones, as well as on the carbon of C ring which is attached on B ring and its saturation and oxidation degree (Panche et al. 2016; Abbas et al. 2017; Liu et al. 2021). Flavonoids are assigned with several functions in plants, including the coloration of flowers or other plant parts which gave them their name (*flavus* means yellow in Latin), and are mostly commonly found in the aerial plant parts, whereas non-flavonoid phenolic compounds are mainly distributed but not limited in seeds, leaves, stems, and roots (Robbins 2003; Tuominen et al. 2013).

Terpenes are the most diverse group of secondary metabolites with more than 40,000 compounds identified so far in all forms of life (Caputi and Aprea 2012). They are synthesized via the mevalonate pathway by using mevalonic acid as the

intermediate compound and consist of repeating units of isoprenes (2 units in the case of monoterpenes and more than 8 units in polyterpenes) (Habtemariam 2019; Kallscheuer et al. 2019). Terpenes are also the building blocks of steroids which are formed from triterpenes, while along with terpenoids which contain additional oxygen-containing groups they are the most abundant class of volatile compounds commonly found in plant species (Derbassi et al. 2022). Their synthesis follows a spatio-temporal pattern regulated by specific genes which result in the numerous volatile compounds that are found in plant species (Kallscheuer et al. 2019).

Alkaloids are nitrogen-containing compounds which depending on their structure are diversified in non-heterocyclic and heterocyclic alkaloids (Lichman 2021). Recent classification of alkaloids considers the similarities in carbon skeleton as the distinguishing parameter in order to include specific compounds in this group (Ziegler and Facchini 2008). Another distinction refers to the precursor compounds, which can be amino acids in the case of "true alkaloids" or other nitrogen-containing compounds in the case or "pseudoalkaloids" (Lichman 2021). Most alkaloids have medicinal properties, therefore its use is limited to the pharmaceutical and drug industry (Aniszewski 2015).

Glucosinolates are another group of sulfur and nitrogen-containing secondary metabolites which are usually founds in species of the Capparales order especially in the Brassicaceae family (Di Gioia et al. 2020). Their biosynthesis consists of three phases, namely the elongation of the side group (R) attached to the core compound (*β*-D-glucopyranose + (Z)-*N*-hydroximinosulfate ester + R) through the addition of methylene groups, the formation of the core structure of glucosinolates, and finally the modification of this side group (Di Gioia and Petropoulos 2021). Glucosinolates are indirectly involved in plant defense mechanisms as deterrents of various pests and pathogens and stress alleviators, through the formation of biologically active compounds (e.g., isothiocyanates) after being hydrolyzed (Vig et al. 2009; Justen et al. 2013; Esfandiari et al. 2017). There are numerous glucosinolates which are highly specified on a species level (Di Gioia et al. 2020), while depending on the precursory amino acids they are classified into three main groups, namely aliphatic, aromatic, and indole glucosinolates (Redovniković et al. 2008).

23.3 Food Industry Applications of Secondary Metabolites

Modern food industry must fulfill consumers' demands for healthy/functional foods, on the one hand, and the substitution of synthetic compounds with naturally derived ones, on the other hand. Additionally, these two requirements have to be fulfilled without compromising food security which is under threat considering the increasing population and the ongoing climate crisis (Carpentieri et al. 2022), while the sustainability of the whole chain has to be addressed within the circular economy context (Cole et al. 2018; Knorr et al. 2020). For this purpose, secondary metabolites are attributed with interesting bioactive properties which can be used in the food industry, especially for the design of new functional foods and natural food

preservatives (Durazzo et al. 2019). The reduction of food waste due to losses throughout the value chain (e.g. crop production, post-harvesting, and processing) could be partly achieved with the use of novel preservatives extracted from plant matrices that could extend shelf life of food products (Cole et al. 2018). The extraction of secondary metabolites from plant tissues (either the primary crop products or the by-products) is also gaining great interest from the scientific community and the food industry sector since it contributes to the added value of crops and reduces the environmental burden (Hartmann 2007). Moreover, the integration of such molecules in food products could increase their overall bioactive properties and contribute to the prevention of chronic diseases and the improvement of consumers' well-being (Ahmad et al. 2019; Carpentieri et al. 2022).

23.3.1 Polyphenols

Among these metabolites, polyphenols are the most widely distributed and can be found in plant species and plant-based foods in various forms (Abbas et al. 2017). Their most common uses include the flavoring, coloring, or texturizing of food products (Kallscheuer et al. 2019), as well as their use as preservatives and food additives due to their antimicrobial properties (Wu and Zhou 2021; Oulahal and Degraeve 2022). The great diversity of polyphenols offers many choices to select in terms of biostability, bioavailability, and functionality. The amount of extracted polyphenols accounted for 16,380 tons in 2015 and is expected to be doubled by 2024, while the majority of these compounds is utilized in the beverages and functional food sectors of the food industry (Adebooye et al. 2018). However, the extraction process of secondary metabolites faces many challenges due to the complexity of the obtained mixtures and similarities in chemical structures between the target compounds (Robbins 2003; Kallscheuer et al. 2019). Moreover, the seasonal fluctuation of these compounds in plant tissues, as well as the impact of growing conditions on the final concentration makes the extraction task more challenging. Therefore, the tailored production of bioactive molecules from engineered microorganisms is gaining ground toward a more sustainable and cost-effective recovery of such compounds (Kallscheuer et al. 2019). The recent advances in food science allowed the microencapsulation of such molecules and their incorporation directly in food products or in food packaging aiming to control food-borne pathogens and increase the shelf life of food products without affecting the sensorial properties of the final product (Hintz et al. 2015).

Several examples of polyphenols utilization highlight their importance in the food industry sector. There is sufficient evidence that polyphenol-rich extracts can inhibit food spoilage from bacteria and fungi infections and oxidation (Olszewska et al. 2020). The use of such extracts either through direct incorporation in healthy/functional food products or in food packaging shows a great potential for the food industry with several benefits such as the consumer acceptance and the lack of microbial resistance (Hintz et al. 2015). There are several paradigms of successful

application of polyphenol-rich extracts obtained from plants in various food products. The fortification of food products with "protected" polyphenols via encapsulation may increase their stability to oxidation and thermal processing and improve their bioavailability (Cao et al. 2021). Moreover, binding polyphenols with specific molecules (e.g., lipids, proteins, carbohydrates) may increase their bioavailability through the reduced oxidation in the upper part of the gastrointestinal tract, although these effects depend on the molecules (Jakobek 2015). The polyphenols extracted from cocoa hulls were integrated in bakery products via microencapsulation and improved the total phenolic content of the final product (Papillo et al. 2019). The direct incorporation of grape-derived polyphenols in spaghetti significantly improved the bioavailability of bioactive compounds and decreased the glycemic index (Marinelli et al. 2018), while the integration of carrot pomace into pasta also improved the overall phenolic compounds content (Gull et al. 2015). Melon peels are also a good source of polyphenols and the addition of peels flour in food products could increase the total and individual phenolic compounds content as well as the prebiotic effects and the gut microbiota diversity (Gómez-García et al. 2022). Moreover, the addition of herbal mixtures containing *Laurus nobilis*, *Curcuma longa*, and *Zingiber officinale* Roscoe improved the nutritional value, the total phenolics and flavonoids content, as well as reduced the bacterial content in extruded corn snacks (Amer and Rizk 2022).

Non-dairy products and beverages are another category of food products where the application of phenolic-rich extracts shows great interest, since there is a market demand for alternatives to milk-based products, especially for consumers that suffer from allergies to milk protein, lactose intolerance, or those who have to follow special diets due to obesity or high blood cholesterol levels (Fidelis and Granato 2021). Some examples (see also Table 23.1) include the addition of polyphenol-rich flaxseed oil cake in a vegan analog of Camembert cheese (Łopusiewicz et al. 2020); the carob-fruit extracts in meat products (Macho-González et al. 2020); the production of non-dairy yogurt with nanoemulsions of rice bran oil and soy protein (Sengupta et al. 2019); the preparation of dairy free creams with the addition of grapes (Cropotova et al. 2017); the addition of germinated brown rice in a multifunctional non-dairy yogurt (Cáceres et al. 2019); the production of non-dairy ice creams with riceberry and sesame seeds milk (Kemsawasd and Chaikham 2020). Phenolic compounds obtained from flower extracts are also a promising material to incorporate in beverages as coloring or flavoring agents, e.g. the anthocyanin-rich extracts from cornflower used to create a model beverage (Escher et al. 2018), or the use of aqueous extracts from butterfly pea petals used as a coloring agent (Escher et al. 2020). Moreover, the extracts from acerola by-products were used to produce a kombucha-like beverage (Leonarski et al. 2021), while Migliorini et al. (Migliorini et al. 2019) suggested the use of red chicory leaves extracts as a promising coloring agent for beverages with great stability and bioactive properties. Other by-products that could be used for that purpose include those obtained from maize processing commonly known as nejayote (Buitimea-Cantúa et al. 2019), the brewers' spent grains produced during beer production (Tan et al. 2020), or purple and red colored potato tubers (Sampaio et al. 2021). Fermented

Table 23.1 Examples of incorporation of phenolic compounds in dairy, non-dairy products, and beverages

Source	Product	Composition	Reference
Flaxseed oil cake	Vegan analog of Camembert cheese	700 g of flaxseed oil cake mixed in 2000 mL of distilled water; then added 0.5 g of MST Cheese-Tek® + 0.5 g of lactic acid bacteria	Łopusiewicz et al. (2020)
Carob-fruit extracts	Restructured meat products	4 g/kg of restructured meat	Macho-González et al. (2020)
Nanoemulsions of rice bran oil and soy protein	Non-dairy yogurt		Sengupta et al. (2019)
Grapes puree	Dairy free creams	72.0%, 80.0%, and 90.0% of grape puree (w/w)	Cropotova et al. (2017)
Flour of germinated brown rice	Non-dairy yogurt	Seeds were germinated for 48 or 96 h, then dried, ground to flour, and fermented	Cáceres et al. (2019)
Riceberry and sesame seeds milk	Non-dairy ice creams	75% pasteurized seed milks (w/w)	Kemsawasd and Chaikham (2020)
Anthocyanin-rich extracts from cornflower petals	Model beverage	0.25 g of dried petals per 25 mL of acidified aqueous solution	Escher et al. (2018)
Butterfly pea petals	Model beverage	0.125 g of dried petals per 25 mL of ultrapure water	Escher et al. (2020)
Acerola by-products	Kombucha-like beverage	1, 3, and 5% w/v of acerola by-product	Leonarski et al. (2021)
Red chicory leaves extracts	Coloring agent for beverages		Migliorini et al. (2019)
Nejayote (maize processing by-product)	Maize-based beverages	3, 6, or 9%, w/w	Buitimea-Cantúa et al. (2019)
Brewers' spent grains	Novel beverage	Submerged fermentation of 10 g of brewer's spent grain with *Bacillus subtilis*	Tan et al. (2020)
Purple and red colored potato tubers	Soft drink formulation	1 g fresh tuber per 2 mL citric acid solution	Sampaio et al. (2021)
Prosopis alba pods	Aloja and añapa beverages	Cut pods fermented in tap water (4: 96 pods:water w/v)	Rodríguez et al. (2020)
Saccharum officinarum L. molasses	Fermented beverage	500 g of molasses were added to 1.5 L of water and fermented with yeasts	Samaniego-Sánchez et al. (2020)
Okara (soy-bean processing by-product)	Novel symbiotic beverage	Okara was hydrolyzed with cardoon enzymes and fermented with lactic bacteria	Voss et al. (2021)

(continued)

Table 23.1 (continued)

Source	Product	Composition	Reference
Mulberry juice	Dried minced pork slices	2% of mulberry juice	Cheng et al. (2019)
Grape marc powder	Fettuccini	0, 25, 50, and 75 g/kg	Nakov et al. (2020)
Grape pomace	Yoghurt	1, 3, and 5% of grape pomace	Demirkol and Tarakci (2018)
Elderberry juice concentrates	Pasta	Elderberry juice concentrates:water (1:1 v/v)	Sun-Waterhouse et al. (2013)
Grape juice, skin, and seed extract	Petit Suisse cheese		Pasini Deolindo et al. (2019)
Buckwheat flour and bran	Durum spaghetti	Between 10 and 40 g/100 g of buckwheat flour and bran	Pasini Deolindo et al. (2019)
Grape pomace extracts	Fermented milk	Different volumes of aqueous grape extracts	de Souza de Azevedo et al. (2018)
Coffee pulp	Probiotic beverage using kefir		Patil et al. (2022)

beverages rich in polyphenols have also been suggested by Rodríguez et al. (Rodríguez et al. 2020) who studied the polyphenols composition of two beverages obtained from *Prosopis alba* pods (namely, Aloja and añapa), Samaniego-Sánchez et al. (Samaniego-Sánchez et al. 2020) who developed a new fermented beverage from *Saccharum officinarum* L. molasses, and Voss et al. (Voss et al. 2021) who proposed the development of a novel symbiotic beverage produced by okara (by-product of soy-bean processing) hydrolyzed by cardoon enzymes and fermented by the prebiotic bacteria *Lactobacillus rhamnosus* R11 and *Bifidobacterium animalis* ssp. *lactis* Bb12. Moreover, instead of extracts the use of individual or mixtures of pure compounds showed promising results, as for example in the case of green tea flavanols epigallocatechin gallate, epigallocatechin, and epicatechin gallate which were incorporated in a catechin-free model beverage (Xu et al. 2021b).

The incorporation of polyphenols has also found applications in baked products, e.g. breads, cookies, muffins, doughnuts, and other products which are consumed more or less on a daily basis. The most common source of polyphenols are fruit and vegetables and their processing by-products which are also a good and underutilized source of bioactive compounds and nutrients. The application methods include the incorporation of raw or processed fruit and vegetable directly in the baked products or the addition of purified polyphenols (Ou 2021). Several examples of practical applications are reported (see also Table 23.2), such as the incorporation of fruit and vegetable pomace obtained from grapes in fortified breads, cookies , muffins (Hayta

Table 23.2 Examples of incorporation of fruit and vegetable by-products in baked products

Source	Bakery product	Composition	Reference
Red and white grapes pomace	Fortified breads, cookies, muffins	5, 10, and 15% of pomace for bread; 10, 15, 20, and 25% of red grapes pomace for brownies; 5, 10, and 15% red grapes pomace or 10, 15, and 20% of white grapes pomace for muffins	Walker et al. (2014)
Grape pomace powder	Bread	2, 5, and 10% (w/w, flour basis) of grape pomace powder	Hayta et al. (2014)
Pomace of various fruit berries	Muffins	Strawberry, black currant, raspberry and sour cherry pomace (50 g of pomace per kg of muffins)	Górnaś et al. (2016)
Tomato pomace	Flat bread	0, 1, 3, 5, and 7% of pomace (w/w flour basis)	Majzoobi et al. (2011)
Raspberry juice	Muffins		Rosales-Soto et al. (2012)
Peach puree	Cookies	10.5% of peach pulp	Blanco Canalis et al. (2020)
Bergamot and orange juice	Cakes	Bergamot juice substituted coconut milk by 25%, 50%, and 75% (w/w); 200 mL of orange juice added in cake mixture	Silva et al. (2006)
Lime and sea-buckthorn pulp	Bread	0, 10.2, or 20.4 g of lime juice per 100 g of flour; 5% or 10% of sea-buckthorn pulp on a flour basis	Guo et al. (2019; Scarton et al. (2021)
Grape seed micropowder	Bread	1% of grape seed micropowder	Valková et al. (2021)
Mango-processing by-product	Muffins	50 and 75% of mango-processing by-product	Ramírez-Maganda et al. (2015)
Grape pomace powder	Cakes	4, 6, 8, and 10% grape pomace powder	Nakov et al. (2020)
Grape marc extract	Biscuits	225 mL of grape marc extract	Pasqualone et al. (2014)
Goji berry by-product	Muffins and cookies	0, 10, 20, 30, and 40 g of goji berry by-product per 100 g for muffins and cookies	Bora et al. (2019)
Carob flour	Cookies	0 and 50% of carob flour	Babiker et al. (2020)
Pomegranate peels	Cookies	5 g of pomegranate peels extracts	Colantuono et al. (2016)
Mango, potato, and onion peels powder	Biscuits	6% of wheat flour substituted with mango, potato, and onion peels powder	Hallabo et al. (2018)
Carrot powder	Tortillas	10% w/w dry weight of carrot powder	Santana-Gálvez et al. (2016)
Spent coffee grounds	Cookies	5 g of spent coffee grounds	Castaldo et al. (2021)

et al. 2014; Walker et al. 2014); the enrichment of muffins with pomace of various fruit berries (Górnaś et al. 2016) or the addition of tomato pomace in flat bread (Majzoobi et al. 2011). Apart from the rich polyphenols content, the high fiber content of pomace may be capable of partially substituting fat in bakery products and design functional foods with low fat and calories content (Sudha et al. 2015; Quiles et al. 2018). However, due to great differences in polyphenols profile of the various pomaces, further studies are needed to evaluate the effects of incorporation on the sensorial quality of the final baked products (Majerska et al. 2019).

Fruit and vegetable juices, pulps, and jams can also be directly incorporated in baked products, helping to improve the nutritional value and the health beneficial effects while at the same time reduce the fat and energy content of the final products (Ou 2021). The various examples include the use of raspberry juice in muffins (Rosales-Soto et al. 2012); peach pulp in cookies (Blanco Canalis et al. 2020); bergamot and orange juice in cakes (Silva et al. 2006; Lertnirundon and Mahidsanan 2020); or lime and sea-buckthorn pulp in breads (Guo et al. 2019; Scarton et al. 2021). Moreover, the application of dried plant tissues (fruit, leaves), vegetable powders and fruit purees and jams as fillings has been tested as an alternative option to enrich baked products in bioactive compounds and particularly in polyphenols, while at the same time reducing the post-harvest losses of perishable fruit and vegetables. For this purpose, dried fruit and powders from several species have been incorporated in various baked products (e.g., beans, fruit berries, melon, pumpkin, citrus fruit, etc., as recently reviewed by Betoret et al. (Betoret and Rosell 2020) and Salehi et al. (Salehi and Aghajanzadeh 2020).

There is also the option to incorporate individual polyphenols after purification and the most commonly applied compounds include various phenolic acids, e.g. caffeic acid, gallic acid, ferulic acid, chlorogenic acid, and rosmarinic acid (Mildner-Szkudlarz et al. 2019; González-Montemayor et al. 2021; Xu et al. 2021a); flavonoids (catechin, epigallocatechin gallate, naringenin, hesperidin, and quercetin (Goh et al. 2015; Fu et al. 2018; Lin and Zhou 2018; López et al. 2019; Mayneris-Perxachs et al. 2019), phenylethanoids (hydroxytyrosol) (Navarro and Morales 2017), or stilbenoids (resveratrol) (Ou 2021) (see also Table 23.3). The aim of this practice is to increase the content of targeted compounds with specified health benefits rather than improving the overall total phenolic compounds content (Ou 2021). Moreover, it is very common that compounds such as chlorogenic acid are used as food preservatives aiming to increase the shelf life of the final products due to its antimicrobial and antioxidant properties, the inhibition of lipid peroxidation, or to improve its prebiotic properties. Other functions of phenolic compounds related to their antimicrobial properties refer to the inhibition of quorum sensing which is the communication system of bacteria that regulates cell functions and the interactions with the host organism (Grandclément et al. 2015). The recent study of Santos et al. (Santos et al. 2021) evaluated the quorum sensing inhibition properties of various polyphenols such as curcumin, capsaicin, gallic acid, phloridizin, and resveratrol and suggested that curcumin and resveratrol showed quorum sensing inhibition activities against *Chromobacterium violaceum* and biofilm inhibition properties against *Aeromonas hydrophila*, *Salmonella enterica* serovar Montevideo,

Table 23.3 Examples of incorporation of phenolic compounds in food products

Compound	Source	Product	Composition	Reference
Caffeic acid	Legume flours: green bean, pea, and mesquite flours	Sourdough bread	20% of total flour	González-Montemayor et al. (2021)
Chlorogenic acid	Pure compound	Grape juice	1:1 of chlorogenic acid and β-cyclodextrine	Shao et al. (2014)
Chlorogenic acid	Pure compound	Blackberry juice		Kopjar et al. (2012)
Ferulic acid	Co-extruded wheat/okra composite	Bread	Native and extruded okra and wheat flour	Xu et al. (2021a)
(−)-epigallocatechin gallate	EGCG powders	White bread	3.3, 6.6, and 9.9 g/kg	Fu et al. (2018)
Epigallocatechin gallate, epigallocatechin, and epicatechin gallate	Flavanols added in chrysanthemum beverage	A catechin-free model beverage	Single compounds (800 μg/mL) or mixtures of two (400 μg/mL) or three compounds (266.67 μg/mL)	Xu et al. (2021b)
Green tea catechins	Green tea extract	Baked and steamed bread	0.45, 1, and 2%	Goh et al. (2015)
Quercetin	Pure compound	Bread	0.05, 0.1, and 0.2%	Lin and Zhou (2018)
(+)-catechin, quercetin, gallic, ferulic, caffeic acids	Pure compounds	Bread	0.1, 0.5, 1.0, and 2.0% (w/w)	Mildner-Szkudlarz et al. (2019)
Cyanidin-3-*O*-glucoside	Extract obtained from *Arbutus unedo* L. fruit	Wafers	5.50 g of extract	López et al. (2019)
Hesperidin and naringenin	Pure compounds	Biscuits	100 mg of hesperidin and 50 mg of naringenin	Mayneris-Perxachs et al. (2019)
Hydroxytyrosol	Olive tree leaves	Biscuits	0.25–0.75% (w/w)	Navarro and Morales (2017)
Naringenin, quercetin, epicatechin, chlorogenic acid, and rosmarinic acid	Pure compounds	Cookies	0.25% (w/w) of individual phenolic compounds	Zhang et al. (2014)
Resveratrol, epicatechin, and rosmarinic acid	Pure compounds	Cookies	0.02 and 0.2% of individual phenolic compounds	Ou et al. (2018)

and *Serratia marcescens*. Other examples include the incorporation of catechin and nisin in gelatin films used to wrap minced pork meat and extended its shelf life from 1 to 4 days (Kaewprachu et al. 2018); edible coating enriched with gallic acid and chitosan increased shelf life of pork stored at modified atmospheres (Fang et al. 2018).

23.3.2 Terpenes

Terpenes are a class of numerous compounds widely distributed in the plant kingdom (Masyita et al. 2022). Terpenes are commonly used in the cosmeceutical industry as fragrances; however, they have also found applications in the food industry as flavoring agents or as natural preservatives in the form of direct incorporation in the food products or through integration in film packaging (Maurya et al. 2021; Wang et al. 2022). Essential oils are usually obtained from medicinal plants such as *Salvia officinalis* L. (Cutillas et al. 2017), lemongrass (Gao et al. 2020), anise (Topuz et al. 2016), cinnamon (Yildirim et al. 2017), thyme (Abdollahzadeh et al. 2014; Gonçalves et al. 2017), basil (Beier et al. 2014), oregano (Hernández-González et al. 2017), clove (Devi et al. 2010), and several other species (Oussalah et al. 2007). The pure essential oils or their mixtures (oils from different species) have been applied as natural preservatives or as flavoring agents in various food products so far, including baked products (Gonçalves et al. 2017; Ju et al. 2018), fresh-cut vegetables and fruit (Kwon et al. 2017; Park et al. 2019), juices (Sarkar et al. 2017; Lee et al. 2020), meat products, and non-dairy products (Cui et al. 2015). However, despite the promising preservating properties of essential oils or pure terpenes and derivatives, there are several drawbacks that hinder their wide application in the food industry due to their high volatility and lability, the potential toxic effects, the low solubility to water, as well as their strong flavor and aroma that may alter the sensorial characteristics of the final product (Salvia-Trujillo et al. 2015).

In the case of direct incorporation, nanoemulsions of essential oils are recently suggested as an eco-friendly alternative to synthetic antimicrobial agents that improves the shelf life and safety of food products without affecting their flavor and taste (Hintz et al. 2015). For example, the co-encapsulation of essential oils obtained from *Pimpinella anisum* and *Coriandrum sativum* showed a great antifungal activity and very promising results as a natural preservative of food products (Das et al. 2022). Moreover, nanoemulsions can be used as ingredients in edible and biodegradable coatings aiming to protect the labile volatile compounds of essential oils from oxidization and allow their gradual release throughout the shelf life of stored products (Arrieta et al. 2013; Das et al. 2021). The direct incorporation of essential oils is also applied in the case of hops (*Humulus lupulus* L.) which are used in the brewery industry as a flavoring agent of beer products (Lamberti et al. 2021). In the study of Abdollahzadeh et al. (Abdollahzadeh et al. 2014), the application of thyme essential oils at 0.8 and 1.2% on cooked minced fish inhibited the growth of *Listeria monocytogenes* 6 days after storage, while the combined application of

thyme essential oils and nisin showed even better results by inhibiting bacterial growth 2 days after storage. Moreover, apart from antimicrobial effects the application of essential oils could improve the sensorial properties and the quality of the final product, as for example the improvement in quality traits of marinated chicken meat with the addition of a blend of thyme and orange essential oils (Rimini et al. 2014) or the increased organoleptic properties of ground meat after the combined application of Chinese cinnamon and cinnamon bark essential oils (Ghabraie et al. 2016). An integrated approach where essential oils are combined with other compounds such as antibiotics, organic acids, and plant extracts or processing methods (vacuum-packaging, irradiation, etc.) is also suggested for increasing the shelf life of sea-food and meat products (Pateiro et al. 2018; Huang et al. 2021). However, the application of essential oils in food products in various countries is permitted within specific amounts depending on the country and the food product, while the synergistic or antagonistic effects of essential oils with other compounds of the food product should also be considered (Pateiro et al. 2018) (Table 23.4).

Apart from the use of essential oils *per se*, the use of pure compounds also shows promising results in the food industry, mostly as flavoring and antimicrobial agents (Burt 2004). For example, (R)-limonene is a monoterpene with several applications in the food industry due to its antimicrobial properties (Ciriminna et al. 2014; Khelissa et al. 2021), while other terpenes include eugenol and isoeugenol (Zhang et al. 2017a), (+)-limonene (Hąc-Wydro et al. 2017; Zahi et al. 2017), thymol (Moon and Rhee 2016), 1–8 cineole (Simsek and Duman 2017), geraniol (Tomadoni et al. 2015; Yegin et al. 2015; Syed and Sarkar 2018), carvacrol (Ultee et al. 1998; Martínez-Hernández et al. 2017), citral (Gao et al. 2020), linalool (Liu et al. 2020), α-terpineol (Cosentino et al. 1999), and several other compounds (Table 23.5).

23.3.3 Alkaloids

Alkaloids are naturally found in various food products, the most common are the vegetables of the Solanaceae family, e.g. tomato, potato, pepper, etc., although their presence is usually associated with toxic and anti-nutritional effects. The use of alkaloids in the food industry is not widely applied compared to the pharmaceuticals sector where these compounds usually find applications due to their important medicinal properties. One common alkaloid which is widely used in energy and soft drinks due to its stimulating effects is caffeine, while quinine is also used in tonics as a flavoring agent (Kurek 2012). Coffee tree species (e.g. *Coffea arabica* and *C. canephora*) are a rich source of caffeine which can be detected in various plants tissues and coffee beans in particular. Coffee processing generates greats amounts of by-products which can be valorized for caffeine extractions and further application in the food industry via microencapsulation or addition in beverages, yogurt, bread, and other food products (Martinez-Saez et al. 2014; Aguiar et al. 2016; Bertolino et al. 2019; Guglielmetti et al. 2019). The recent review of Klingel

Table 23.4 Examples of incorporation of essential oils in food products

Source	Food product	Composition	Reference
Chicory essential oil	Beef slices	*Lepidium perfoliatum* seed mucilage (2 g) + Tween 80 (1 mL) + Chicory essential oil (0%, 0.5, 1.0, and 1.5% v/v)	Alizadeh Behbahani et al. (2021)
Lemon peels, clove buds, and juniper berries	Fermented cream	0.0, 12.5, 25.0, 50.0, and 100.0 μg of essential oil added in 100 mL of cream	Kozłowska et al. (2022)
Cinnamon essential oil	Vacuum-packaged common carp	0.1% food grade cinnamon essential oil	Zhang et al. (2017b)
Cinnamon and clove essential oil	Cakes	Diluted essential oils at 1.562–50,000 μL/mL	Ju et al. (2018)
Lippia alba essential oil	Fillets of silver catfish	30 and 40 μL/L of essential oil	Veeck et al. (2013)
Plantago major seed mucilage + dill oil	Edible coating of refrigerated beef	Seed mucilage + 0, 0.5, 1, and 1.5% of essential oil	Behbahani et al. (2017)
Basil-seed gum + oregano oil	Edible coating of fresh-cut apricots	Seed-gum + 0, 1, 2, 3, 4, 5, and 6% (v/v)	Hashemi et al. (2017)
Sodium alginate + galbanum gum + *Ziziphora persica* essential oil	Edible coating of chicken fillet	Sodium alginate + galbanum gum + 1.25 to 50 mg/mL of essential oil	Hamedi et al. (2017)
Plantago major seed mucilage + *Citrus limon* essential oil	Edible coating of buffalo meat	Seed mucilage + 0, 0.5, 1, 1.5, and 2% of essential oil	Noshad et al. (2021)
Orange oil	Salacca fruit	Orange oil at 0.02, 0.04, 0.06, 0.08, 0.12, 0.16% w/w	Phothisuwan et al. (2021)
Thyme essential oil + nisin	Minced fish meat	0.4, 0.8, and 1.2% of essential oil + 500 and 1000 international units (IU) of nisin	Abdollahzadeh et al. (2014)
Thyme oil	Bakery products	1 mg/mL of essential oil +2.5% DMSO[a] + 0.1% Tween 80	Gonçalves et al. (2017)
Thyme and oregano oil	Tomato juice	Thyme and oregano oil (at 1:2 and 2:1 ratios) were added to 9.9 mL of tomato juice	Lee et al. (2020)
Oregano essential oil	Film packaging of cherry tomatoes	1, 2, and 3% of essential oil	Kwon et al. (2017)
Oregano essential oil	Chicken pâté	5 g essential oil/100 g nanoemulsion	Moraes-Lovison et al. (2017)
Lemongrass essential oil	Wheat bread, carrot, and celery	62.5, 125, 250, and 500 μL/L	Valková et al. (2022)

(continued)

Table 23.4 (continued)

Source	Food product	Composition	Reference
Clove oil	Encapsulation in tofu	2.0, 3.0, 4.0, 5.0, and 6.0 mg/mL of clove oil	Cui et al. (2015)
Clove bud essential oil	Fresh-cut pak-choi	0.02% of essential oil	Park et al. (2019)

[a]*DMSO* dimethylsulphoxide

Table 23.5 Examples of incorporation of terpenes in food products

Compound	Product	Recipe	Reference
(+)-Limonene	Spraying on strawberries	0.2% limonene + 0.4% Tween®80	Vu et al. (2011)
D-Limonene	Dipping of blueberries	50 mM of limonene	Umagiliyage et al. (2017)
Limonene	Nanocoating of strawberries	50 mM of limonene	Dhital et al. (2018)
Thymol and nisin	Cantaloupe juice	2.0 g of thymol + nisin at 400 μg/mL	Sarkar et al. (2017)
Thymol-loaded nanofiber	Coated cheese	0.01% free thymol	Tatlisu et al. (2019)
Thymol	Apple cider and milk	0.3, 0.5, 0.75, and 1.0 g/L of thymol	Shah et al. (2012)
Geraniol and vanillin	Strawberry juice	0.2 to 2.4 μL/mL of geraniol + 0.2 to 4.0 mg/mL of vanillin	Tomadoni et al. (2015)
Geraniol and vanillin	Strawberry juice	0.04% (v/v) of geraniol and/or 0.18 g/100 mL of vanillin	Cassani et al. (2016)
Geraniol and carvacrol	Raw goat meat	Geraniol and carvacrol at ratios of 1:0, 2:1, 1:1, 1:2, and 0:1 (2.5% v/v)	Syed et al. (2020)
Carvacrol and thymol	Soy sauce	0.25, 0.5, or 1 mM of carvacrol and thymol	Moon and Rhee (2016)
Carvacrol	Fresh-cut carrots	0.5% of carvacrol	Martínez-Hernández et al. (2017)
Carvacrol	Cherry tomatoes, lychees, and table grapes	Halloysite nanotubes/carvacrol at a ratio of 1:2 w/w	Shemesh et al. (2016)
Carvacrol nanoemulsions	Shredded cabbage	3.5% (w/w) oil phase	Sow et al. (2017)
Carvacrol and methyl cinnamate	Edible films of strawberry fruit puree	Carvacrol and methyl cinnamate at 0.75% (w/w) of strawberry puree	Peretto et al. (2014)
Citral and eugenol	Coating of wheat bread	Eugenol and citral in a ratio of 1:1 in 5 mL	Ju et al. (2020)
Citral, linalool, and β-pinene	Citrus-based beverages	Different combinations of terpenes	Belletti et al. (2010)

et al. (2020) proposes several uses of coffee tree plant parts and coffee processing by-products in the EU food industry for the production of novel foods, although the authors suggest that the approval from authorities is needed before the legal marketing of such products. Other uses include the addition of alkaloid-rich plant tissues in various teas, infusions, and beverages, e.g. lotus seeds and leaves (Ding et al. 2017; Cheng et al. 2021). Mulberry leaves are a good example of an alkaloid-rich natural matrix which has been suggested for various food products, e.g. beverages, yogurts, teas, seasonings, and healthy foods (Ma et al. 2022). Considering the important pharmacological properties of these compounds, further research is needed aiming to design new functional foods and further valorize these valuable compounds. However, extensive trials are needed to eliminate toxicity effects and to establish the risk-free dosages for applications as food additives.

23.3.4 Glucosinolates

Glucosinolates are also a group of compounds with well evidenced positive health effects that are widely distributed in various plant species that are commonly consumed. The Brassicaceae family is the most common source of glucosinolates and several studies suggest the use of *Brassica* species crops by-products in food products in various forms. For example, Dominguez-Perles et al. (Dominguez-Perles et al. 2011) suggested the application of minimally processed broccoli by-products in organic green tea for the production of a novel functional beverage, while other authors proposed the use of by-products from the same species in gluten-free sponge cakes (Drabińska et al. 2018), in fortified tortilla chips (Vazquez-Duran et al. 2014), bread (Zambelli et al. 2017), etc. Apart from by-products, there is also great potential in using the raw materials *per se* for the design of novel functional foods and according to Oliviero et al. (Oliviero et al. 2013) three different drying approaches of broccoli were suggested aiming at final products with diverse composition in glucosinolates and by-products. Moreover, there is high interest in using extracts or essential oils obtained from *Brassica* species as antimicrobial and food preservatives agents in various food products or in film packaging (Pereira et al. 2015; Salehi et al. 2021), while the hydrolysis of glucosinolates produces compounds with prominent antimicrobial activities (Sanchez Maldonado et al. 2015). Selected examples of applications of *Brassica* by-products in the food industry are listed in Table 23.6.

Moringa oleifera is another interesting species with great potential in designing new food formulations, since many of its health beneficial effects are associated to the presence of glucosinolates (Giuberti et al. 2021). There are already commercially available Moringa-based products in various categories, e.g. protein shakes, porridge, pasta, bread, juice, and chocolate (Teclegeorgish et al. 2021). Another example is the incorporation of powders of *Moringa oleifera* leaves in a maize grain non-alcoholic beverage called mahewu (Olusanya et al. 2020) or in snacks (Zungu et al. 2020).

Table 23.6 Examples of incorporation of glucosinolate-rich by-products or pure compounds in food products

Source	Food product	Composition	Reference
Broccoli flour	Tortilla chips	2, 4, and 8% of broccoli flour	Vazquez-Duran et al. (2014)
Broccoli leaf powder	Gluten-free sponge cakes	0, 0.9, 1.8, and 2.7% of broccoli leaf powder	Drabińska et al. (2018)
Leaves and stems powder	Functional tea beverage	0, 33, 50, 67, and 100% of broccoli concentrates	Dominguez-Perles et al. (2011)
Broccoli and carrot juice by-products	Bread	50%/50% (w/w) blend of by-products powder	Zambelli et al. (2017)
Oriental mustard flour or allyl isothiocyanate	Pizza crust	170, 425, or 850 mg of oriental mustard flour	Quiles et al. (2015)
Deodorized oriental mustard extract or allyl isothiocyanate	Chicken breasts	250 mg/g of oriental mustard extract or 50 μL/g allyl isothiocyanate	Olaimat and Holley (2015)
Deodorized oriental mustard extract or allyl isothiocyanate	Cooked cured chicken breasts	100–250 mg/g oriental mustard extract or 25–50 μL/g allyl isothiocyanate	Olaimat and Holley (2016)
Allyl isothiocyanate	Refrigerated ground beef	0.7 mL of allyl isothiocyanate added in 0.3 mL of corn oil	Muthukumarasamy et al. (2003)
Allyl isothiocyanate	Pears	5 mg of allyl isothiocyanate per L of air	Mari et al. (2002)
Ground mustard seeds	Antimicrobial packaging	Different particle sizes of ground seeds	Bahmid et al. (2020)
Borage and Indian mustard aqueous extracts	Various food models	200 mg/mL for borage and 500 mg/mL for Indian mustard	Miceli et al. (2014)
Essential oils of clove and/or mustard	Strawberry fruit	0, 7.71, 15.42, 30.85, 61.71, and 92.56 μL of essential oil per L of air	Aguilar-González et al. (2015)
Essential oil of black mustard	Bread-type product	0.0, 11.7, 17.6, 23.5, 29.4, 41.1, 47.0, 53.0, or 58.8 mL of essential oil per liter of air	Mejía-Garibay et al. (2015)
Essential oil of mustard	Cooked and acidified chicken meat	0.1% w/w of essential oil	Lemay et al. (2002)
Essential oil of mustard	Film packaging of bread	1, 10, or 100 μL of essential oil	Nielsen and Rios (2000)
Distillates from horseradish roots	Pre-cooked roast beef	0, 2000, 4000, and 20,000 nL of horseradish distillate per L of air	Ward et al. (1998)
White cabbage, cauliflower, and Brussels sprouts extracts	Avocado pulp	10 g of lyophilized extracts per 100 g of avocado pulp	Bustos et al. (2015)
Kale	Fermented kale juice	Fermentation with *Lactobacillus* strains	Oguntoyinbo et al. (2016); Kim (2017)

(continued)

Table 23.6 (continued)

Source	Food product	Composition	Reference
Pak-choi and kale leaves and microgreens	Wheat bread	10.7% freshly harvested pak-choi and kale	Klopsch et al. (2019)
Curly kale leaves	Apple juice	13% of frozen or 3% of freeze-dried kale leaves	Biegańska-Marecik et al. (2017)

23.4 Conclusions and Future Remarks

The use of plant-based extracts rich in secondary metabolites is a very promising and challenging task to be addressed by the food industry. Despite the successful paradigms of industrial applications of such extracts, further research and efforts are needed to overcome the hindrances related to: (a) bioavailability issues, since secondary metabolites such as polyphenols may interact with other components in food products (e.g., carbohydrates, proteins, lipids, etc.) and alter the overall nutritional value of the final product, (b) biostability issues, since the increased content of plant-based extracts in targeted secondary metabolites does not ensure the stability of these compounds in the final product, (c) the standardization of extraction protocols due to the complexity in the composition of natural matrices and the use of "green" and non-toxic solvents, (d) the effectiveness of extractability due to fluctuations in the content of secondary metabolites in plant matrices due to crop production, post-harvesting, and processing effects, (e) toxicity issues, since plant matrices may contain toxic substances therefore attention is needed prior to integration in industrial scale, (f) the cost-effectiveness of the process which should be addressed to make the final products affordable, (g) the effects of the addition of extracts on the sensorial quality of the final product, (h) the use of raw material instead of processed and purified ones. Addressing these issues would broaden the use of secondary metabolites in the food industry making the design of new functional products easier and cost effective, while increasing the added value of crops and minimizing the environmental burden. Moreover, the affordability of these newly developed products is of high importance to increase the market penetration and the adoption of consumers with direct impacts on the whole food value chain and the well-being of the general public.

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Chapter 24
Applications of Fungi Secondary Metabolites in the Food Industry

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Abstract The increasing consumers' awareness about natural vs. synthetic food additives/ingredients has challenged the food sector to make microbial fermentation economically feasible and scalable. Several fungal volatile compounds with a wide range of fruity, floral, musty, fatty, malty, almond-like, mushroom-like, and alliaceous-like flavors can be produced by fermentation, and incorporated into processed foods such as breads, cheeses, cured meats, soy products, and beverages, besides inhibiting phytopathogens and spoilage organisms that decrease foods and crops shelf life. Fungal pigments that can replace synthetic food dyes are sustainable and independent of seasonal changes, but stability is still a challenge, while essential fatty acids with oxidative stability can be produced in high yields by oleaginous fungi. Moreover, bioactive fungal metabolites may contribute to the development of functional foods and supplements with remarkable anti-inflammatory, antitumor, antiviral, among other activities. Using bio-wastes as substrates for fungal fermentation allows cost-reduction, which associated with "green" extraction processes reinforces the sustainability of fungal metabolites. Modern techniques such as genetic engineering, CRISPR/Cas9, chemical modulation, mutagenesis, and co-cultivation can be harnessed for optimization and diversification of secondary metabolites production, as well as spectroscopical and chromatographic tools can be used for screening and characterizing new molecules useful for food purposes in complex samples.

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24.1 Introduction

The global market for food additives reached 50.6 billion dollars in 2020. Even with the uncertainties brought with the COVID-19 pandemic, this market is expected to grow 5.6% by 2026 (Imarc 2020). The North American market represents 30% of the entire processed food market (Mordor Intelligence 2021a) and, in this niche, an increase of 5.5% in the sale of additives is expected until 2025 (Mordor Intelligence 2021b). In Europe, 53.8% of food products contain at least one additive, while 11.3% contain at least five, as ultra-processed foods usually contain a mixture of substances. Around 330 compounds are authorized by the EU for use in this type of food, while only 48 substances are allowed in organic foods.

Some ingredients and additives used in the food industry as thickening, binding or gelling agents, flavor enhancers, acidulants, flavor compounds, vitamins, essential amino acids, and polyunsaturated fatty acids can be produced by well-described microbial fermentation. However, synthetic compounds are still the industry's first choice, since their production is more economically viable in face of the growing demand for food additives. Even though the substances used in the food industry are widely studied, the cumulative effect of using more than one additive is usually unknown, and there are scarce reports related to some of them in the literature. Nonetheless, some broadly used additives, such as nitrates/nitrites, carrageenans, glutamate, bixin, artificial sweeteners, phosphates, emulsifiers, caramel, titanium dioxide, tartrazine, and butylated hydroxyanisole/butylated hydroxytoluene have been linked to metabolic disorders, gut microbiota or endocrine perturbations along with carcinogenic, inflammatory, and/or oxidative stress effects (Chazelas et al. 2020).

Natural food additives overcome the technological function of delaying oxidative reactions, improving color and texture, and inhibiting microbial growth, as they also provide health benefits. Natural ingredients are generally considered as a safer option by consumers, since the perception that "natural is good" is widely accepted, although natural compounds must also be studied in terms of toxicity. It has been reported that most educated consumers tend to prefer foods with natural over synthetic ingredients (Munekata et al. 2020). Synthetic compounds are not compulsorily more toxic than their natural counterparts, but those regarded as artificial are often refused by consumers, and these perceptions usually turn into demand. Individuals aged over 65 years also tend to choose healthier products, pay more attention to the label, and appreciate foods with additional functional ingredients. Since life expectancy is rising in most countries, the food industry is searching for innovation to meet the requirements of this age group (Oeser et al. 2018). In addition, regardless of age and educational level, sensory evaluation studies show that consumers have a smaller "willingness to accept" foods with artificial additives (Zhong et al. 2018). With the growing knowledge about the benefits of natural products for health improvement, natural colorants such as carotene, turmeric, and anthocyanins are now gaining increasing attention. In this scenario, there is a current demand for functional foods and ingredients of natural origin to supply the food industry

(Santeramo et al. 2018). This is a latent claim, which means that the industry is aware of the technology and innovation demands but has been slow in offering a clear solution for the issue (Muscio et al. 2010).

Several natural pigments, such as β-carotene, lycopene, lutein, astaxanthin, and other carotenoids used as food additives have been produced primordially by microalgae and plants. However, plant metabolites yields are usually determined by seasonal variations. Although microalgae can be cultivated all over the year without seasonal limitations, the process becomes expensive in countries where sunlight needs to be replaced by artificial lighting in order to keep stable production. Alternatively, the production of a wide range of food additives by fungi is well-documented, although microbial production requires expensive conversion in technology, infrastructure and must meet regulatory requirements (De Mejia et al. 2019; Dufossé 2018). Moreover, finding the best suited natural additives can pose specific challenges, for example stability, since natural pigments are usually unstable at neutral and high pH values, while synthetic dyes tend to be very stable in a wide pH range (De Mejia et al. 2019).

Furthermore, beyond the requirements of good hygienic practices along the food chain necessary to ensure food safety, microbiological and chemical safety are also mandatory. The latter means that food should not contain non-food grade additives and/or contaminants (Fung et al. 2018). Other issues must be taken into consideration, among which the most cited is the production of mycotoxins like citrinin, during fungal fermentation. Mycotoxin production not only poses a threat to food safety, but fungal issues may represent 30% of crop loss through diseases and spoilage processes (Avery et al. 2019). There are reports of minor eyes, nose and throat irritation after exposure to 10 mg/m^3 of volatilized fungal metabolite 1-octen-3-ol for 2 h. In some circumstances, low concentrations of this compound can be neurotoxic and cytotoxic (Bojke et al. 2018). Therefore, even natural aromas must be fully studied with respect to safety aiming to determine their suitability for use as food additives.

Although there are challenges, natural products from fungi with different aromas, colors, physicochemical characteristics, and functional properties are conquering consumers and industry. The awareness of consumers about the benefits and the current international scenario is pressuring the food industry sector for more research primordially focusing the development of economically viable supplies of natural products applicable to the food industry.

Some fungi are already used to convert multiple carbon sources into organic acids with high yield, providing industrial production of organic itaconic, lactic, fumaric, malic, tartaric, succinic, oxalic, and citric acids. The most versatile of them in terms of food applications is the citric acid. More than 95% of global production of citric acid is obtained by fermentation of glucose or sucrose by *Aspergillus niger*, although other species of filamentous fungi such as *Aspergillus wentii* and *Penicillium citrinum* also produce this acid (Copetti 2019). About 70% of the produced citric acid is used as additive in the food industry as acidulant, preservative, pH regulator, flavor enhancer, chelating agent, stabilizer, antioxidant, and also acts as a preservative, delaying the growth of spoilage organisms (Sahasrabudhe and Sankpal 2001).

24.2 Fungi Historically Employed in Food Manufacture

Metabolites produced by higher fungi have long been used as providers of flavor, color, and texture to several traditional foods, as exemplified by some *Aspergillus*, *Monascus*, and *Penicillium* species. *Aspergillus oryzae*, a filamentous fungus recognized as safe by the Food and Drug Administration (FDA), has been used in China for over 3000 years, for manufacture of soy sauce and other soy fermented products, in association with microorganisms such as *Saccharomyces* spp. and *Lactobacillus* spp. (Zhao et al. 2020). Volatile flavor compounds including aldehydes, alcohols, esters, pyrazines, and furans are formed during fermentation of soybeans, mainly due to enzymes produced by *A. oryzae*. For example, glycoside hydrolases, amylases, and peptidases release glucose and amino acids, while copper amine oxidases catalyze the oxidation of primary amines to aldehydes, then converting into flavor aldehydes, including phenylacetaldehyde (**1**), with almond/caramel flavor and isovaleraldehyde (**2**) with fruity/malty/cocoa flavors. In addition, alcohol dehydrogenase and aldehyde dehydrogenase from *A. oryzae* produce ethanol, isobutanol, among other alcohols, that add slight rose/sweet ethanol aroma and reduce bacterial contamination of soy sauce (Zhao et al. 2020). *A. oryzae* is also the dominant fermenting mold in the rice fermentation process for sake production, where its main role is to produce enzymes, especially α-amylases, that decompose rice starch into glucose, providing a suitable substrate for subsequent *Saccharomyces cerevisiae* fermentation (Zhang et al. 2020) (Fig. 24.1).

The major secondary metabolite produced by *A. oryzae* is kojic acid (**3**) (5-hydroxy-2-(hydroxymethyl)-4-pyrone) (Dao et al. 2021). Kojic acid (**3**) is used in the food industry as a precursor of the flavor enhancers maltol and ethyl maltol, antioxidative agents used in fruits and vegetables, color preservative for seafoods and meats, and in manufacture of composite films for development of antimicrobial packages (Goldberg and Rokem 2009; Liu et al. 2020a). Besides kojic acid (**3**), L-malate produced by *A. oryzae* can be converted into malic acid, an acidulant and flavor enhancer additive used in foods and beverages, including candies, soft drinks, and jellies (Daba et al. 2021; Kövilein et al. 2020). *A. oryzae* freeze-dried powder has been studied as a probiotic and functional feed for horses and poultry, due to the presence of bioactive secondary metabolites, such as aspirochlorin (**4**) (antimicrobial and antifungal), asperfuran (**5**) (antifungal and anticancer), tocopherol (**6**)

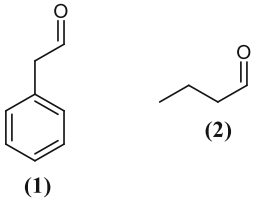

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Fig. 24.1 Flavor aldehydes produced by *A. oryzae*

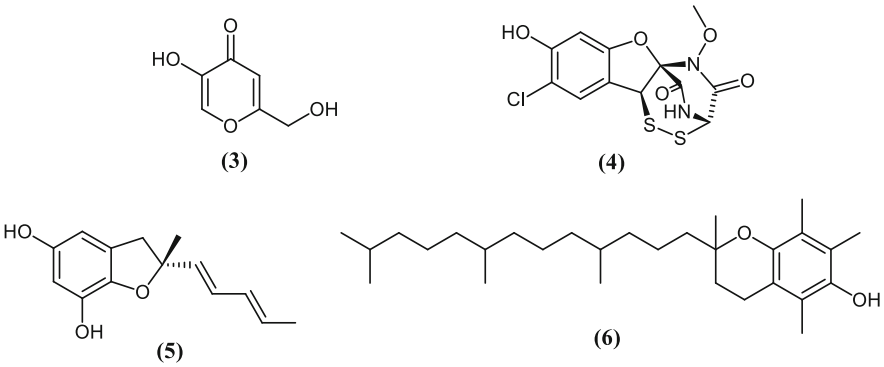


Fig. 24.2 Bioactive secondary metabolites produced by *A. oryzae*

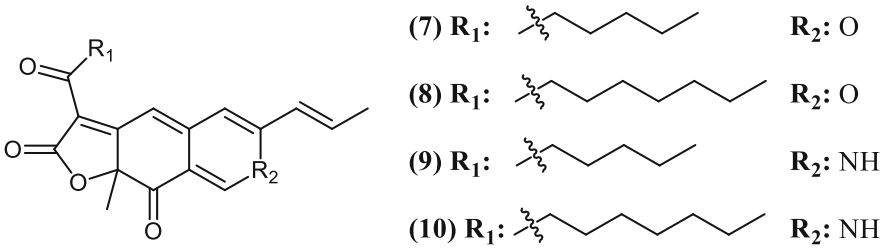


Fig. 24.3 Polyketide pigments produced by *M. purpureus*

(antioxidant), and kojic acid (**3**) (melanogenesis inhibition, antimicrobial, insecticide, pain killer, among others) (Daba et al. 2021) (Fig. 24.2).

Monascus purpureus is another useful filamentous fungus. It is popular in China, Japan, and other East-Asian countries since at least the ninth century, owing to its use for production of the long-established fermented red rice, also called red *koji*, and the soybean curds *tofunyu* and *tofuyo* (Fukami et al. 2021; Le Bloc'h et al. 2015). The natural red (rubropunctamine (**7**) and monascorubramine (**8**)) and orange polyketide pigments (rubropunctatin (**9**) and monascorubrin (**10**)) produced by *M. purpureus* and other species of *Monascus* have been employed as coloring agents in processed meats, seafoods, and beverages in Asian food industries since the first half of the twentieth century, as alternative to synthetic pigments (Fukami et al. 2021) (Fig. 24.3).

In addition to pigments, *M. purpureus* produces the cholesterol-lowering compound monacolin K, commercially known as the drug lovastatin, also produced by *Aspergillus terreus*. This metabolite inhibits the enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase, consequently avoiding formation of mevalonic acid, an important intermediate in cholesterol synthesis (Le Bloc'h et al. 2015; Mohankumari et al. 2021). This property led to approval by European Union regulations of fermented red rice as a hypolipidemic food supplement, as long as its consumption provides a daily intake of 10 mg of monacolin K. According to Le

Bloc'h et al. (2015), since the prescription drug lovastatin is available at a minimal dosage of 10 mg, red fermented rice containing higher quantity of monacolin K would have pharmacological action, and therefore should be classified as a medicinal drug (Le Bloc'h et al. 2015).

Other remarkable examples of foods made with higher fungi and their metabolites are the widely appreciated moldy cheeses. The earliest records of the well-known French blue-veined cheeses Roquefort and Gorgonzola date from the ninth and eleventh centuries, although other mold-ripened cheeses may have existed 4000 years ago (Dogan and Tekiner 2021; Fox et al. 2017). *Penicillium roqueforti* is inoculated in blue cheeses as a secondary starter, growing inside the cavities formed by gas-releasing *Leuconostoc* bacteria, thus producing melanin-rich spores responsible for the characteristic blue/green veins (Caron et al. 2021). Proteases (aspartyl protease, metalloprotease, alkaline metalloaminopeptidase, aminopeptidase, alkaline and acid carboxypeptidases) and lipases (acidic and alkaline) produced by *P. roqueforti* have a central role in the ripening process of blue cheeses, leading to the formation of flavor compounds (Cerning et al. 1987; Coton et al. 2020). Hydrolysis of α_s-casein, β-casein, and triglycerides generates peptides and fatty acids, further converted into methyl ketones, alcohols, acids, esters, lactones, and aldehydes, which give the typical texture and aroma of blue cheeses, for example the fruity notes and piquant flavor of Roquefort cheese (Caron et al. 2021; Martín and Coton 2017; Xia et al. 2020). Variations on volatile and non-volatile compounds concentration may occur depending on strain, type of milk, and time of ripening (Martín and Coton 2017).

Similar to blue cheeses, the maturation process of soft white cheeses like Camembert and Brie relies on proteolytic and lipolytic enzymes produced by bacteria, yeasts, and molds, where methyl ketones, notably nonan-2-one and heptan-2-one, are the most abundant volatile flavor compounds, giving fruity, floral, musty, and blue cheese notes (Spinnler 2017). However, *Penicillium camemberti*, the main mold in this type of cheese, produces only an extracellular alkaline lipase and, unlike *P. roqueforti*, grows on the surface of the cheeses, since it is less tolerant to low levels of oxygen, catabolizing the lactic acid produced by the primary starter and producing CO_2, thus accelerating the ripening process (Cerning et al. 1987; Spinnler 2017).

In the beginning of the eighteenth century, fungal fermentation of dry cured sausage and salami was already reported in Italy. Although variations between geographical regions may occur, species of the genera *Aspergillus*, *Eurotium*, and *Penicillium* present in the processing plants and cure rooms are often isolated from artisanal sausages, salami, and hams, while *Penicillium nalgiovense* and *Penicillium chrysogenum* are the main starter cultures added to cured meats in large-scale production with the aim of improving sensorial characteristics, whitish appearance, and prevention of pathogenic, toxic, or spoilage microorganisms (Perrone et al. 2015). As in cheeses, complex biochemical reactions are involved in flavor development of dry cured meats where selected molds contribute to amino acid degradation and lipid oxidation, thus leading to formation of many volatile compounds, such

as alkyl-branched aldehydes with malty aroma, unsaturated ketones with animal fat odor, among other classes of compounds (Flores 2018).

24.3 Fungal Secondary Metabolites in the Food Industry

24.3.1 Volatile Natural Products

Aroma and flavor are commonly added to foods in order to improve or intensify taste, which is a fundamental sensory characteristic for food acceptability. A large number of aromas are provided by volatile organic compounds (VOCs), which are small molecules with low vapor pressure that therefore easily evaporate at room temperature. All fungi produce blends of VOCs that may contain mixtures of alcohols, aldehydes, acids, ethers, esters, ketones, terpenes, thiols, and their derivatives. The volatile pattern produced by a fungus in laboratory vary between species and depends on intrinsic and extrinsic factors like medium composition, temperature, and age of the culture (Bennett and Inamdar 2015). The term "volatome" or "volatilome" is used to describe the volatile profile of an organism (Inamdar et al. 2020).

More than a hundred VOCs responsible for aroma are currently present on the market and are classified as natural if they are taken from natural sources such as microorganisms (Bojke et al. 2018). Fungal VOCs contribute to the taste and smell of fermented food like bread, cheeses, soy products, and alcoholic and nonalcoholic beverages, like wine, beer, and coffee (Kües et al. 2018). Volatile metabolites produced by fungi are much less studied than those produced by bacteria. The fungal VOCs known so far have been used for their flavor properties as indirect indicators of fungal growth in agriculture, or due to their role as semiochemicals for insects (Hung et al. 2015). VOCs produced by endophytic fungi in plants are essential for interspecies signaling, including other organisms present above and below the ground (Siddiquee 2017).

The classification of fungal VOCs as secondary metabolites is not a consensus. Some authors point that VOCs produced by fungi are not restricted to a particular species or genus, as for 1-octen-3-ol, produced by a wide number of fungal species and also found in plants and animals (Bennett et al. 2012). It is also pointed that only a few VOCs, if any, are exclusive of fungal metabolism (Inamdar et al. 2020). Secondly, some VOCs are not directly produced by the fungal metabolism. Many of them are either products of metabolic transformation of lipids, proteins, heterocyclic metabolites, and other components, or consist in degradation waste products of fungal catabolic pathways (Bennett et al. 2012).

There are a few ways to categorize volatile metabolites produced by fungi. A common method is by the carbon chain size, with VOCs classified as C-6, C-8, and so on. Functional group classification is also used, as fungi can produce plain hydrocarbons, heterocyclic compounds, thiols, alcohols, phenols, acids, and isoprenoids and their respective derivatives. Chirality must always be considered,

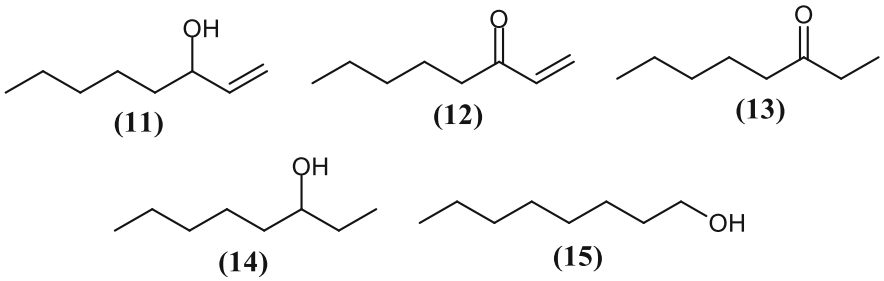


Fig. 24.4 Most commonly found fungal C-8 oxylipins

as it directly influences the perception of the molecules' aroma. The volatile metabolite most commonly found in the kingdom Fungi, 1-octen-3-ol, has its characteristic mushroom aroma only in the *R* form, while the *S* form has a grassier and moldier aroma (Inamdar et al. 2020). Some authors divide mushrooms into three groups, according to the profile of VOCs emitted by the organism: those producing C-8 oxylipins, those rich in terpenoids, and, finally, those secreting sulfur-containing volatiles (Fraatz and Zorn 2010).

Eight carbon oxylipins are present in almost all species of fungi and are considered the characteristic aroma substances of this group. The main examples are 1-octen-3-ol (**11**), 1-octen-3-one (**12**), 3-octanone (**13**), 3-octanol (**14**), and octanol (**15**) (Fig. 24.4) (Combet et al. 2006). The metabolites **11**, **13,** and **14** represent 70% of all VOCs produced by the genera *Aspergillus*, *Penicillium*, *Fusarium*, and *Alternaria* (Herrera et al. 2015). The most reported VOC produced by fungi, 1-octen-3-ol (**11**), is also called "mushroom alcohol" or "matsutake alcohol" (Bennett et al. 2012). Oxylipins are derived from lipid oxidation and include fatty acid hydroperoxides, hydroxyl fatty acids, epoxy fatty acids, keto fatty acids, volatile aldehydes, and cyclic compounds. Usually, some oxylipins are associated with off-odors in many types of foods (Bennett and Inamdar 2015). Volatile and non-volatile oxylipins also function as hormones and regulate fungal growth, morphological differentiation, and secondary metabolite production (Holighaus and Rohlfs 2019). For example, 1-octen-3-ol (**11**) is a growth hormone for several fungi, and a breakdown product of linoleic acid (Inamdar et al. 2020). Regarding fungi producing oxylipins, many examples can be found in the literature, as the production of **11**, **15**, hexanol, and benzaldehyde by *Metarhizium anisopliae*, along with other species (*Batkoa* sp., *Isaria fumosorosea*, and *Hirsutella danubiensis*) (Bojke et al. 2018). The production of the aforementioned metabolites 1-octen-3-ol (**11**) and benzaldehyde, along with 3-methyl-1-butanol is reported in three species of endophytic fungi of *Nodulisporium* genus (Schoen et al. 2017).

There are also volatile metabolites of terpene class. As an example, the fungus *Hypoxylon anthochroum* produces a blend of VOCs with high activity in inhibiting the growth of the phytopathogen *Fusarium oxysporum* in cherry tomatoes. The main compound produced by the fungus and associated with this activity is the monoterpene eucalyptol, or 1,8-cineole (Macías-Rubalcava et al. 2018), a metabolite also

produced by fungi of *Nodulisporium* genus (Schoen et al. 2017). Sesquiterpenes produced by fungi can contain different types of carbon skeletons, the most common being those derived from farnesol-pyrophosphate that can generate several different molecular configurations according to the action of different cyclases (Kramer and Abraham 2012).

Sulfur-containing VOCs are widely found in plants, especially from the genus *Allium* (chive, garlic, onion, etc.) and they are responsible not only for the characteristic flavor, but also for the health benefits of this group. These so-called alliaceous compounds are also an important part of the odor profile of truffles, mushrooms like *shiitake* (*Lentinula edodes*), and other fungi from the genera *Tricholoma* and *Marasmius* (Bennett et al. 2012). Some thiols produced by *shiitake* mushroom are similar to those found in garlic and onions. Since most VOCs produced by fungi are degradation products, it is suggested that volatile thiols are derived from degradation of amino acids (Inamdar et al. 2020).

24.3.1.1 VOCs in Industrial Food Products

The presence and size of blue areas in Roquefort cheese is associated with the fungus *P. roqueforti*. The microorganism population is directly linked to the efficiency of cheese proteolysis and lipolysis, which increases the production of VOCs. Among the main compounds produced by the fungus, there are casein degradation products, 3-methyl-butanal (**16**), 3-methyl-butanol (**17**), isopropyl-alcohol (**18**), and 3-methylbutanoic acid (**19**), which are responsible for fruity (banana), cheesy, and alcoholic aroma. In addition to these, several compounds derived from the degradation of lipids are formed, the main ones being the methyl ketones 2-pentanone (**20**) and 2-heptanone (**21**), the latter being responsible for the characteristic aroma of blue cheese, the alcohol 2-heptanol (**22**), responsible for the fruity aroma, as well as other compounds of these classes, in addition to various acids and esters (Fig. 24.5) (Caron et al. 2021).

Fermentation of Chinese black tea leaves (*Camellia sinensis*) with *A. niger* resulted in increased concentration of the metabolites geraniol (**23**), linalool oxide (**24**), 9,12-octadecadienoic acid (**25**), and β-ionone (**26**). Geraniol (**23**) is known as the characteristic aroma of rose and geranium, and it is considered the typical aroma compound of green tea as well. On the other side, the fermentation of the same black tea using *Eurotium cristatum* led to appearance of the metabolites cis-jasmone (**27**), α-terpineol (**28**), β-ionone (**26**), nonanal (**29**), and 2-pentylfuran (**30**). It is known that these compounds are fundamental to the flavor profile of tea, since cis-jamone (**27**) is related to floral aroma and 2-pentylfuran (**30**) is related to sweet and burnt odor (Fig. 24.5) (Cao et al. 2018). Therefore, the aforementioned species have potential to produce specific aromas demanded by the food industry.

Coffee aroma can be modified and improved using fungi. Bean fermentation significantly alters the volatile profile, with emphasis on the increase of pyrazine molecules such as trimethylpyrazine (**31**) and 2-ethyl-3,5-dimethylpyrazine (**32**) in coffee fermented with *A. oryzae*. The increase of molecules with furan nucleus,

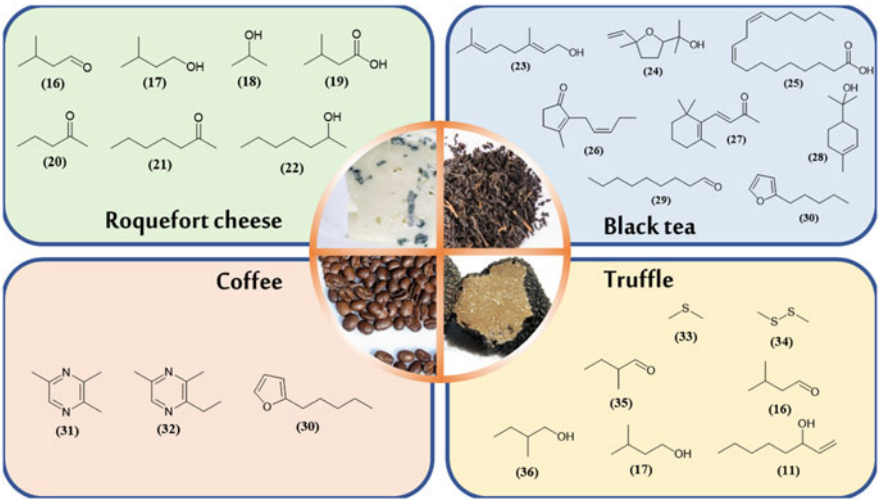


Fig. 24.5 Main volatile compounds of Roquefort cheese, black tea, coffee, and truffle

mainly 2-pentylfuran (**30**), is reported in grains fermented by *Mucor plumbeus* (Fig. 24.5). Pyrazine and furan metabolites are degradation products of coffee beans macromolecules and give the beverage its typical aroma (Tang et al. 2021). *Penicillium brevicompactum*, *Aspergillus luchuensis*, and *Penicillium* sp. naturally found in coffee were also screened and, despite producing VOCs, mainly oct-1-en-3-ol (**11**), these substances did not negatively interfere in the sensory quality of the beverage. Actually, coffee beans infected with *P. brevicompactum* resulted in increased sensory quality of the beverage (Iamanaka et al. 2014).

In truffles, although some volatiles are produced by the fungus itself, the interaction with subterranean bacteria and yeasts contributes to the flavor profile (Inamdar et al. 2020), and a single species can produce 30–60 VOCs. Dimethyl sulfide (**33**) and dimethyl disulfide (**34**), in addition to 2-methylbutanal (**35**), 3-methylbutanal (**16**), 2-methylbutan-1-ol (**36**), 3-methylbutanol (**17**), and oct-1-en-3-ol (**11**) are present in almost all white and black truffles (Fig. 24.5). In addition to these, each truffle has its specific pool of volatile metabolites, assuring a species-specific aroma profile. Black truffles, such as *Tuber melanosporum*, considered a "black diamond of cuisine," have a high content of VOCs, making this truffle more aromatic and, therefore, more appreciated (Mustafa et al. 2020).

Some fungi are specifically used to produce aroma compounds through biotransformation. Strains of *Ischnoderma* spp. convert L-phenylalanine to benzaldehyde, the second most important bitter compound for food industry, after vanillin (Copetti 2019). Another example is the production of 2-phenylethanol, a compound responsible for the aroma of roses, with antibacterial properties, by *A. oryzae*, when it is cultivated using L-phenylalanine as the only source of nitrogen. It has been demonstrated that the microorganism uses more than one enzymatic mechanism to convert the amino acid into the volatile derivative (Masuo et al. 2015).

24.3.1.2 Indirect Food Benefits of Fungal VOCs

Fungal VOCs can indirectly benefit the food industry in processes like mycofumigation, which consists in using volatile fungal metabolites or mixtures of these compounds to control crops, industrial products, and building materials against bacterial growth or insect infestation (Kües et al. 2018). VOCs have the capacity of producing intracellular reactive oxygen species (ROS), cause lipid peroxidation and electrolyte loss in microorganisms, which explains its biocontrol suitability (Don et al. 2021). Some species are already commercialized, such as the well-known biocontrol agent, *Aureobasidium pullulans*. The VOCs produced by the fungus *A. pullulans*, 3-methyl-1-butanol (**17**), 2-methyl-1-butanol (**36**), 2-methyl-1-propanol, and 2-phenethyl alcohol, especially the latter, have effect on controlling infection by fungi of the genus *Monilinia* in fruits including cherries, peaches, and apricots (Di Francesco et al. 2020). The aforementioned compounds also have effects in controlling other phytopathogenic fungi, such as *Botrytis cinerea* and *Alternaria alternata* (Don et al. 2021).

The main VOCs of the basidiomycetes *Coriolopsis gallica*, *Megacollybia platyphylla*, and *Lentinus arcularius,* identified as 1-octen-3-ol (**11**), 3-hexanol, 3-methyl-1-butanol (**17**), 3-octanone (**13**), 2-hexanone, benzaldehyde, and limonene, showed antifungal activity against phytopathogens (Petre et al. 2017). VOCs produced by the biocontrol fungus *Trichoderma* sp. have effect on plants of the *Arabidopsis thaliana* species. For example, 3-methyl-1-butanol (**17**), 1-decene, and 2-heptylfuran induced plant growth as well as chlorophyll production, while 1-decene has been shown to induce the expression of genes related to cell wall modification, auxin induction, stress, and defense responses (Lee et al. 2019).

Entomopathogenic fungi produce compounds, usually VOCs, that cause disruption in the normal functioning, disease or even death of insects. Therefore, these fungi and/or their secondary metabolites are used for pest control in crops (Bojke et al. 2018). Three compounds, usually C-8 VOCs, act as infochemicals, regulating the interaction between endophytic fungi and insects. These volatiles directly repel phytopathogenic insects and induce the plant to produce C-6 VOCs that will play the same role (Holighaus and Rohlfs 2019). The metabolites 1-octen-3-ol (**11**), 3-octanol (**14**), and 3-octanone (**13**) repel the insect *Sitophilus zeamais* during maize storage, in addition to inhibiting the growth of the phytopathogenic fungus *Fusarium verticillioides*, as well as the production of its mycotoxin fumonisin B1 (Herrera et al. 2015).

Fungi can also produce compounds such as 3-methyl-1-butyl acetate, 2-methyl-1-butyl acetate, and 2-phenylethyl acetate which attract the most destructive pest in Europe, the spruce bark beetle (*Ips typographus*), thus can be used in biocontrol (Kandasamy et al. 2019). The VOCs profile in seeds and crops can be used to predict fungal contamination and to identify the pathogen. Many companies use the so-called E-nose for this purpose (Gancarz et al. 2017). Peach contamination by the fungi *B. cinerea*, *Monilinia fructicola*, and *Rhizopus stolonifer,* for example, can be identified and monitored by electronic nose through detection of the main VOCs.

Hexanal, E-2-hexenal, and benzaldehyde are examples of specific VOCs used to predict fungal contamination and identify the phytopathogenic species in peaches (Liu et al. 2018).

24.3.1.3 Industrial Production of Fungal VOCs

The process of scaling up fungal VOCs production faces many challenges, mainly due to the physicochemical properties of these compounds. VOCs are generally small, non-polar, and unstable to acids and oxygen. Furthermore, there may be lost due to natural volatilization during the cultivation process. To solve these problems and increase yields, the composition of the culture medium can be modified to avoid degradation and volatilization, and adsorbents have been employed around or within the culture medium (Berger 2015). The use of alternative media, like bagasse, during the growth of *L. edodes* mushroom is proven to be a cheap alternative to maintain the fungal volatile profile (Li et al. 2019).

Conversely, maintaining the truffle aroma, by avoiding the loss of VOCs, is still a challenge for the food industry, although freeze-drying, refrigeration, irradiation, modified atmosphere packaging (MAP) with microperforated films, and combination of treatments have been used (Mustafa et al. 2020). In fact, some loss of VOCs is expected in the industrial treatment, especially during the drying step. In *Ganoderma lucidum*, drying process decreases the content of alcohols and ketones, but increases the yields of esters, alkane compounds, and especially aldehydes. This effect is much more noticeable using hot air drying than other methods such as freeze-drying, sun-drying, and vacuum-drying. Specifically, sun-drying increased the concentration of 2-methyl-cyclohexanone, vacuum-drying increased the amount of 2,3-butanediol and ammonium acetate, and hot air drying increased the amount of 1-propen-2-ol, acetate, and hexanoic acid. This data can be used to determine the suitable drying process for use on fungal commercial species (Yi-Jun et al. 2021).

Decreasing cultivation temperature is also an alternative, but this change can increase carbon chain average size of the VOCs emitted by the fungus, especially those related to fatty acid metabolism (Schoen et al. 2017). Reducing salt concentration in food fermentation increased the amount of VOCs, reduced osmotic stress, and favored the accumulation of nutrients, especially amino acids during the fermentation of *doubanjiang* (broad bean paste) by some fungal species (Yang et al. 2021). Finally, co-cultivation of different fungal species stimulates fungal metabolism, increasing VOCs production, as reported for growing together the basidiomycetes *Hypholoma fasciculare* and *Resinicium bicolor*. Some metabolites elicited when the two species were grown together were not detected in the isolated cultures of each strain (Hynes et al. 2007).

24.3.2 Pigments

Although plants, algae, and animals are sources of natural pigments, the production of these metabolites by fungi is considered more sustainable and the production process (cultivation and harvesting) is simpler (Kumar et al. 2015). In addition, fungal pigments are more cost-effective, can be produced in high yields, and shortcomings are minimized, as production does not depend on seasonal features (Chatragadda and Dufossé 2021).

Pigments produced by superior fungi have been the subject of a significant number of researches in recent years aiming to develop natural pigments in alternative to synthetic ones (Dufossé et al. 2021; Valenzuela-Gloria et al. 2021). Arpink Red, a hydroxyanthraquinoid produced by *Penicillium oxalicum,* and β-carotene (**37**) synthesized by *Blakeslea trispora* are examples of pigments already employed in food industry (Poorniammal et al. 2021; Sen et al. 2019). Other fungal pigments, such as lycopene (**38**), are considered safe as novel food ingredients by the European Food Safety Authority (EFSA 2005). Most fungal pigments fall into the class of carotenoids, quinones, flavonoids, melanins, and azaphilones, of which some examples are presented below.

24.3.2.1 Carotenoids

Carotenoid derivatives are yellow, orange, and red food colorants constituted by an aliphatic polyene chain composed of eight isoprene units (Lin and Xu 2020; Sen et al. 2019). These natural products are unstable when exposed to oxygen or light and present solubility issues in water. Although carotenoids are mainly extracted from plants, several fungi have been subjected to engineering strategies to host and overexpress carotenoids synthesis. Some of the current techniques include control of oxygen transfer rate, deletion of a transcriptional regulator located in the gene cluster (crtR) and integration of crt pathway genes, while other approaches such as mining pathways and manipulation of microbial hosts arise as new opportunities in the field (Wang et al. 2019).

β-carotene (**37**) is produced by several organisms, including the fungus *B. trispora* that also produces the red pigment lycopene (**38**). The latter is used in the USA, Australia, and New Zealand as meat colorants (Sen et al. 2019). Lycopene (**38**) is also industrially employed as a flavor modifier and intensifier in foods, such as sauces and ketchup (Bhatt and Patel 2020). The yield of lycopene (**38**) produced by fungal fermentation has been improved using metabolic accelerators. Treatment of *B. trispora* with the cyclase inhibitor tripropylamine during 2 days significantly improved the total carotenoids expression from 1.7 to 90.1%. The production of β-carotene (**37**) by *B. trispora* was 77.7% higher compared to the control group, after adding acetate during the fermentation process (Liu et al. 2021a). Accumulation of pigments in *B. trispora* varies according to the pH; β-carotene (**37**) is preferentially produced in acidic pH, while neutral or alkaline pH drives the fungus biosynthesis

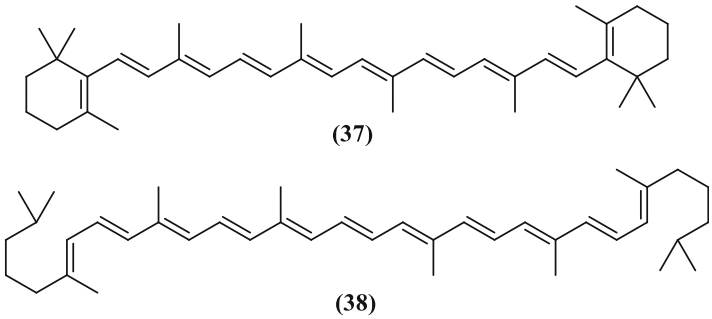


Fig. 24.6 Carotene and lycopene carotenoids produced by fungi

toward the production of lycopene (**38**). The structures of carotene and lycopene can be found in Fig. 24.6 (Foong et al. 2021).

The human body cannot synthesize carotenoids de novo and therefore these compounds must be acquired from the diet. In vivo, β-carotene (**37**) is converted into vitamin A. This carotene is recognized by its antioxidant and antitumor activities and has beneficial cardiovascular effects (Lin and Xu 2020). Its uptake reduces the risk and improves the prognosis of gastric cancer (Chen et al. 2021). Some carotenoids activate gene expression through transcriptional nuclear factor erythroid-2, with positive effects in decreasing neurological disorders and diabetes (Bhatt and Patel 2020). Oxygenated carotenoids suppress the expression of some cytokines, such as IL-6, TNF-α, and IL-1β and act as anti-inflammatory agents while others are capable of reducing the risk of neurodegenerative diseases such as Alzheimer's, Huntington's, Parkinson's, and amyotrophic lateral sclerosis (Bhatt and Patel 2020).

The wide benefits of carotenoids intake have increased the popularity of functional foods containing these metabolites. They can be incorporated in diverse bakery products, and some seafoods rich in carotenoids such as the seaweed *Undaria pinnatifida* (*wakame*) are incorporated in pasta to improve the bio-functional and nutritional values (Prabhasankar et al. 2009). Nutraceutical formulations containing carotenoids have been commercialized, mainly focusing the antioxidant properties. Enrichment with β-carotene (**37**) decreases some quality-related parameters of extra virgin olive oil subjected to microwave heating and UV light exposition. On the other hand, the addition of β-carotene enhances shelf life and nutritional value of this oil (Murillo-Cruz et al. 2021).

Carotenoids can be produced by synthetic means but will lose the "natural source" label. Therefore, biotechnological production would better meet the ever-growing demand of food industry for carotenoids, a market that is projected to reach two billion dollars by 2022 (Wang et al. 2019). Investments in this area may broaden the number of carotenoids commercially available, currently restricted to a few representatives, mainly β-carotene (**37**), lycopene (**38**), astaxanthin, canthaxanthin, and lutein (Cardoso et al. 2017). The market potential of astaxanthins is estimated to reach 880 million dollars by 2026 (Foong et al. 2021).

24.3.2.2 Other Pigments

Quinone and azaphilone are representative classes of colored natural products of polyketide origin, produced by fungi. They have potential application in the food industry as color, flavor, and fragrance additives, as well as antimicrobial and antioxidant agents to enhance shelf life avoiding food microbial spoilage (Dulo et al. 2021). In addition, these pigments have important biological activities such as antitumor, anti-inflammatory, hepatoprotective, antidiabetic, and laxative (Duval et al. 2016). Therefore, they are also capable of adding bio-functional properties to foods. Dyes with quinone nucleus have been reported to have improved dyeability, stability, and fastness compared to anthocyanins and carotenoids (Dulo et al. 2021).

Anthraquinones are well-known colored natural products produced by fungi (Masi and Evidente 2020). Naphthoquinone, an important class of anthraquinone, is a pharmacophore present in commercial drugs such as doxorubicin, idarubicin, and mitoxantrone, prescribed for metastatic cancer, acute myeloid leukemia, and multiple sclerosis, respectively (Li et al. 2020). These bioactive natural products can be used as food pigments, also including 1,4-naphthoquinones, a subclass more commonly found in plants and animals. These pigments can also be biosynthesized by some superior fungi, with the advantage of producing high-quality colorants after genetic modification. In addition, quinone pigments produced by fungi possess a wide array of shades, including red, which is an important color in the food industry (Dulo et al. 2021; Pimenta et al. 2021). These colored metabolites are biosynthesized by polyketide synthases that also produce azaphilones, natural products widely studied in the search for compounds safe enough to be used as food additives or ingredients.

Azaphilone pigments are structurally characterized by a pyrone-quinone bicycle containing a chiral quaternary center, usually of red, yellow, and orange color, produced by a variety of fungi, such as *Aspergillus*, *Penicillium*, *Chaetomium*, *Talaromyces*, *Pestalotiopsis*, *Phomopsis*, *Emericella*, and *Epicoccum*, as well as *Monascus* and *Hypoxylon* (Lin and Xu 2020; Pimenta et al. 2021). Azaphilone pigments such as monascin (**39**) and ankaflavin (**40**) (Fig. 24.7) produced by fungi from *Monascus* genus have millenary use as food colorants, spice, and food preservatives. These yellow-colored molecules have pronounced biological activities toward non-communicable diseases related to high blood lipids level, diabetes, inflammation, and fatty liver (Lin and Xu 2020). Ankaflavin (**40**) displays selective

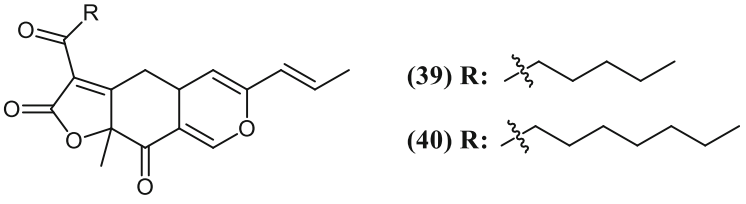


Fig. 24.7 Azaphilones produced by fungi

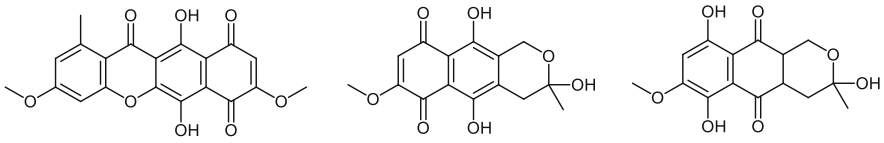


Fig. 24.8 Chemical structures of bikaverin, fusarubin, and dihydrofusarubin

cytotoxicity against cancer cells lines but low toxicity toward normal cell lines. This selective activity encourages the development of ankaflavin-based anticancer drugs.

Several works describe optimizations and color variation according to the pH of the culturing media. A long-chain red hydrocarbon pigment isolated from *Fusarium chlamydosporum*, stable at 97 °C (99% stability) shows decrease in color intensity under acid pH. Under sunlight, the pigment color turned to yellow (Soumya et al. 2018). The production of the antitumor red polyketide metabolite bikaverin (**41**) (Fig. 24.8) by *F. oxysporum* can reach 320.5 mg of pigment per liter in rice medium (20 g/L), after 96 h of incubation at 28 °C, under stirring (Santos et al. 2020). Bikaverin (**41**) has multiple applications in food industry beyond its natural coloring property. It can be used as antibacterial agent to promote extended shelf life, and as ingredient to add functional value to foods. Bikaverin (**41**) is also reported to inhibit the growth of tumor cells and to suppress apoptosis (Santos et al. 2020). Ankaflavin (**40**) is very stable (>94%) under steam at 121 °C and still more stable to direct sunlight exposure. However, under acid pH, color intensity decreases (Krishnamurthy et al. 2020). In the search for improved yields of the red pigments fusarubin (**42**) and dihydrofusarubin (**43**) (Fig. 24.8), Menezes et al. (2020) reported that a concentration of glucose above 20 g/L is ideal for red pigment production by *Fusarium solani*. The increase of glucose in the fermentation broth enhances ethanol production and inhibits the activity of respiratory chain enzymes. Fusarubin (**42**) presented anti-inflammatory activity (Menezes et al. 2020) and was also described as an antitumor metabolite (Chowdhury et al. 2017).

24.3.3 Essential Fatty Acids

Fatty acids containing one (monounsaturated) or more (polyunsaturated) double bonds in the carbon chain are present in phospholipid membranes of human cells, where they participate in cell signaling, protein transport, and other mechanisms. Fatty acids have been linked to many major health-promoting properties, including cardiovascular and nervous system protection (Patel et al. 2021). Dietary monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) are habitually obtained from fish and vegetable oils. Certain species of filamentous fungi, the so-called oleaginous fungi, are cell factories able to accumulate high-quality lipids, mainly in the form of triglycerides, with the advantage of being less fastidious and

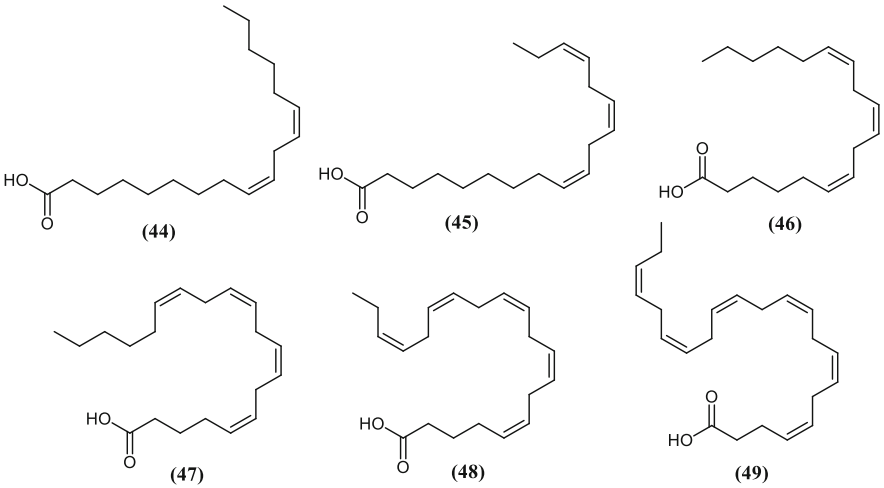


Fig. 24.9 Essential polyunsaturated fatty acids produced by oleaginous fungi

more replenishable than animal and plant sources (Dzurendova et al. 2020a). *F. oxysporum*, *Fusarium equiseti* UMN-1, *Mortierella alpina* LP M 301, *Sarocladium kiliense*, and *Microsphaeropsis* sp. can produce lipids comprising up to 56% of their total mass (Patel et al. 2020). In contrast, 57–63% (11.5–14.1 g/L) lipids have been reported to be biosynthesized by the fungus *Umbelopsis vinacea* (Dzurendova et al. 2020a). Lipid contents and fatty acids proportions vary according to the fungal strain and growing conditions, including nutrients, pH, temperature, agitation, and aeration rates. Unlike the high amounts of MUFA and saturated fatty acids (SFA) desirable in oils intended for biodiesel production, PUFA are the ideal fatty acids for food ingredients, supplements, and nutraceuticals (Chan et al. 2020)

Essential omega-6 (ω-6) and omega-3 (ω-3) PUFA have the first double bond located between the sixth and seventh, and the third and fourth carbon atoms from the methyl end (omega-end), respectively (Patel et al. 2021). These PUFA cannot be synthesized by mammalian cells, hence they must be provided by dietary sources. Elongases and desaturases catalyze the synthesis of other ω-6 and ω-3 PUFA from linoleic (**44**) (LA, C18:2 ω-6) and α-linolenic acid (**45**) (ALA, C18:3 ω-3). For instance, LA is the precursor of γ-linolenic acid (**46**) (GLA, C18:3 ω-6) and the long-chain PUFA arachidonic acid (**47**) (ARA, C20:4 ω-6), while eicosapentaenoic acid (**48**) (EPA, C20:5 ω-3) and docosahexaenoic acid (**49**) (DHA, C22:6 ω-3) can be produced from ALA (Cajka et al. 2016; Oliver et al. 2020). The aforementioned PUFA are precursors of chemical mediators associated with inflammatory response and have important role in the regulation of pathological inflammation that occurs in some chronic conditions, including asthma, rheumatoid arthritis, and inflammatory bowel disease. The structures of the PUFAs can be found in Fig. 24.9 (Innes and Calder 2018).

Oleaginous fungi such as *Mucor circinelloides*, *Umbelopsis* (*Mortierella*) *isabellina*, and *Cunninghamella echinulata* have the advantage of producing GLA

(**46**) and other PUFA from waste material containing xylose, glucose, pectin, starch, and tomato hydrolysate as substrates, making it possible to reduce fermentation costs (Patel et al. 2020). *M. circinelloides* was the first microbial species commercially exploited for production of GLA-enriched oil during the 1980s in the United Kingdom (Patel et al. 2020; Reis et al. 2019). Dietary sources of GLA (**46**) are relatively scarce and include borage, primrose and blackcurrant oils, meat and eggs (Innes and Calder 2018). *M. circinelloides* (ATCC®1216B™) produces 17–28% lipids, from which 14–16% is GLA (**46**), when cultivated in non-supplemented hydrolyzed whey permeate (Chan et al. 2020). *C. echinulata* ATHUM 4411 produced 2.04 g/L total lipids, corresponding to 36.20% of its dry weight, in submerged fermentation using spent sulfite liquor, a paper industry effluent, as carbon source, while fermentation with *U. isabellina* ATHUM 2935 in the same conditions yielded higher total lipids (5.54 g/L, equivalent to 54.34% of its dry weight). In the same conditions, *C. echinulata* oil was richer in LA (**44**) and GLA (**46**) (17% and 12%, respectively) in comparison with *U. isabellina* (16.6% and 3.6%) (Tsouko et al. 2021). Interestingly, lower lipid yield (36.58%), containing 11% LA (**44**) and 5% GLA (**46**), was achieved with the strain *U. isabellina* CCT 3498 when grown in a bioreactor under submerged cultivation in liquid medium containing yeast extract, sucrose, and salts (Somacal et al. 2020). Another strain, *U. isabellina* CCF2412, showed good ability to produce GLA (**46**) (6.4 mg/g fermented substrate), in solid-state fermentation using a blend of corn meal and animal fat as substrate. In addition to GLA (**46**), this fungus produced β-carotene (**37**), suggesting oxidative stability of its PUFA-rich oil (Slany et al. 2021). Other strains also showed ability to produce GLA (**46**) (maximal yield 10.7 mg/g fermented product) and β-carotene (**37**) under solid-state fermentation, such as *Mucor wosnessenskii* CCF2606, *M. circinelloides* CCF2617, *Mucor hiemalis* CCF2698, and *Mucor mucedo* CCF2659 (Klempová et al. 2020).

Some fungal species are able to produce long-chain PUFA and thus have numerous potential applications in the food industry. For example, ARA-rich lipids (1.9–19.0 g/L) from *M. alpina* received GRAS status (Generally Recognized as Safe) by the FDA, and is widely used as ingredient in infant formula in association with DHA (**49**). Also, *M. alpina* lipids have been studied for inclusion in poultry feeding for lipid quality improvement of egg yolk (Chang et al. 2021).

Chang et al. (2021) reviewed environmental factors involved in lipid accumulation by *M. alpina* and other aerobic oleaginous microorganisms. Nitrogen limitation and a carbon/nitrogen ratio ranging from 7 to 15 were reported as key factors to direct carbon flow toward lipid biosynthesis in detriment to amino acids production, consequently improving ARA (**47**) yields after mycelial growth (Chang et al. 2021). Much higher carbon/nitrogen ratios may decrease desaturation reactions and, consequently, decrease SFA conversion to PUFA (Ferreira et al. 2020). Moreover, mycelium aging method, which consists in feeding the fungal biomass with ethanol and KNO_3 after the fermentation period and storing it without agitation, has been considered an efficient strategy for enhancing ARA (**47**) accumulation in *M. alpina* (Chang et al. 2021; Jin et al. 2009).

The fatty acid profile of *M. alpina* revealed higher proportions of ARA (**47**) compared to eight other oleaginous fungi species (*Absidia glauca*, *Cunninghamella blakesleeana*, *Lichtheimia corymbifera*, *Mortierella hyalina*, *Amylomyces rouxii*, *M. circinelloides*, *R. stolonifer*, and *U. vinacea*). In these eight species, the biosynthesis of the MUFA oleic acid (C18:1 ω-9) predominated. In the same study, the use of yeast extract as nitrogen source increased unsaturation of *M. alpina* fatty acids compared with supplementation of inorganic ammonium sulfate, indicating that the organic nitrogen positively influenced the activity of *M. alpina* desaturases (Dzurendova et al. 2020b). On the other hand, the strain *M. alpina* Peyronel 9412 yielded 535.4 mg/g PUFA, mainly composed of ALA (**45**) (403.2 mg/g), in solid-state fermentation using polyurethane foam as support for fungal growth, on a synthetic medium containing glucose supplemented with 10% linseed oil as carbon source. Other essential PUFA, including LA (**44**) (129.1 mg/g), EPA (2.6 mg/g), and DHA (1.5 mg/g) were obtained (Ferreira et al. 2020). In turn, mushrooms are very susceptible to the environment conditions and media supplementation (Cardoso et al. 2020) and they can degrade lignocellulosic biomass, accumulating high-value lipids. For instance, the white-rot fungus, *Trametes versicolor*, cultivated in wheat straws or in rye-grains secreted oil droplets predominantly composed of PUFA containing, in grams per liter of substrate aqueous solution: ALA (**45**) (0.5–6.6), GLA (**46**) (8.6–10.5), ARA (**47**) (0.9–5.8), EPA (**48**) (1.9–4.9), and DHA (**49**) (2.3–17.4) (Hao and Barker 2021).

24.3.4 Bioactive Secondary Metabolites

Some filamentous fungi species such as *Aspergillus* sp., *Tolypocladium niveum*, and *Rhizopus oligosporus* are useful sources of metabolites and ingredients regarded as safe for food utilization and conquered the GRAS label from the FDA. *A. niger* is one of the most prominent examples of a GRAS species with multiple applications in the food industry, including the production of citric acid along with multiple secondary metabolites with functional properties. A strain of *A. niger* (IMBC-NMTP01) isolated from moldy peanuts in Vietnam was grown in PDA to produce fourteen metabolites. One of them, a naphtho-γ-pyrone (**50**), was active against six human carcinoma cell lines (HepG2, KB, HL-60, MCF-7, SK-Mel-2, and LNCaP). This metabolite also showed high NO inhibitory effect of $IC_{50} = 2.1$ μM. Nigerasperone A (**51**), isolated in the same study was also reported as a NO inhibitor, and this activity was linked to the hydroxyl group at C-3. When this position is methylated, the anti-inflammatory activity decreases significantly. Another metabolite isolated from *A. niger* IMBC-NMTP01 (**52**) was selectively active against *Enterococcus faecalis*, a Gram-positive bacterium, and should be further studied as a selective antimicrobial agent (Quang et al. 2020). In another work, chlorogenic acid (**53**) was detected among several metabolites produced by *A. niger*. This compound has several functional properties related to the inhibition of oxidative stress, vasodilatation, cardioprotective effects and has also been widely reported as

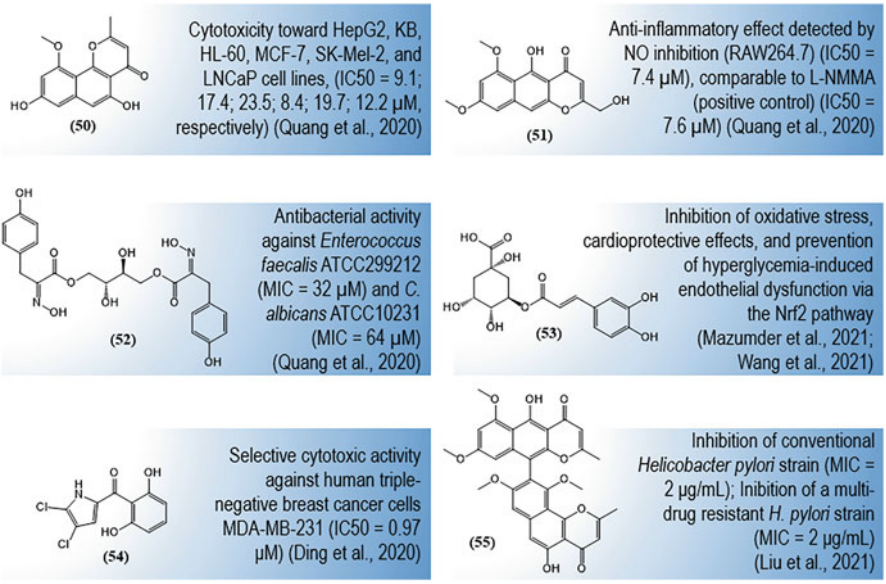


Fig. 24.10 Structures and biological activities reported for some fungal metabolites

an effective agent for control of glucose and lipid metabolic disorders (Mazumder et al. 2021).

Cell viability assay pointed pyoluteorin (**54**), another metabolite produced by *A. niger*, as a promising lead compound for the development of drugs for treatment of triple-negative breast cancer. Pyoluteorin activity was related to ROS accumulation and change of apoptosis-related protein expressions. This metabolite has low cytotoxicity against human breast epithelial cells MCF-10A (Ding et al. 2020). Other metabolites isolated from *A. niger*, such as fonsecinone A and asperazine demonstrated in vitro cytotoxicity against five human tumor cell lines, U87, A549, HCT116, MCF-7, and K562 (Ding et al. 2020). The bis-naphtho-γ-pyrone (**55**) was produced by an *A. niger* strain isolated from the marine sponge *Reniera japonica* collected at Xinghai Bay (China). This metabolite presented promising activity against normal and resistant *Helicobacter pylori* strains, although, in previous studies, it was active against other pathogenic microorganisms such as *Bacillus cereus*, *B. subtilis*, *Escherichia coli*, *Pseudomonas fluorescens*, *Trichophyton rubrum*, and *Candida albicans* (Liu et al. 2021b). The selectivity of metabolite **55** can be further explored in the search for anti-*H. pylori* selective drugs. Structure of compounds **50**–**55** can be found in Fig. 24.10.

Tannin-rich biomasses from fruit processing industries have been utilized for production of gallic acid by *A. niger* under solid-state fermentation. The yield reached 14.5 mg/g of substrate after glucose supplementation, under optimized conditions (Saeed et al. 2020). The use of bio-waste from fruit processing industries seems to be economically viable and can be further explored to increase domestic

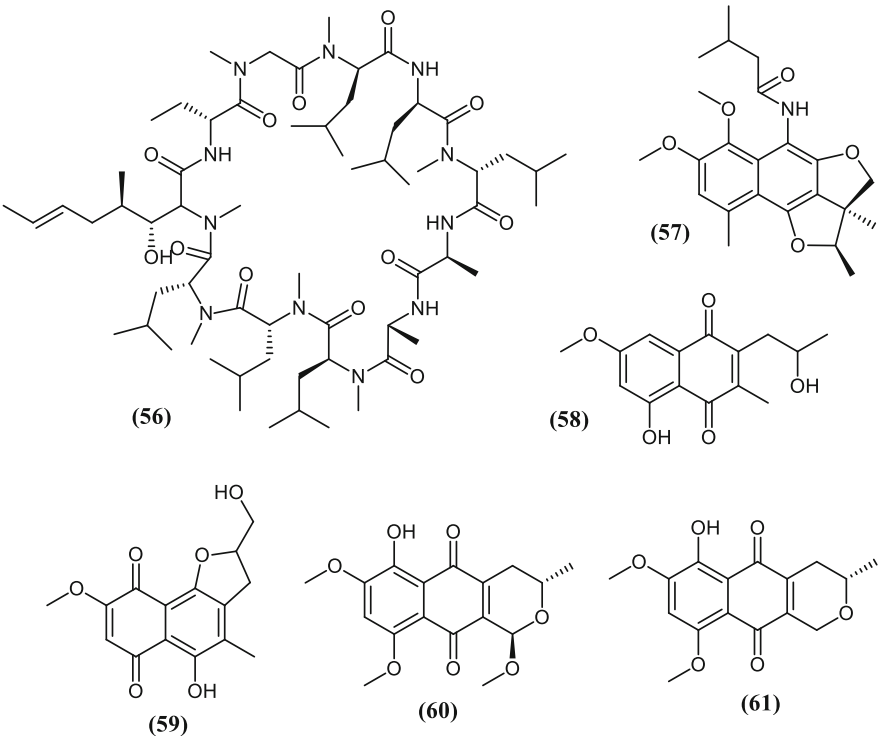


Fig. 24.11 Chemical structures of the bioactive metabolites **56–61**

production of this secondary metabolite in countries that still depend on the international supply of gallic acid (Saeed et al. 2020). Gallic acid has several beneficial effects in human body and therefore the world demand for this metabolite is high, especially for supplying the pharmaceutical industry where it is used as substrate for the synthesis of propyl gallate, a potent antioxidant. Propyl gallate is also an intermediate in the synthesis of trimethoprim, a commercially available antibacterial drug. In the food industry, gallic acid has important role, since this phenolic compound induces the production of tannase under submerged fermentation. Tannase is important for elaboration of instantaneous tea and is used as a clarifying agent in the production of wines, fruit juices, and other beverages (Belmares et al. 2004). As an ingredient of functional foods, gallic acid can help to prevent methylglyoxal-induced diabetes and its effect can be even better than the use of metformin. Besides its antidiabetic properties, anti-inflammatory, anticoagulant, and antitumor effects were also reported for gallic acid (Behdarvand-Margha et al. 2021).

Other GRAS species such as *T. niveum*¸ *R. oligosporus*, and *Agaricus bisporus* also produce secondary metabolites useful in the pharmaceutical and food industries, such as cyclosporins (**56**) (Fig. 24.11). With the growth of food supplements industry, many other fungal metabolites emerge as potential ingredients capable of offering benefits for human health. In this context, one of the fastest growing areas

refers to new cytotoxic metabolites, which can act at different stages of development and in different types of cancers. Long-chain unsaturated red hydrocarbons produced by *F. chlamydosporum*, isolated from Bangalore soil, are capable of selectively inducing death in human breast adenocarcinoma cells MCF-7, without significant toxicity in CHOK 1 non-tumor cells. In addition, this pool of metabolites reduced the lipid peroxidation caused by H_2O_2 in MCF-7 cells (Soumya et al. 2018). Ankaflavin (**40**), isolated from *Penicillium aculeatum*, possesses moderate cytotoxicity over HCT116 and PC-3 cancer cell lines, a promising activity since normal CHOK 1 and HEK 293 cells were much less affected. Selectivity is very welcome for the development of antitumor drugs with less side-effects (Krishnamurthy et al. 2020). Fermentation for 60 days using rice as a substrate resulted in the production of a yellow metabolite by the lichenized fungus *Trypethelium eluteriae*. The yellow pigment denominated trypethelonamide A (**57**) has moderate cytotoxicity against RKO cell line and possesses the unusual 2a,3-dihydro-2H-1,4-dioxapentalen[1,6-ab] naphthalene skeleton with the linkage of a 3-methylbutanamide unit at C-1 (Fig. 24.11) (Basnet et al. 2018). Anti-inflammatory metabolites were reported for *F. solani* BRM054066 and *Talaromyces* sp. SK-S009, endophytic species isolated from the fruit of *Kandelia obovata*. *F. solani* major metabolite isolated by Menezes et al. (2020) was fusarubin (**42**), an anti-inflammatory metabolite capable of reducing the overexpression of the pro-inflammatory cytokines TNF-α, IL-1β, and IL-6. In addition, fusarubin (**42**) promotes the production of anti-inflammatory IL-10 and IL-17, in murine macrophages activated by LPS. A total of twelve metabolites of the 1, 4-naphthoquinone class were produced by *Talaromyces* sp., and two of them were new compounds, talanaphthoquinones A and B (**58** and **59**) (Fig. 24.11). In general, although some metabolites were less toxic, anti-inflammatory activity in LPS-induced inflammation model was observed for all the 1,4-naphtoquinone derivatives. They inhibited NO production in RAW264.7 cell line, being more potent than the positive control, indomethacin (Liu et al. 2020b). Two pigments, 6-hydroxyastropaquinone B (**60**) and astropaquinone D (**61**) (Fig. 24.11) of orange and red color, respectively, produced by *Fusarium napiforme*, an endophyte of *Rhizophora mucronata*, inhibited the bacterial growth of *S. aureus* (6.3 and 12.5 μg/mL) and *Pseudomonas aeruginosa* (6.3 and 6.3 μg/mL) (Supratman et al. 2021). Antibiotic drugs should only be taken as prescription drugs and, therefore, antimicrobial metabolites should be preferably used in the food industry to avoid spoilage and increase shelf life of perishable foods.

Some metabolites such as the polyketide bikaverin (**41**) have multiple applications in the food industry as natural coloring pigments, as antibacterial agents to promote extended shelf life, and as ingredients to add functional value to foods. Bikaverin is also reported to inhibit the growth of tumor cells and suppress apoptosis (Santos et al. 2020). The compounds described in this section represent only a few examples of bioactive fungal metabolites that can be incorporated to foods in order to provide functional properties, making the products more attractive.

24.4 Fungal Metabolites Toward the Food Industry

Natural pigments and other additives to be incorporated in foods must be non-toxic, stable under light, heat, and pH changes, and be economically competitive (Venkatachalam et al. 2018). Water solubility is highly important, and edible pigments cannot be produced by toxin-producing strains (Poorniammal et al. 2021). Although the low cost of the process is a major concern for the industry, the competitiveness of fungal additives greatly relies on the advantage of their natural origin. Sustainability is a major concern of the contemporary food industry, and green processes, appliable to the extraction of pigments from fermentative broth, in suitable yields, have been addressed by several groups (Kalra et al. 2020; Lebeau et al. 2020; Oliveira et al. 2021).

An interesting study conducted on a cold-adapted strain of *Penicillium* sp. (GBPI_P155) isolated from soil of Indian Himalayan region, demonstrate the complexity of parameters to be addressed in yield improvement. The target strain produces a mixture of orange-colored carotenoids, and maximum production was achieved after 15 days at 15 °C, while maximum biomass yield occurred at 25 °C. Carbon and nitrogen sources, presence of mineral salts, and pH modulated the pigment expression, showing the complexity of parameter responses in the technological development of pigments production (Pandey et al. 2018). Industrial byproducts have been targeted as raw materials for production of pigments by filamentous fungi to feed the growing demand for natural pigments. This is highly important to enable bench processes to reach industrial scale, since the substrates used to produce pigments account for up to 70% of the total production cost (Foong et al. 2021). More recently, agri-food byproducts are being described as safe food additives, which can reduce cost of production (Faustino et al. 2019). Under this approach, a broad screening with several agro-industrial residues pointed the potential of pomegranate pulp as solid-state culture medium to produce yellow pigments by *Aspergillus carbonarius* in initial pH 6.5 (Arikan et al. 2020). However, using byproducts to produce fungal additives is an area that still lack research to define fermentation conditions that grant high yields. In addition, raw material may be seasonal, unsafe, contain minor contaminants incompatible with pigments biosynthesis or stability over the fermentation length (Kalra et al. 2020). Therefore, research in this area is necessary and should bring about new industrial processes in the near future.

In the post-COVID-19 era, the food industry will face new challenges, since the food chain supply must stay operational, providing safe foods, while keeping its workers safe and healthy (Nakat and Bou-Mitri 2021). The isolation imposed by COVID-19 led to a notorious increase in the online food purchases (Rodrigues et al. 2021). Although this behavior generated increased consumption of fast and ultra-processed food, a significant portion of consumers felt themselves motivated to reevaluate consumption options, due to an increased perception of their choices concerning food intake. In several sectors, the food industry has observed qualitative

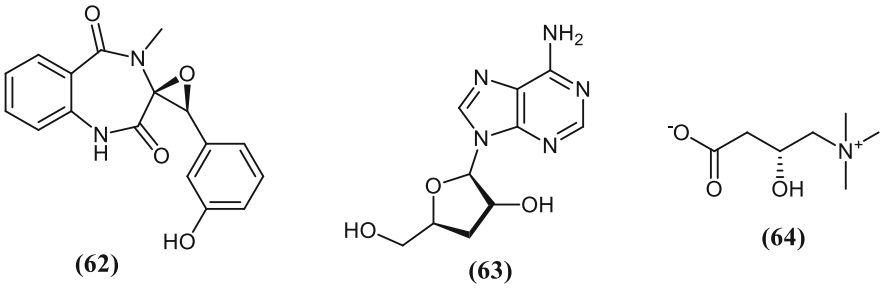


Fig. 24.12 Chemical structures of (–)-cyclopenol, cordycepin, and L-carnitine

changes in product purchase decisions, with preference for healthier products (Xu et al. 2022).

Higher fungi consist of a good option for coping with post-COVID challenges. Chain supply maintenance, one of the most immediate needs, is not an issue in this field. Fungal metabolites, nutrients, additives, and materials associated to the food industry are independent of seasonality but still meet the appeal of natural foods and contribute to waste reduction. Flavors and pigments naturally produced by fungi and new biosynthetic routes can be awakened or introduced, with the aim of producing new ingredients and materials (Al-Obaidi et al. 2021; Poorniammal et al. 2021). The market for nutraceuticals and functional foods, also on the rise, has incorporated natural fungal products and much research is underway in this field (Lagashetti et al. 2022; Takahashi et al. 2020). Recent research in this area showed outstanding outcomes, exemplified by (–)-cyclopenol (**62**), that has notably anti-food allergic property in RBL-2H3 cells, even superior to the activity of the commercial drug loratadine (Xing et al. 2021); cordycepin (**63**), an antidiabetic and antitumor metabolite present in nutraceuticals already marketed (Al-Obaidi et al. 2021); and L-carnitine (**64**) an antioxidant agent, capable of minimizing age-related disorders (Rousta et al. 2021) (Fig. 24.12). The immediate nature of the search for drugs to combat SARS-CoV-2 has propelled research on drug repositioning and screenings via computational assays, such as molecular docking and a significant number of fungal secondary metabolites were included in these studies (Takahashi et al. 2021). Therefore, fungal additives containing antiviral properties are expected to be described in the near future. In addition, fungal biomass is a protein source whose consumption is expected to grow in the coming decades, since this alternative protein is healthy, has a lower carbon footprint, and production technology is already used industrially (Moura et al. 2018).

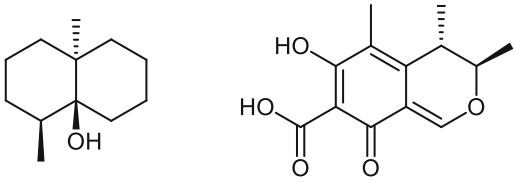
24.5 Evolution of Techniques Used for Obtaining Fungal Secondary Metabolites

24.5.1 Genetic Tools

Domestication is a primary tool for selection of desirable traits in fermenting microorganisms, which consists in breeding wild species able to consume certain nutrients, tolerate industrial processes, or produce specific metabolites (Daba et al. 2021). The reduced competition in nutrient-rich environments provided by industrial food systems favors downregulation of metabolites associated with competitiveness and survival. In this way, strains of *A. oryzae* currently used in the food industry, and *Penicillium* sp. used for cheese manufacture, have been phenotypically selected for production of enzymes and secondary metabolites along with minimal production of spores, pigments, and toxins (Bodinaku et al. 2019; Daba et al. 2021). Interestingly, production of certain secondary metabolites, including VOCs might be improved in domesticated strains. For instance, cheeses produced with ancestral *Penicillium* sp. had musty smell due to the presence of geosmin (**65**) (Fig. 24.13) as the only VOC, while cheeses produced with domesticated phenotypes smelled cheesy due to methyl ketones and other VOCs responsible for fatty, fruity, and green-plant aromas (Bodinaku et al. 2019).

Genetic engineering of filamentous fungi can also be employed for improving the yield and diversity of secondary metabolites. However, this approach is still incipient as the genome of these microorganisms was elucidated just over 10 years ago, and contain twice as much genes compared to yeasts and bacteria genomes. Nevertheless, filamentous fungi are superior in terms of secretory capacity, metabolic versatility, and tolerance to industrial cultivation conditions (Meyer 2021). Introduction of foreign DNA into fungi through protoplasts, electroporation, or *Agrobacterium*-mediated techniques, and the use of selectable markers to indicate that inserted genes are being expressed by the host cell, are among the most common genetic manipulation techniques used for obtaining biotechnologically important heterologous proteins by fungi such as *A. oryzae*, that are able to secrete proteins in high concentrations (Daba et al. 2021).

Several industrial filamentous fungi are composed of hyphae and spores containing more than one nucleus (polykaryotic), another challenge to genetic engineering. In these cases, the Clustered Regularly Interspaced Short Palindromic



(65) **(66)**

Fig. 24.13 Chemical structures of geosmin and citrinin

Repeats (CRISPR) Associated Protein 9 (Cas9) system has been used for genome editing, including for large genome fragments (Liu et al. 2020c). CRISPR/Cas9 was used for deletion of 15-kb citrinin (**66**) biosynthetic gene cluster in *M. purpureus* KL-001, a strain used for industrial production of the food colorant Monascus Red. The homokaryotic mutants generated expressed improved production of the pigments, with color values 2.3–4.6% higher than the parent strain, indicating that disruption of citrinin (**66**) production redirected metabolic pathways to pigment production (Liu et al. 2020c) (Fig. 24.13).

Chemical modulators including butylated hydroxyanisole (BHA), abscisic acid (ABA), naphthoxyacetic acid (BNOA), ethanolamine (ETA), 2-chlorodracylic acid, and salicylic acid (SA) are useful supplements in fungi cultivation media to induce overexpression of genes associated with production of desirable metabolites, such as the long-chain PUFA DHA (Diao et al. 2020). Equally, inducing microbial mutations by treatments with UV light, ethyl methane sulfonate and *N*-methyl-*N*-nitrosourea have been useful for improving EPA and DHA yields in algae and could potentially be applied to oleaginous fungi (Diao et al. 2020). Mutagenesis has the advantage of rapidly producing efficient mutants and does not require knowledge about microbe genomes.

Controlled co-cultivation is also an important tool for inducing changes in metabolic pathways involved in the production of fungal secondary metabolites. Cutting-edge technologies such as microfluidics are available to study fungal–fungal, bacterial–fungal, and fungal–nematode interactions at the cellular and intracellular levels, including enzymes, antibiotics, and other substances produced as part of the inhibition mechanism in the interaction zone, which can be helpful during high-throughput screenings and transcriptome profiling studies (Gimeno et al. 2021).

24.5.2 Spectroscopical and Chromatographic Analysis of Fungal Metabolites

Research regarding fungal metabolism, whether it targets the discovery of new molecules or the improvement in production yields, depends on the use of modern analytical techniques (Keller 2019). The recent advances in this area reported in the literature will certainly speed up the development of fungal additives for the food industry. As an example, the Fourier Transform Infrared Spectroscopy (FTIR) technique is a well-described fast and non-destructive method for characterizing complex samples through molecular absorption of infrared radiation. Direct readings of fungal samples generate spectra that are unique for each sample, which can be used to identify metabolites or to characterize species. The FTIR technique is capable of detecting intra and extracellular metabolites, as shown for *M. circinelloides*, *U. isabellina*, and *Penicillium glabrum* (Kosa et al. 2017). This analysis, in addition to being fast, does not require time-consuming extraction

methods and allows quick metabolites identification, as demonstrated for several *Mucoromycota* fungi (Dzurendova et al. 2020b).

Nuclear magnetic resonance (NMR) spectroscopy, primarily used for structural elucidation of fungal metabolites, has also been evolving to help with the study of fungal metabolome. The data obtained refer to the intramolecular magnetic field and its direct data analysis or comparison with a database in the literature provides important information about the electronic structure and functional groups of a metabolite, as described to identify colletochlorin A from *Colletotrichum gloeosporioides* (Masi et al. 2018). This technique is also used for the characterization of fungal degradation products, as already demonstrated in the biotransformation of the synthetic cannabinoid UR-144 by *Cunninghamella elegans*. In this way, along with detecting new potentially bioactive compounds directly in fungal extracts, or even, in fungal fermented broth, this technique is useful to follow the fermentation process, allowing understanding fungal metabolism and detecting the apex of metabolites production (Watanabe et al. 2018).

The NMR spectroscopy data have also been used in computational simulation models, to confirm some predicted structures, as used to identify new metabolites produced by *Aspergillus fumigatus* (Zhang et al. 2021). The use of quantum mechanics simulation in conjunction with spectroscopy, mainly electronic circular dichroism (ECD), optical rotation (OR), and vibrational circular dichroism (VCD), has become increasingly common, mainly for the determination of molecule chirality and establishment of absolute configuration. The advance of computational techniques, mainly the wide use of quantum mechanics calculations, allows the determination of the spatial configuration of metabolites of variable molecular weight (Superchi et al. 2018).

The Matrix Assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF) MS has been used for the identification of clinically related fungal species, detection of antibiotic resistance, and even the detection of microorganisms directly in patients. The use of this technique has also been adopted by agriculture and food industry, as an identification method to substitute processes that rely on DNA detection, that are still time-consuming. MALDI-TOF uses simple and easy preparation methods, reduces time spent with sample preparation, and has low cost. Furthermore, it is possible to carry out the analysis using whole cells or raw extracts to accompany the fermentation (Drissner and Freimoser 2017).

Chromatographic techniques, in turn, focus mainly on the identification, isolation, and quantification of metabolites. Column chromatography has been traditionally used as the method of choice for the isolation of metabolites of several fungi such as *Trichoderma* sp. (Hateet 2017) and *A. fumigatus* (Abdel-Aziz et al. 2018). However, modern liquid and gas chromatography techniques have brought practicality, speed, and precision to the analysis, mainly combined with modern detection methods. The High-Performance Liquid Chromatography (HPLC) coupled with Electrospray Ionization Mass Spectroscopy (ESI-MS) technique, was efficiently utilized, for example, to identify new pigments produced by *Talaromyces albobiverticillius*, for industrial use. The detection techniques, allied with

metabolites databases, facilitate the identification of metabolites directly in fungal culture media (Venkatachalam et al. 2018).

Dereplication has been growing as an important step in the identification of new compounds in extracts, requiring reliable of methods, to add practicality and speed to the studies, as demonstrated for *Clonostachys rogersoniana*, using HPLC-ESI-MS (Paguigan et al. 2017). The rapid identification of metabolites with antibiotic activity produced by *Aspergillus clavatonanicus* was demonstrated using the same technique, in addition to the use of Thermal Desorption Gas Chromatography coupled to Mass Spectroscopy (TD-GC-MS) (Mishra et al. 2017).

The quality of chromatographic analysis is highly dependent on the quality of the metabolite's extraction, especially in the detection of intracellular substances, in which the interruption of fungal metabolism and the breakdown of cell membranes are mandatory. This step can be performed chemically, with the use of liquid solvents and acidified solutions, or mechanically, using techniques such as pressurized liquid or microwave extraction (Pinu et al. 2017). The success in identifying pigments from several ascomycete fungi after extraction by pressurized liquid is reported. The mechanical methods have the advantage of being faster, greener, and better at preserving the metabolites' structure. However, the choice of extraction method is specific to each process and must consider the characteristics of the fungus and the culture medium (Lebeau et al. 2017).

Much of the literature regarding instrumental analysis of fungal metabolites focus on the detection of mycotoxins, as it is a major concern of the food industry and an important limiting factor for the commercialization of metabolites produced by fungi (Andrade et al. 2017; Durmus et al. 2017). Therefore, there is an increasing demand for rapid on-site detection methods that can be used to detect mycotoxins in industrial routine. Electrochemical sensors and biosensors use a variety of nanomaterials and allow on-site monitoring of mycotoxins in solutions, already used to detect a wide variety of mycotoxins (Goud et al. 2018). More recently, electronic nose (e-nose) technology has been shown to be able to detect volatile metabolites, making it an instantaneous method for identifying food contamination by pathogenic fungi and the production of mycotoxins (Brenet et al. 2018; Sanaeifar et al. 2017)

Finally, the advancement of analysis techniques of metabolites, together with computational techniques and database research, led to the development of the interdisciplinary techniques known as "omics" (Fouillaud and Dufossé 2022). Metabolomics is based on the simultaneous identification of several metabolites in fungal culture medium, which is of great help in dereplication studies to discover novel substances. Even small modifications in fungal growing conditions can cause great changes in the metabolites profile, and this can be easily identified by metabolomics. This strategy was effective in discovering new metabolites produced by *Penicillium ubiquetum* and *Fusarium graminearum* in altered media (Adpressa et al. 2019; Hoang et al. 2018).

24.6 Conclusion and Perspectives

Secondary metabolites including volatile compounds, pigments, essential fatty acids, and bioactive compounds produced by fungi have broad application in the food industry along with primary metabolites (e.g., enzymes, organic acids, and proteins) that have longer been used for manufacture of traditional food products worldwide. This suggests that fungal secondary metabolites will contribute not only to the expected growth of food additives market, but also to the development of new functional foods, supplements, and nutraceuticals in the coming years.

Once the non-toxicity of a fungal strain cultivated under certain submerged or solid-state condition is confirmed, studies are necessary to enable high yields of the desired metabolite to be released with minimal production costs, for example, through modulation of parameters like type of substrate, pH range, temperature, light, and agitation. Genetic approaches are useful for enhancing productivity and variability of fungal compounds, and spectroscopic and chromatographic techniques can help in the identification of new compounds of interest in fungal extracts.

It is conceivable that the post-COVID-19 food industry will see consumers more alert to the quality of their food choices and more likely to value natural food products, additives, and ingredients, instead of their synthetic counterparts, which opens up a promising field for fungal metabolites in the food sector. However, in vivo studies on health improvements provided by fungal metabolites are necessary, and fungal cultivation and metabolites extraction technologies need to evolve for achievement of food-grade fungal metabolites in an environmentally sustainable way.

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Part V
Pharmaceutical Industry

Chapter 25
New Trends from Plant Secondary Metabolism in the Pharmaceutical Industry

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Abstract Plants constitute nowadays an important source for the discovery of new products of medicinal value for drug development, food additives, and cosmetic ingredients. High-value plant derivatives include phenolic compounds, terpenes and terpenoids, alkaloids, phytoalexins, among others, all of which biosynthesized as a defensive strategy by plants to respond to perturbations under certain environmental conditions. Although plants produce a number of therapeutical products, either already on the market or under clinical trial, the amounts obtained from natural sources are still very minute or difficult to synthesize at an industrial level because of the complex chemical composition and chirality exhibited by these compounds. Nonetheless, as natural bioactive-oriented researches intensify—from large-scale epidemiological and interventional studies to innovative biotechnological tools pursuing compound refinement and production at the industrial level—, one may expect the upcoming years to be determinant for plant secondary metabolites to achieve their highest value in the most diverse technological and pharmacological domains. In this chapter, we discuss various aspects of plant secondary metabolites, including their biosynthetic pathways, health-promoting properties, and sources of medical products for commercial and pharmaceutical applications.

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25.1 Phenolic Compounds

25.1.1 Overview into the Chemistry and Biosynthesis of Phenolic Compounds

Phenolic compounds represent one of the most versatile classes of plant secondary metabolites as more than 8000 different molecules have been identified as belonging to it. From simple to more complex structures, these compounds are characterized by the presence of aromatic rings with one or more hydroxyl group. Due to the large number of molecules identified, phenolic compounds are usually divided into different categories, namely, flavonols, flavanols, flavones, anthocyanins, chalcones, isoflavones, stilbenes, lignins, lignans, xanthones, coumarins, or phenolic acids (Vuolo et al. 2019; Araújo et al. 2021). As secondary metabolites—mainly involved in the defense of plants against external aggressions to promote growth and development—several polyphenols have shown promising health benefits against different pathologies (Tuladhar et al. 2021; Dias et al. 2021). Several pathways are involved in the biosynthesis of phenolic compounds. From this step on, we will focus on two of the main classes of food phenolics: phenolic acids and flavonoids.

25.1.1.1 Phenolic Acids

The term "phenolic acids" is normally associated with aromatic molecules containing a carboxylic acid group. This class is amidst the main phenolic compounds widespread in nature. And although it is present in a lot of different natural sources, this group is mainly composed by hydroxybenzoic and hydroxycinnamic acids (Fig. 25.1). Such compounds are synthesized in plants through the shikimate pathway (Fig. 25.2) via the action of chorismate mutase to form the precursors aromatic amino acids L-tyrosine and L-phenylalanine, involving three main reactions (deamination, hydroxylation, and methylation) that operate through the actions of different enzymes in a chain reaction fashion to give origin to the different known phenolic acids (Maeda and Dudareva 2012; Heleno et al. 2015). It is not difficult

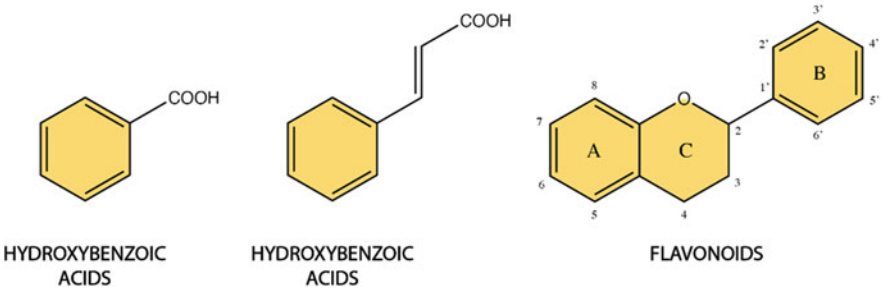

Fig. 25.1 The core structures of phenolic acids and flavonoids

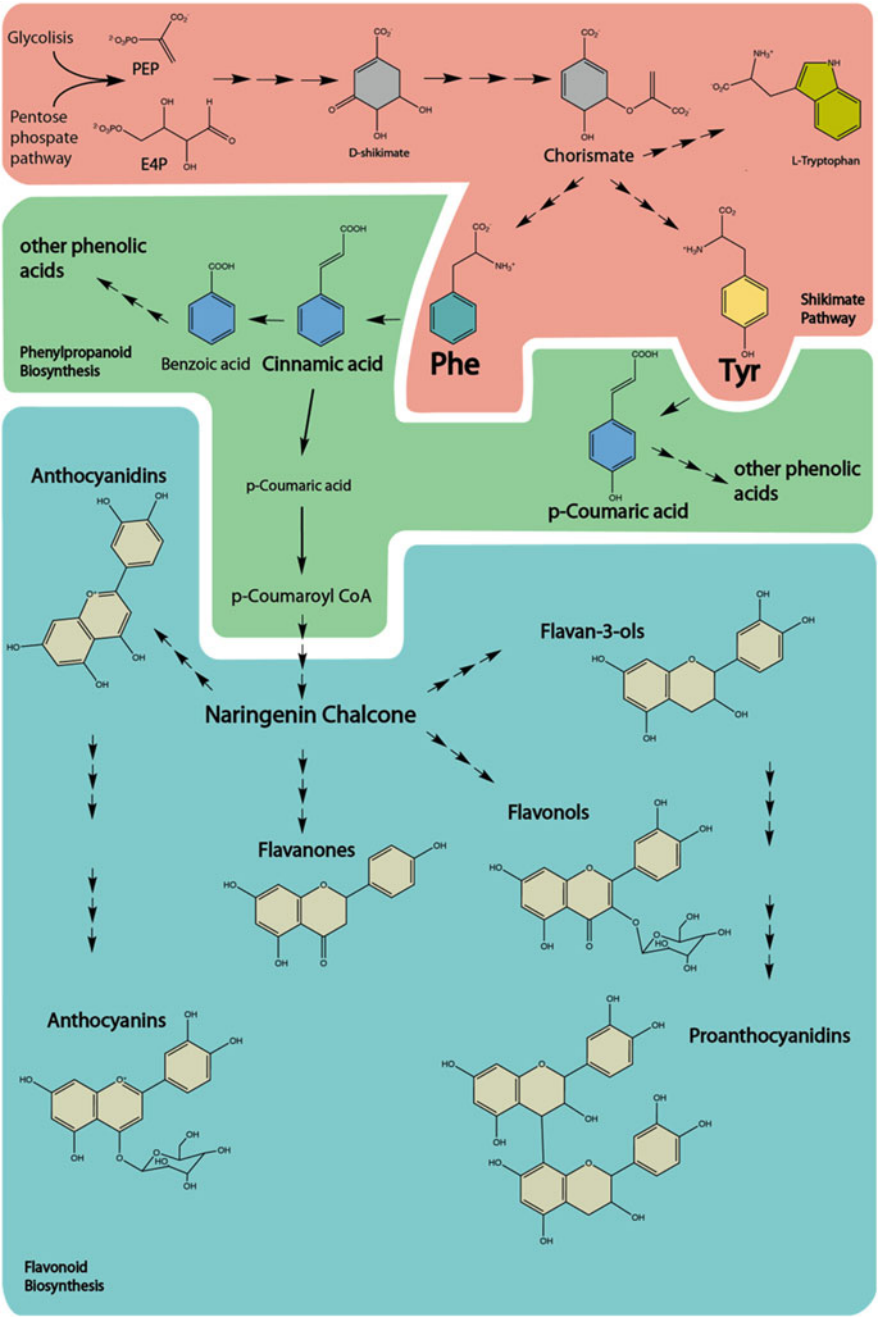

Fig. 25.2 The shikimate/phenylpropanoid pathway for the biosynthesis of phenolic acids and flavonoids. PEP—phosphoenolpyruvate; E4P—erythrose-4-phosphate. The three-arrow sequence means multiple enzymatic reaction steps to form the molecule indicated

then to comprehend the similarity between such compounds. Indeed, phenolic acids are derivatives of cinnamic acid and benzoic acid (resulting from the first chain reaction steps). Briefly, the deamination process of L-phenylalanine originates cinnamic acid (by the action of the phenylalanine ammonia lyase) and that of L-tyrosine originates *p*-coumaric acid (by the action of the tyrosine ammonia lyase) (Maeda and Dudareva 2012). After these first steps, the removal of the ethyl side chain of cinnamic acid originates benzoic acid, and further hydroxylations and/or methylations, these molecules give rise to the different derivatives (Heleno et al. 2015). The specific patterns of hydroxylation/methylation depend on the enzyme recruited for the biosynthesis. Furthermore, different enzymes have specificity for the same downstream chain reaction structure, which result in a diverse range of phenolic acids. For instance, the formation of protocatechuic acid depends on three previous reactions—deamination of L-phenylalanine to originate cinnamic acid, the removal of the side chain to form benzoic acid, and the hydroxylation to form hydroxybenzoic acid—however, protocatechuic acid itself can originate vanillic acid or gallic acid depending on the enzyme that acts upon it structure (protocatechuic acid 5-hydroxylase for gallic acid and protocatechuic acid 3 *O*-methyltransferase for vanillic acid) (Heleno et al. 2015).

25.1.1.2 Flavonoids

Flavonoids constitute one of the most characteristic groups of compounds in higher plants, many of which easily recognized as flower pigments in most angiosperm families. Endowed with a basic C6-C3-C6 carbon skeleton—combining a benzo-y-pyrone structure and a phenyl ring (Fig. 25.1)—, flavonoids are abundantly found in fruits, vegetables, and beverages of plant origin such as tea or wine. Depending on carbon atom to which the B ring is linked to the tetrahydropyran ring C, as well as on the degree of unsaturation and oxidation of C, flavonoids may be divided into six different subclasses (Fig. 25.3). Distinctions are thus made between flavones (e.g., luteolin 7-*O*-glucoside), flavanones (e.g., naringenin 7-*O*-neohesperidoside), isoflavones (e.g., genistein 7-*O*-glucoside), anthocyanidins (e.g., cyanidin 3-*O*-glucoside), flavonols (e.g., quercetin 3,4′-*O*-diglucoside), and flavanols (e.g., epicatechin). Other flavonoid subclasses that are usually of minor dietary relevance include the chalcones, dihydrochalcones, and dihydroflavonols. Within each subclass, structural variations may occur in the type, substitution, and oligomerization pattern of aglycones, due to, among others, hydration, methylation, glycosylation, acylation, prenylation, and/or oxidative reactions. Flavonoids are essentially all bio-generated through the shikimate/phenylpropanoid pathway (Fig. 25.2). Starting from a stepwise conversion of L-phenylalanine to *p*-coumaroyl-CoA, the biosynthesis of flavonoids proceeds toward the formation of the first key intermediate metabolite—chalcone (4,2′,4′,6′-tetrahydroxychalcone)—, by condensation of one molecule of *p*-coumaroyl-CoA with three molecules of malonyl-COA in a reaction catalyzed by chalcone synthase. Next, chalcone is isomerized to the flavanone naringenin. From this point, naringenin can originate other flavanones, isoflavones,

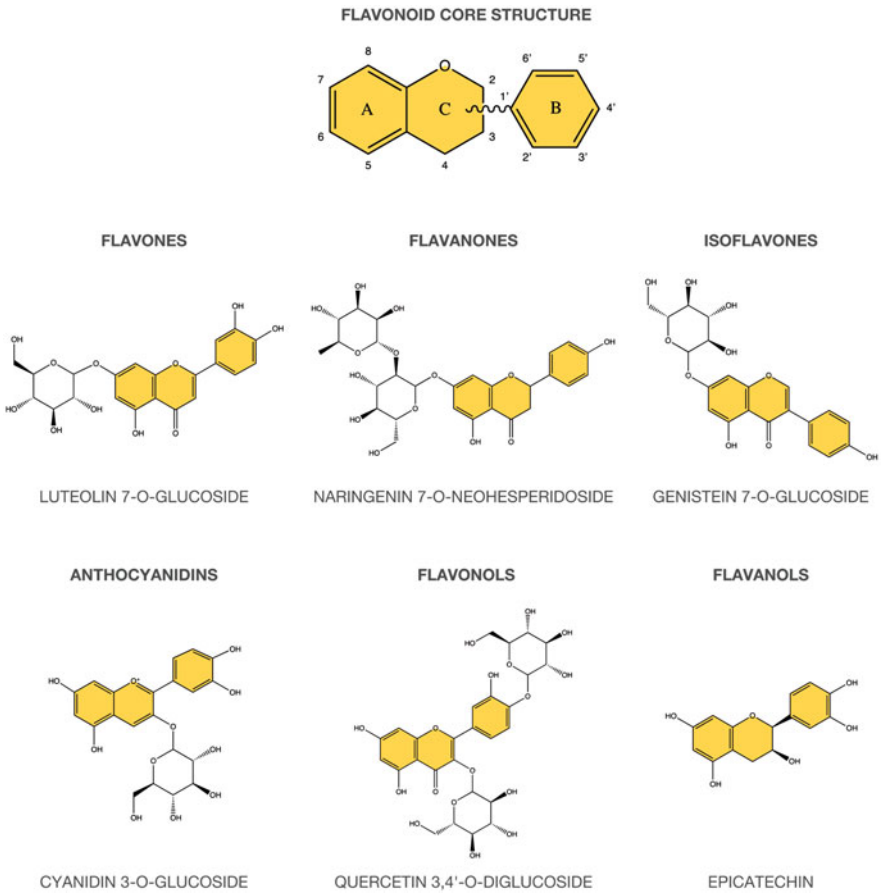

Fig. 25.3 Representative structures of some basic flavonoids

and be converted to dihydrokaempferol. The flavonoids biosynthesis pathway is then activated to originate the remain flavonoids, namely, anthocyanidins, anthocyanins, flavonols, flavanols, and proanthocyanidins. The specific pathways for each class will obviously depend on the action of different enzymes to catalyze the several reactions.

25.1.2 Distribution of Phenolic Compounds

Phenolic compounds are widely distributed in nature and virtually present in all the species of plant kingdom known to mankind.

25.1.2.1 Phenolic Acids

Phenolic acids are abundant secondary metabolites, present in a very large variety of edible and non-edible natural sources (Table 25.1). They have important roles on plants metabolism and development: they are components of the cell wall, they may enhance the uptake of the different nutrients, they can act as signal mediators, support seed germination among others (Cheynier et al. 2013; Khokhani et al. 2013; Mandal et al. 2010; Vicente and Plasencia 2011). For instance, chlorogenic acid can be found in apples, artichoke, betel, burdock, carrots, coffee, eggplants, Eucommia, grapes, honeysuckle, kiwi fruit, pears, plums, potatoes, tea, tobacco leaves, tomatoes, and wormwood (Santana-Gálvez et al. 2017). Caffeic acid, the main hydroxycinnamic acid of human diet, can be found in olives, coffee beans, potatoes, carrots, and propolis, among others (Espíndola et al. 2019). *p*-Coumaric acid is also present in a lot of different fruits, vegetables, and plants including rice, berries, wine, ginseng, apples, bananas, and particularly high in mushrooms (Pei et al. 2016). With such a wide distribution, it becomes virtually impossible to assess all phenolic acids and their respective natural sources. However, some plants, fruits,

Table 25.1 Structures of some basic polyphenol's representatives (Escobar-Cévoli et al. 2017; Zamora-Ros et al. 2013)

Polyphenol class	Examples	Food sources	Estimated dietary intake
Phenolic acids	E.g., hydroxybenzoic acid, caffeic acid, ellagic acid	Wine, fruit juices, vegetables, coffee, olives, berries	158–1265 mg/day
Flavones	E.g., apigenin, luteolin	Parsley, celery, capsicum, pepper, broccoli	1–20 mg/day
Flavanones	E.g., naringenin, eriodictyol, hesperetin	Lemon, orange, oregano	5–60 mg/day
Anthocyanidins	E.g., pelargonidin, cyanidin, delphinidin, petunidin, malvidin, peonidin	Black elderberry, blackberry, blackcurrant, sweet cherry, red wine	20–50 mg/day
Isoflavones	E.g., daidzein, genistein	Soy tempe, soybean, soy yogurt, beer	<2 mg/day (Western countries) 30–40 mg/day (Asian countries)
Flavonols	E.g., kaempferol, quercetin, myricetin	Red onion, spinach, asparagus, capers	20–40 mg/day
Flavanols	E.g., catechin, epicatechin, gallocatechin, epigallocatechin	Dark chocolate, cocoa powder, almond, black/green tea, apples	50–600 mg/day
Chalcones, dihydrochalcones, dihydroflavonols	E.g., xanthohumol, phloretin, dihydroquercetin	Beer, cider, apple juice, wine	0.01–5 mg/day

and vegetables have exquisitely high amounts of certain phenolic acids. For instance, pomegranates are a very good source of ellagic acid (Wu et al. 2021). While corn, bamboo, and whole-grains of oat or rye bran are good sources of ferulic acid (Zhao and Moghadasian 2008). On the other hand, the species of rice *Oryza sativa* contain different phenolic acids such as protocatechuic, syringic, gallic, vanillic, or sinapic acids (Shao et al. 2014).

25.1.2.2 Flavonoids

In *planta*, flavonoids are used for growth, signaling, and defense agents against biotic and abiotic stresses, including UV irradiation, plant freezing, pathogen infection, and insect feeding. Consequently, they are also widely distributed in nature (Table 25.1). Anthocyanins are a great example of that (Cruz et al. 2022). They are present in an enormous number of fruits, roots, flowers, or leaves. Due to their chromatic characteristics, anthocyanins are responsible for the bright blue to red colors of their sources, consequently, anthocyanin-rich sources are easily spotted. Indeed, anthocyanins play an important role in plant reproduction due to the attraction of pollinators and seed dispersers for their colors (Hoballah et al. 2007). In addition they protect plants for biotic and abiotic stress (Ahmed et al. 2014). Therefore, anthocyanins can be found in red fruits, purple vegetables, and several types of fruits. Depending on the food source, different types of anthocyanins are present, however, some have a higher distribution than other, such as the case of cyanidin-3-*O*-glucoside, the most common anthocyanin present in nature. For instance, red and purple fruits are normally rich in higher concentrations of monoglucosylated anthocyanins (Herrera-Balandrano et al. 2021; Castañeda-Ovando et al. 2009; Krga and Milenkovic 2019; Khoo et al. 2017). On the other hand, vegetables and roots are normally rich in polyglycosylated anthocyanins with acylation in the different sugars (Jokioja et al. 2021). Highly complex structure anthocyanins can be found in certain types of flowers, such as the heavenly blue (Mendoza et al. 2018). The hydroxylation/acylation patterns also depend on the source. The main anthocyanin in red wine and blueberries is malvidin-3-*O*-glucoside, while in blackberries is cyanidin-3-*O*-glucoside and strawberries pelargonidin-3-*O*-glucoside (Seeram et al. 2006; Silva et al. 2007). Colored sweet potatoes and colored cabbages are rich in acylated anthocyanins with multiple sugars attached, mainly derived from cyanidin and peonidin anthocyanidins (Oliveira et al. 2019; Fenger et al. 2020). A great number of red, blue, and purple-colored flowers contain anthocyanins from different types, from simple monoglucosylated to high acylated anthocyanins with multiple sugar units (Vankar and Srivastava 2010). This may be determined by the different biosynthetic strategies and the environmental modulation.

25.1.3 Structure–Activity Relationship of Phenolic Compounds

The biological activities of phenolic compounds are virtually infinite as more than 8000 different molecules have been identified as belonging to this group of natural compounds. However, some patterns can be found that help to assess the potential of the overall group. The case of the antioxidant properties of phenolic acids and flavonoids is a good example.

25.1.3.1 Phenolic Acids

The activity of phenolic acids will highly depend on their substitution patterns. Namely, their antioxidant activity depends on the number of hydroxyl groups in the molecule that would be theoretically strengthened by steric hindrance (Dziedzic and Hudson 1983). Indeed, the number and position of hydroxyl groups in phenolic compounds is directly related to their free radical scavenging capacity and therefore to their bioactivity. In a recent study, 18 phenolic acids were evaluated for their antioxidant capacity using DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) and FRAP (ferric reducing antioxidant power) assays in ethanol and water systems, respectively. The carboxyl group contribution to the antioxidant features of phenolic acids showed to be affected by its electron-donating ability. This study also reinforced the role of methoxy groups for the antioxidant capacity, stating that the higher the number, the better. The hydroxyl in the position *ortho* showed a better antioxidant performance (Chen et al. 2020). Different studies have also stated that hydroxycinnamic acids have a better antioxidant capacity than that for hydroxybenzoic and derivatives (Nattela et al. 1999; Teixeira et al. 2013). It was also reported that 3,4-dihydroxyphenylacetic acid had stronger antioxidant activity than caffeic acid and protocatechuic acid (Siquet et al. 2006). However, this is not the only factor that will determine their efficiency. The specific target will also define their higher or lower activity. Consequently, the structure–activity relationship should be also target-oriented rather and not only, compound-oriented. For instance, in the case of β-lactoglobulin, hydroxylation at the C3-position increased the affinity of the phenolic acids, while hydroxylation at the C2- or C4-positions reduced it. Complete methylation of all hydroxy groups, except at the C3-position, enhanced the binding affinity. Replacing the hydroxy groups with methyl groups at the C2-position also had a positive effect. This binding to β-lactoglobulin improved the antioxidant activity of the phenolic acids (Wu et al. 2018). The highest reduction of breast cancer in postmenopausal women was associated with the intake of chlorogenic acid in comparison to other hydroxycinnamic and hydroxybenzoic acids, prompting for the substitution pattern of such molecule (Romanos-Nanclares et al. 2020). The antioxidant capacity of phenolic acids in lipophilic systems showed that a double hydroxylation at the *ortho* and *para* positions (caffeic and protocatechuic acids) significantly enhances the antioxidant capacity. Also, the

presence of one or two methoxy groups in position *ortho* in relation to –OH such as in ferulic acids further enhances this capacity (Pokorny 1987; Cholbi et al. 1991; Chimi et al. 1991). In an aqueous phase, ferulic acid showed to be 150% as efficient as caffeic and chlorogenic acids (Rice-Evans et al. 1996). The antimicrobial activity of phenolic acids, on the other hand, seems to be slightly decreased with the number of pendant hydroxyl/methoxyl substituents against three different bacteria strains *Escherichia coli*, *Staphylococcus epidermidis*, and *Staphylococcus aureus* (Liu et al. 2020).

25.1.3.2 Flavonoids

As far as the antioxidant activity of flavonoids is concerned, four structural features were shown to be fundamental for flavonoids to exhibit enhanced radical scavenging properties: (1) a 3′,4′-dihydroxy structure in ring B, favoring electron delocalization along C, (2) an unsaturated 2–3 bond in conjugation with a 4-oxo function, (3) a free hydroxyl group at position 3 conjugating—by hydrogen bonding—both rings A and B (essential for coplanarity), and (4) lack glycosidic moieties—known to enhance flavonoid bioavailability but decrease antioxidant effectiveness (the opposite occurs for oligomeric species) (Heim et al. 2002). In the case of anthocyanins, the activity will depend on three main key features: the B-ring substitution pattern; the number and type of sugars attached to the flavylium core; and the number and type of acyl groups. This will determine their ability to exert specific functions within specific environment or against specific targets. Also, the number of sugars attached to its core seems to affect their stability in a positive way, probably due to the higher number of potential acylation points and the formation of intramolecular interactions (Oliveira et al. 2019; Zhao et al. 2014).The antioxidant features of anthocyanins relative to their structure are also well documented. Regarding B-ring, *o*-hydroxylation and methoxylation greatly increase the antioxidant capacity of anthocyanins (Kharadze et al. 2018). Anthocyanidins are more antioxidant than anthocyanins, suggesting that the presence of sugar moieties negatively influences this property (Khoo et al. 2017). However, this strongly depends on the glycosylation site, the sugar type, and the number of sugars (Zhao et al. 2014). On the other hand, acylation of the sugar moieties significantly increases the antioxidant features of anthocyanins (Matera et al. 2015; Stintzing et al. 2002; de Morais et al. 2020). Anthocyanins exists in a dynamic structural equilibrium dependent on pH, consequently in solution, these compounds may present more than one structure at a given time period (Pina et al. 2015). Chalcones and quinoidal bases contribute to a higher scavenger and antioxidant capacity, potentially due to the presence of conjugated double bounds between the keto group and the phenolic aldehyde (Tena et al. 2020).

25.1.4 *Bioactivity of Phenolic Compounds: Commercial Bioactives and Disease Targets*

Recent decades have witnessed a considerably renewed interest on transformation of current food systems to improve availability, affordability, and uptake of nutritious, safe, and sustainable diets, capable of tackling malnutrition in all its forms and associated, diet-related, non-communicable diseases (NCDs). Within the singular elements that compose the healthy eating pyramid, over the last few years, WHO authorities have been emphasizing the importance of increasing the consumption of plant-based foods such as fruits, vegetables, legumes, nuts, and wholegrains. Why? Because plant-based foods are an exclusive and rich source of bioactive phytochemicals (including flavonoids) with specific groups of compounds being particularly abundant in some foods for which specific physiological effects and health implications have been attributed. The increasing recognition of the benefits brought by natural bioactives to human health has sparked a new appraisal of foodstuffs and value-added agri-food "*wastes*" including all kinds of fruit juices, cocoa, coffee, tea, wine, apple pomace, grape seeds, etc., whose content in health-promoting compounds has been driving numerous investigations focused on understanding their functional and pharmacological potential against development of low-grade inflammatory conditions (Fig. 25.4). Phenolic acids, for instance, have been described to act in a wide range of different diseases. The intake of such compounds has been associated with a reduced risk of many oxidative stress related diseases, including cancer, diabetes, or cardiovascular disorders, mainly due to their well reported anti-inflammatory, antimicrobial, anti-mutagenic, or anticancer properties (Rashmi and Negi 2020). The examples for this and other diseases are enormous, making this

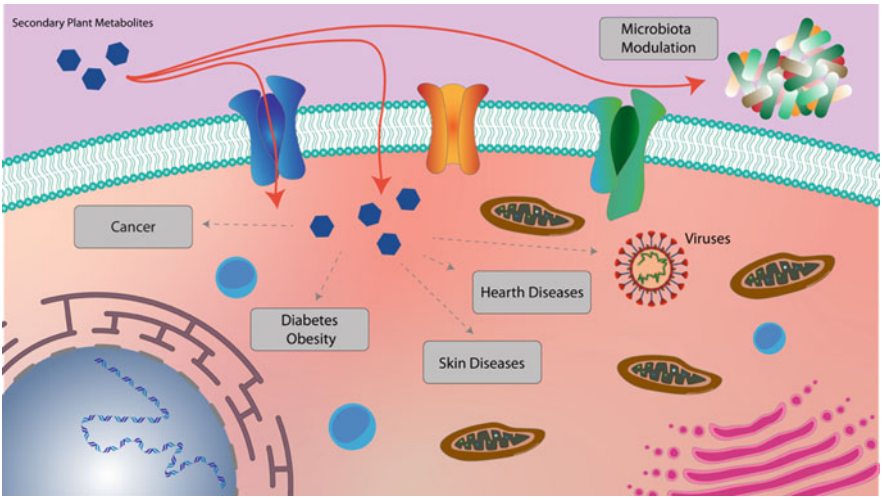

Fig. 25.4 The actions of secondary plant metabolites after ingestion and their role in different pathologies

class of natural secondary metabolites one of the most bioactive groups. Like their bioactivities, phenolic acids have many applications, from therapeutics to cosmetics and food industries. Ferulic acid is an interesting example of the commercial usage of phenolic acids. It is commercialized in the form of serums, mainly for skin applications (Dermatology 2014). On the other hand, trans-cinnamic acid is used in dyes, pharmaceuticals for disease like tuberculosis and malaria, and even in the manufacture of flavors. This acid is also the precursor of aspartame, used as a sweetener in food industry.

Flavonoids also constitute one of the main classes of bioactive phytochemicals with a proven track of success as either dermatological (e.g., resveratrol for anti-aging skincare) or functionalized dietary supplements (*elaVida, AppleActiv DAAP*); from a pharmaceutical perspective, however, there is still some resistance concerning the use of flavonoids at the clinical level, likely because of controversial in vitro and/or observational evidences supporting their benefits in humans. The latter relate to (1) the wide variability of flavonoid structures and bioactive effects, making it difficult to assess which specific compound is responsible for a specific biological action, (2) difficulties in determining polyphenol content and antagonistic/synergistic interactions within natural/processed food products, and (3) lack of a comprehensive understanding of flavonoids absorption and metabolism at the human body, hindered by the high inter-individual variability following acute/chronic polyphenol consumption (Peluso and Palmery 2015). Still, there are some exceptions, i.e. examples of flavonoid compounds that already made their way into the (para)pharmaceutical market, or are, instead, in clinical trials (e.g., *genistein*, a soy-derived isoflavone and phytoestrogen, currently examined as an alternative to classical hormone therapy to help prevent cardiovascular disease in postmenopausal women) (De Gregorio et al. 2017). Among the successful cases, one may find, for instance, *flavoxate*, a muscarinic antagonist and spasmolytic flavone used for the symptomatic relief of conditions associated with a lack of muscle control in the bladder, such as dysuria, urgency, and nocturia (Zor et al. 2015). *Diosmin* (diosmetin 7-*O*-rutinoside, Fig. 25.4), on the other hand, is a citrus flavone used for the improvement of capillary fragility or venous insufficiency, including chronic venous insufficiency and hemorrhoids (Feldo et al. 2019). The same goes for mixtures of proanthocyanidin oligomers, extracted from pine tree bark or grape seeds, and approved years ago as vasculoprotecting and venotonic drugs (e.g., *Pycnogenol*) and *Crofelemer* (*Fulyzaq*), used in the management of non-infectious diarrhea in adult patients with HIV/AIDS on antiretroviral therapy (Frampton 2013). Anthocyanins have been commercially in the form of food supplements such as the MEDOX® capsules, rich in anthocyanins from bilberries and black currants for blood vessel health, although it is known that these compounds act on a wide variety of other health issues (Tian et al. 2021).

Notwithstanding the advances made so far—most based on emerging scientific evidences correlating flavonoids intake with decreased blood pressure, reduced cholesterol levels, and fine-tuning of energy metabolism—, the design of novel flavonoid-based or flavonoid-inspired drugs against specific protein targets is still limited (Williamson 2017). Nonetheless, as flavonoid-oriented researches are

intensifying—from large-scale epidemiological and interventional studies to innovative biotechnological tools pursuing flavonoid refinement and production at the industrial level (e.g., by reconstruction of biosynthetic gene clusters in plants or through metabolic engineering of industrially relevant microbes)—, one may expect the upcoming years to be determinant for flavonoids to achieve their highest market value in the most diverse technological and pharmacological domains (Cravens et al. 2019; Polturak and Osbourn 2021).

25.2 Terpenes and Terpenoids

25.2.1 Biosynthesis

Terpenes constitute one of the largest and structurally diverse groups of naturally occurring compounds. To date, more than 50,000 terpenoids have been found in nature and most of them are isolated from plants (Li et al. 2020a). These are known as secondary metabolites since they are formed due to the enzymatic resections of primary metabolites (amino acids, sugars, vitamins, etc.) and basically consist of five carbon isoprene structural units which are assembled to each other by thousands of ways (Fig. 25.5).

Terpenes are simple hydrocarbons that based upon the number of isoprene units can be classified into monoterpene (C10), sesquiterpene (C15), diterpene (C20), triterpene (C30), tetraterpene (C40), and polyterpene (C>40). Terpenoids are modified class of terpenes with different functional groups (alcohols, aldehydes, carboxylic acids, ketones, esters, and glycosides) and oxidized methyl group moved or removed at various positions.

The synthetic pathways for terpenoids include the mevalonic acid (MVA) pathway and the 1-deoxy-D-xylose-5-phosphate (DXP) pathway (Fig. 25.6). The MVA pathway exists in the cytoplasm and the secondary metabolites such as sesquiterpenes, sterols, and triterpenes are mainly synthesized through this way; the DXP pathway is mainly present in plastids, and monoterpenes, diterpenes, and tetraterpenes are synthesized by this way. The basic building blocks of all terpene constructs are isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). DOXP-MEP pathway, that is expressed in the plastids of plants, green algae, some bacteria, and parasites such as the malaria parasite *Plasmodium*.

The biosynthesis of the major terpenoid classes in plants is outlined in Fig. 25.6. The first step in terpene synthesis involves the condensation of IPP and DMAPP by

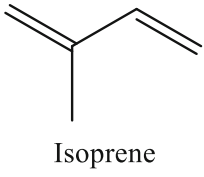

Fig. 25.5 Structural unit of terpenoids

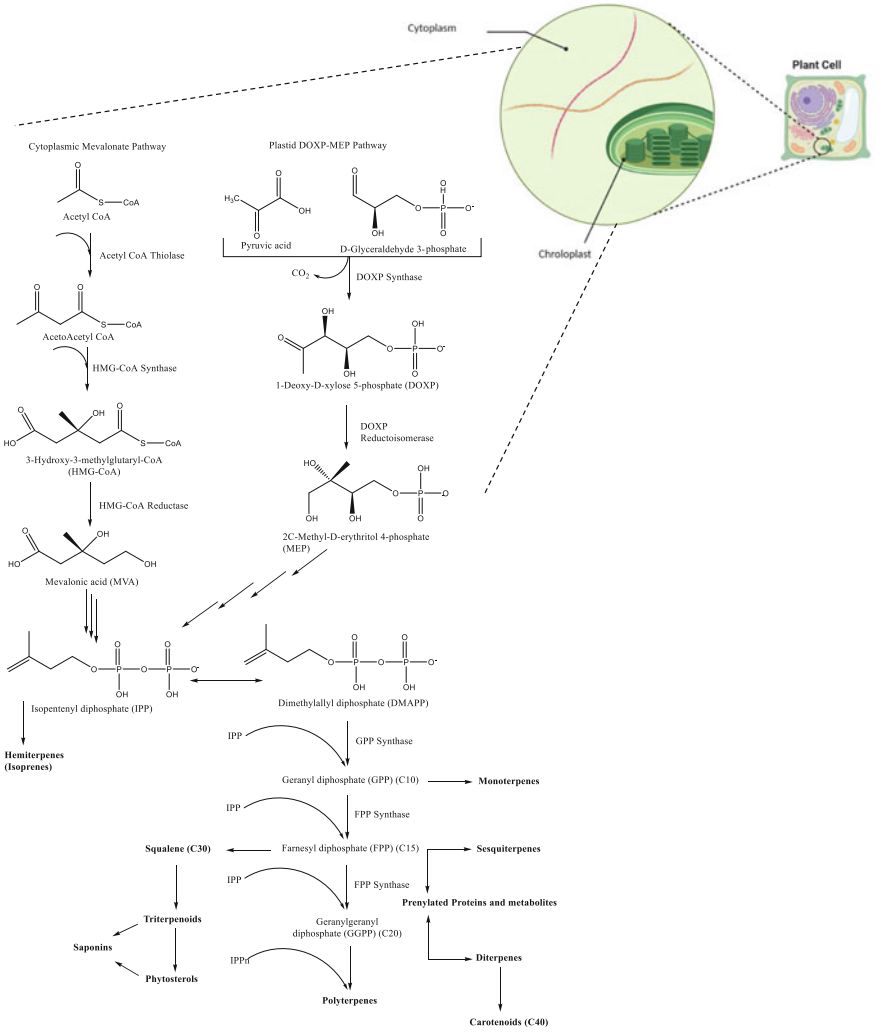

Fig. 25.6 The cytoplasmic mevalonic acid pathway and the plastid DOXP-MEP pathway for the synthesis of the terpene precursors isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) and the various terpene classes

geranyl diphosphate (GPP) synthase to form the GPP precursor from which monoterpenes (C10) are synthesized. Farnesyl diphosphate synthase adds an IPP moiety to GPP to form farnesyl diphosphate (FPP), the precursor of the sesquiterpenes (C15) and the triterpenes (C30). The addition of IPP to FPP forms geranylgeranyl diphosphate (GGPP) the precursor of the diterpenes (C20) and carotenoids (C40).

Monoterpenes, sesquiterpenes, diterpenes, and carotenoids are primarily products of the plastid DOXP-MEP pathway. Monoterpenes and sesquiterpenes are often

flavor and fragrance components of essential oils. The triterpenoids and their derivatives, the phytosterols and saponins are produced via the cytoplasmic mevalonic acid pathways and are often components of cell membranes. Some sesquiterpenoids and the isoprenoids involved in the prenylation of ubiquinone and other proteins are also products of the cytoplasmic synthetic pathways.

In addition to natural routes, synthetic routes for non-natural precursors have also been reported (Kang et al. 2016; Chatzivasileiou et al. 2019; Clomburg et al. 2019). Core structures of terpenes are then post-modified by cytochromes P450s (P450s) that play a vital role in endowing various bioactivities to terpenoids.

25.2.2 Natural Sources and (Bio)chemical Synthesis

Several terpenoids have their roles in plant defense against biotic and abiotic stresses or they are treated as signal molecules to attract the insects of pollination (Singh and Sharma 2015). Terpene can ward off pathogens, predators, and competitors. Living organisms use terpene for multiple reasons like medicine and biotechnology (Singh and Sharma 2015). Wormwood (*Artemisia annua L.*, or *qinghaosu*, *Asteraceae*) is native to China and is the source of the sesquiterpene endoperoxide artemisinin, shown to be highly effective at treating malaria (Nosten and White 2007).

The noted anti-neoplastic agent paclitaxel (also known by its trademarked name Taxol®) was first isolated from the bark of the pacific yew tree (*Taxus brevifolia*) and structurally elucidated in 1971 (Wani et al. 1971).

There are commonly used plants like tea (*Melaleuca alternifolia*), thyme, Cannabis, *Salvia lavandulifolia* (Spanish sage), citrus fruits (lemon, orange, mandarin), etc., that provide a wide range of medicinal values. Tea tree oil has increased in popularity in recent years when it comes to alternative medicine (Carson et al. 2006). Tea tree oil is a volatile essential oil and is famous for its antimicrobial properties, and acts as the active ingredient that is used to treat cutaneous infections (Carson et al. 2006). Apart from the flavour that gives to food, essential oil contain antimicrobial properties (Carson et al. 2006). Thyme is one of plants that synthesize terpene alcohols and phenols which contain powerful antibacterial and antifungal properties (Bound et al. 2015). Terpene synthesized from cannabis also long served as medicines. "Cannabinoid" originally referred to the group of prenylated phenolic compounds from Cannabis spp. (*Cannabaceae*) but now includes any ligand capable of specifically binding to the human cannabinoid receptors, even endogenously produced cannabinoids with no structural similarity to their plant-derived, terpene phenolic counterparts (Devane et al. 1992).

Salvia lavandulifolia is known for anti-dementia (current memory-enhancing) drugs by enhancing cholinergic activity via inhibition of cholinesterase (Porres-martinez et al. 2013).

Monoterpenes are C10, plastid derived terpenoids, often with appreciable volatility. As such, they are frequent constituents of plant essential oils. Their antimicrobial action has been attributed to their general membrane disrupting properties

(Bakkali et al. 2008). The Mentha genus (*Lamiaceae*) provides several *p*-menthane monoterpenoids of pharmacological interest. (–)-Menthol, a major constituent of the essential oil of peppermint (Mentha x piperita), has been known since the 1950s to act as a full agonist of the Cold and Menthol Receptor 1 (Hensel and Zotterman 1951).

Sesquiterpene are frequently responsible for familiar scents and flavors; for instance, ginger (gingerol), clove, cannabis, rosemary (β-caryophyllene), patchouli (patchoulol), sandalwood (α-santalene), and rain (geosmin, a bacterial sesquiterpene). They are the heaviest of the volatile terpenes under standard conditions (diterpene hydrocarbons ordinarily require heating to form gases). Plant families known to be principal producers of sesquiterpene volatiles include the *Lamiaceae, Geraniaceae, Rutaceae, Myrtaceae, Cannabaceae, and Zingiberaceae.* The use of these essential oils in traditional herbal medicine such as aromatherapy and Ayurvedic medicine is well documented (Sharifi-Rad et al. 2017).

Production of terpenoids from natural resources may encounter technical challenges, including the low yields of extraction. For instance, ginsenoside Rh2 content in dried *Panax ginseng* roots is less than 0.01% (Wang et al. 2019). However, using the synthetic biology approach in engineered yeast, the yield of ginsenoside Rh2 has reached 2.25 g/L in fed-batch fermentation (Wang et al. 2019).

Problems associated with their purchasing and adaptation for human use can be solved using chemical synthesis, which is an increasingly economical option in the modern era of chemistry with strategies for reducing the time and cost of terpene synthesis for drug discovery. Chemical synthesis strategies have been recently reviewed and include: stereochemistry-based, structure-goal, transform-based, functional group-based (Jansen and Shenvi 2014).

The rapid advances in synthetic biology suggest an alternative sustainable approach to achieve the industrial scale of terpenoids production (Bian et al. 2017; Clomburg et al. 2017; Wang et al. 2018).

Synthetic biology is presenting possibilities for sustainable and efficient production of high value-added terpenoids in engineered microbial cell factories, using *Escherichia coli* and *Saccharomyces cerevisiae*. There is a growing interest in utilizing the acetyl-CoA pool in mitochondria for the biosynthesis of value-added compounds. For example, the production of geraniol in *S. cerevisiae* mitochondria was sixfold increase compared to cytosolic producing strains (Yee et al. 2019). Recently, a lipophilic lycopene production strategy in *S. cerevisiae* by using lipid droplets accumulation resulted in lycopene continuous accumulation to 2.37 g/L in 5 days (Ma et al. 2019).

Expansion of the endoplasmic reticulum (ER) stimulated the production of triterpene biosynthesis enzymes and increased triterpenoid and triterpene saponin accumulation (Arendt et al. 2017). Engineered *S. cerevisiae* by overexpressing a key ER size regulatory factor resulted in the overproduction of squalene by 71-fold (Kim et al. 2019).

Cell free biosynthesis (CFB) systems (purified enzyme system and crude cell extracts) are easy to use multiple enzyme pathways sourced from various organisms,

and also overcome the challenges of precursor supply and products toxicity (Dudley et al. 2015; Li et al. 2018).

However, several significant challenges remain in microbial biosynthesis, including (1) the biological parts for genetic circuits construction have not been sufficiently characterized; (2) the post-modifications of terpenoids remain inefficient; and (3) the toxic accumulation of intermediate products and insufficient supply of precursors.

25.2.3 Structure–Activity Relationship

Hemiterpenes are the simplest, with a single isoprene unit (e.g., prenol, angelic acid, isovaleric acid, tiglic acid). Hemiterpenes are being investigated as potential sources of biofuel (Reynolds and Enriquez 2017).

Monoterpenes have two isoprenoid units (Magnard et al. 2015). They are odoriferous compounds that partly account for the scent of many flowers and fruits. Monoterpenes include acyclic (citral, myrcene, ocimene, etc.), monocyclic (carvone, menthol, D-limonene, etc.), and bicyclic forms (α-pinene, α-thujone, β-thujone, . . .). They are components of essential oil compounds that give plants aroma and flavor and are active ingredients for agricultural, pharmaceutical, cosmetic, and food application (Kang and Lee 2016).

Sesquiterpenes are an abundant natural compounds with 3-isoprene (Ashour et al. 2018). They share the same ring classification with monoterpenes, plus tricyclic terpenes. This terpene diversity arises from the arrangement of the 15-carbon skeletons, the layering of the functional groups, and the substituents on their backbone (Lorigooini et al. 2020). This compound group includes both hydrocarbons (humulene, farnesene), aldehydes (farnesal and lepidozenal), oxygenated hydroxyl or carbonyl derivatives, esters (torilin and ejaponines), and also alcohols (δ-elemanol and β-germacrenol) (Lorigooini et al. 2020). They are antimicrobial, antifungal, antitumor, and anti-inflammatory agents. They have wide plant defense applications against herbivores and are active constituents in the perfumery industry (Li et al. 2020b).

Diterpenes are non-volatile C20 hydrocarbons derived from four isoprene units (Reynolds and Enriquez 2017). They include linear, bicyclic, tetracyclic, pentacyclic, or macrocyclic forms. Diterpenes are characterized by polyoxygenated keto and hydroxyl groups (Ashour et al. 2018). Diterpenes from various sources have exhibited inhibitory effects against pathogenic microbes, herbivore pests, and weeds. These promising biological activities place them among the essential agricultural secondary metabolites with potential in the production of biopesticides (De Sousa et al. 2018).

Triterpenes are derivatives of the C30 precursor, squalene, with over 20,000 known members, most in the plant kingdom. However, bacteria and sea cucumbers produce defense-related triterpene glycosides (Thimmappa et al. 2014). Two sesquiterpene molecules form triterpenes by linking in a head–head fashion (Thimmappa et al. 2014). Cyclic triterpenes (1–5 rings) are the most significant

members. They are primarily alcohols, aldehydes, or carboxylic acids (Ashour et al. 2018). A cyclopentane perhydrophenanthrene ring system defines sterols and phytosterols as triterpenes (Ashour et al. 2018). Glycosylated triterpenes, such as saponins, protect plants against pathogenic microbes and insect pests. Some simple triterpenes are signaling molecules that are also constituent ingredients in the food, health, and biotechnology industries (Thimmappa et al. 2014).

Tetraterpenes (carotenoids) are 8-isoprene units consisting of C40 (Maoka 2020). Carotenoids are the most studied tetraterpenes, with more than 750 members (Maoka 2020). Terrestrial plants, algae, and cyanobacteria all produce tetraterpenes. Their biological roles include light trapping, antioxidative function, and plant protection against free radicals. They are also involved in plant hormone synthesis and form the structural components of cell membranes. They are active ingredients in the pharmaceutical and food industries (Sozer et al. 2010; Domonkos et al. 2013).

25.2.4 Active Principles/Target Diseases

Not only do terpenes demonstrate broad structural diversity, but they also exhibit a wide array of biological actions. In recent years, with the deepening of research on terpenoids (especially terpenoids in medicinal plants), it has been found that such compounds play an increasingly prominent role in the field of medicine and have various biological activities such as antitumor, anti-inflammatory, antibacterial, antiviral, antimalarial, promoting the transdermal absorption, preventing and treating cardiovascular diseases, lowering blood sugar, and other effects. In addition, some terpenoids also have insecticidal, immunomodulatory, antioxidant, anti-aging, and neuroprotective effects. These activities are summarized in Table 25.2. Out of these natural compounds, several terpenes are under studies of clinical trials.

In phase I clinical trial of orally administered D-limonene, 17 women and 15 men aged 35–78 years old with advanced metastatic solid tumors received an average of three treatment cycles of 21 days at dose ranging from 0.5 to 12.0 g/m^2 body surface area (Saldanha and Tollefsbol 2012). Carcinoma's regress was observed, when D-limonene is added to the diet either when the tumor is small or still capable of spontaneously regressing, probably by cytostatic action.

In humans, 1,8-cineole inhibits sensory irritations caused by octanol and methanol with sensitive volunteers (Takaishi et al. 2012). 1,8-cineole not only reduces exacerbation rate but also provides clinical benefits as manifested by improved airflow obstruction, reduced severity of dyspnea and improvement of health status (Juergens et al. 2003). Therefore, it can provide a useful treatment option for symptomatic patients with chronic obstructive pulmonary disease (COPD).

Extracts of resin enriched in pentacyclic triterpenoid known as boswellic acid have been employed as anti-inflammatory drugs (Anthoni et al. 2006). Clinical trials have demonstrated promising benefits from boswellic acids in rheumatoid arthritis, chronic colitis, ulcerative colitis, Crohn's disease, and bronchial asthma in addition to benefits for brain tumor patients (Singh and Sharma 2015). The effects of

Table 25.2 Terpenes and their pharmaceutical properties

Plant source	Compound	Function	References
Anticancer			
Citrus peels, orange peels, and several other citrus fruits	Limonene	Strong cancer inhibition activity both in vitro and in vivo	Miller et al. (2013); Lee et al. (2017)
Limonene metabolite	Perillyl alcohol	Broad-spectrum anticancer	Chen et al. (2015a); Sobral et al. (2014)
Rose oil, citronella, lemongrass, lavender	Geraniol	Antilung cancer, colon cancer, prostate cancer, pancreatic cancer, and liver cancer	Galle et al. (2014); Carnesecchi et al. (2001); Cho et al. (2016); Kim et al. (2012)
Costus (*Saussurea lappa Clarke*) root, among others	Costunolide	Antibladder cancer, ovarian cancer, leukemia cells, prostate cancer, non-small cell lung cancer, esophageal cancer	Hassan et al. (2018); Rasul et al. (2013); Yang et al. (2011); Hsu et al. (2011)
Herbaceous plant *Nigella sativa* (black cumin)	Thymoquinone	Anticancerous against several cancers such as breast cancer, skin cancer, non-small cell lung cancer, bile duct cancer, and brain cancer	Sobral et al. (2014); Majdalawieh et al. (2017)
Artemisia annua L. (sweet wormwood)	Artemisinin and its derivatives	Antileukemia, melanoma, colon cancer, non-small cell lung cancer, lung cancer, prostate cancer, breast cancer, and ovarian cancer	Hsu et al. (2011); Efferth and Kaina (2010); Crespo-Ortiz and Wei (2012)
Pacific yew tree (*Taxus brevifolia*)	Paclitaxel	Treatment of breast, lung, and ovarian cancer, as well as Kaposi's sarcoma	Song et al. (2015); Sun et al. (2018a)
Mirabilis jalapa plant and many fruits and herbs	Ursolic acid	Antiliver cancer, breast cancer, osteosarcoma, prostate cancer, and cervical cancer	Chang et al. (2016); Lewinska et al. (2017); Wang et al. (2017)
Cucurbitaceous plants (*Bryonia, Cucumis, Cucurbita, Luffa, Echinocystis, Lagenaria, and Citrullus*)	Cucurbitacin	Antibladder cancer, liver cancer, pancreatic cancer, breast cancer, and leukemia	Duangmano et al. (2010)
Antidiabetic			
Leaves of the herbaceous plant *Andrographis paniculata. A. paniculata*	Andrographolide	Reduces plasma glucose and increasing the utilization of glucose by	Gupta et al. (2007)

(continued)

Table 25.2 (continued)

Plant source	Compound	Function	References
		the body in diabetes mellitus rats	
Curcuma longa	Curcumin	Acts by quashing the oxidative stress and inflammation. Reduces the plasma glucose and levels of glycosylated hemoglobin	Nabavi et al. (2015)
Plant stevia (*Stevia rebaudiana* (Bertoni) Hemsl.)	Diterpenoid stevioside (SVS)	Improves the hyperglycemia of rats	Jeppesen et al. (2006)
Antidepressant			
Litsea glaucescens and *Tagetes lucida* and flowers of lavender	Linalool and β-pinene	Interact with serotonergic and adrenergic receptors	Guzmán-Gutiérrez et al. (2015)
Oil from the stems and flowers of Syzygium aromaticum (cloves), of Cannabis sativa, rosemary, and hops	β-caryophyllene	Ameliorates the depressive symptoms in mice	Bahi et al. (2014)
Extracts of *Hypericum perforatum*	Hyperforin	Inhibits the neuronal uptake of serotonin, dopamine, norepinephrine, GABA, and L-glutamate	Subhan et al. (2010)
Valeriana wallichii	Maaliol, patchouli alcohol, and 8-acetoxypatchouli alcohol	Reduces the stress and anxiety levels but also improves the symptoms of depression in humans	Bhattacharyya et al. (2007)
Anti-inflammatory			
Roots of *Paeonia lactiflora* Pall	Paeoniflorin	Inhibit the production of inflammatory factor nitric oxide (NO), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF-α) induced by lipopolysaccharides (LPS)	Bi et al. (2017)
Inula flower (*Inula japonica* Thunb.)	IVSE	Inhibits the production of NO induced by LPS	Chen et al. (2015b)
Inula flower (*Inula japonica* Thunb.)	JEUD-38	Attenuated LPS-induced NO production	Wang et al. (2015)
Tripterygium wilfordii Hook	Triptolidenol	Found to treat various autoimmune and inflammation-related disorders, mainly by	Graziose et al. (2010)

(continued)

Table 25.2 (continued)

Plant source	Compound	Function	References
		inhibition of the production of inflammatory cytokines	
Ginseng	Ginsenoside	Ginsenoside-Rb1 (G-Rb1) is a potential anti-inflammatory agent that inhibits the activation of NF-κB; Ginsenoside-Rb2 (G-Rb2) and ginsenoside-Rd (G-Rd) exhibited neuroprotective effects	Kim et al. (2017)
Antibacterial			
Salvia and Eucalyptus leaves	1,8-eucalyptus	*Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, and *Streptococcus*, *Listeria*, *Bacillus cereus*	Domonkos et al. (2013); Dogan et al. (2017)
Citrus peels, orange peels, and several other citrus fruits	Limonene Geranialdehyde	*B. subtilis*, *S. aureus*, *Streptococcus mutans*, *E. coli*, *Candida albicans*, methicillin-resistant *S. aureus*, and *Saccharomyces cerevisiae*	Lee et al. (2007)
Oils of plants including Marjoram, holm oak (*Quercus ilex*) and Norway spruce (*Picea abies*)	Sabinene	*S. aureus* (Gram-positive) and *E. coli* (Gram-negative)	Wagner et al. (2017)
Mentha arvensis (wild mint) and Mentha × piperita (peppermint) leaves	Menthol	*S. aureus*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Haemophilus influenzae*	Park et al. (2016)
Oil of several plants e.g., *Juniperus sabina L.*	Sabinol	Oral bacteria	Wang et al. (2016)
Oils from seeds of caraway (*Carum carvi*), spearmint (*Mentha spicata*), and dill	Carvone	*S. aureus*, *E. coli*, *B. subtilis*, *Salmonella typhimurium*, *Listeria*	Chan et al. (2016); Shahbazi (2015)
Leaves of *Pogostemon cablin* (Blanco) Benth. (*Lamiaceae*)	Patchouli alcohol	*Helicobacter pylori*	Xu et al. (2017)
Artemisia annua L. (sweet wormwood)	Artemisinin	Various pathogens such as *B. subtilis*, *E. coli*, *Pseudomonas aeruginosa*,	Appalasamy et al. (2014); Kim et al. (2015a)

(continued)

Table 25.2 (continued)

Plant source	Compound	Function	References
		S. cerevisiae, *S. aureus*, *Mycobacterium tuberculosis*	
Leaves of the herbaceous plant *Andrographis paniculata. A. paniculata*	Andrographolide	*P. aeruginosa*	Zhang et al. (2020)
Olives (*Olea europaea L.)*	Oleanolic acid	*S. aureus*, methicillin-resistant *S. aureus*, *S. mutans* and *Listeria monocytogenes*, *Enterococcus faecium*, *Enterococcus faecalis*	Kim et al. (2015b)
Antiparasitic			
Artemisia annua Linn	Artemisinin	Antiplasmodium activity in the red blood cell phase	Jeppesen et al. (2006)
Citrus peels, orange peels, and several other citrus fruits	Limonene	Antiplasmodial activity against *Plasmodium falciparum*	Jordão et al. (2011)
Pine trees	Pinene (α, β isomers)	Effective against the W2 strain of *Plasmodium falciparum*	Boyom et al. (2011)
Oil from the stems and flowers of Syzygium aromaticum (cloves), of Cannabis sativa, rosemary, and hops	Caryophyllene	Active component of insect repellents especially for mosquitoes	Maia and Moore (2011)

boswellic acid on central signaling pathways in human platelets and on various platelet functions have been investigated (Poeckel et al. 2005). Clinical trials of gum-resin of *Boswellia serrata* have shown to improve symptoms in patients with osteoarthritis and rheumatoid arthritis (Poeckel and Werz 2006).

β-sitosterol is used to prevent and relieve prostate symptoms and has been tested for thousands of years in Asia and Mediterranean (Richelle et al. 2004). Taking β-sitosterol at the dose of 60–110 mg/day significantly improve urinary symptoms. It increases the maximum urinary flow and decreases the volume of the urine left in the bladder. β-Sitosterol is also used to lower cholesterol, and therefore incorporated in cholesterol-lowering margarine (Berges et al. 2000). β-Sitosterol has been recommended as natural supplement to promote prostate health (Bent et al. 2006). A randomized controlled trial of 47 patients with pulmonary tuberculosis investigated adjuvant β-sitosterol therapy vs placebo (de Cássia da Silveira e Sá et al. 2013). The β-sitosterol treatment group (average dose 60 mg/day) demonstrated increased weight gain, higher lymphocyte and eosinophil count, and a generally faster clinical recovery.

Cannabis has been used for millennia to reduce pain. Herbal cannabis is currently strongly promoted by some patients and their advocates to treat any type of chronic pain. A recent review of randomized, double-blind controlled trials of cannabis against placebo with chronic neuropathic pain in adults, concluded that cannabis-based medicines may increase the number of people achieving 50% or greater pain relief compared with placebo (Mücke et al. 2018). However, moderate to high certainty evidence shows that non-inhaled medical cannabis or cannabinoids (administered orally or topically) results in a small to very small improvement in pain relief, physical functioning, and sleep quality among patients with chronic pain, along with several transient adverse side effects, compared with placebo (Wang et al. 2021).

In the 1960s, the National Cancer Institute (NCI) discovered a crude extract from the bark of the Pacific yew, *Taxus brevifolia*, that showed activity against many tumor types (Wani et al. 1971). Some years later the active ingredient, paclitaxel, was isolated. By the mid-1980s, the NCI had initiated the clinical Phase I trials (Oberlies and Kroll 2004). Paclitaxel for ovarian cancer is the current standard front-line therapy along with carboplatin (Kumar et al. 2010). Several newer forms of paclitaxel have been manufactured, aimed at decreasing toxicity, increasing efficacy, and improving ease of administration, including Abraxane®, Taxoprexin®, Paclitaxel poliglumex, and ANG1005 (Kumar et al. 2010).

Artemisinin (ART), bearing a peroxide bridge in its sesquiterpene lactone structure, can be extracted and separated from *Artemisia annua L.* (sweet wormwood). ARTs which are already established as safe drugs for treating malaria, possess a host of advantages that make them worthy of development as novel anticancer agents. Outcomes from clinical trials provide encouraging evidence for their excellent antitumor activities (Xu et al. 2020). However, some problems such as poor solubility, toxicity, and controversial mechanisms of action hamper their use as effective antitumor agents in the clinic. In order to accelerate the use of ARTs in the clinic, researchers have recently developed novel therapeutic approaches including developing novel derivatives, manufacturing novel nano-formulations, and combining ARTs with other drugs for cancer therapy (Xu et al. 2020).

25.3 Nitrogen-Containing Compounds

Nitrogen is part of the structure of a huge number of plant secondary metabolites, the so-called nitrogen-containing (N-containing) compounds. This class is a wide and heterogeneous class of compounds that apart from the nitrogen content, do not present similarities among the compounds from any angle, whether biochemical, chemical, or physiological. N-containing compounds are synthesized in the tricarboxylic acid cycle pathway (Jamwal et al. 2018). However, their biosynthesis is indirectly linked to the shikimate pathway by the production of phenylalanine, tyrosine, and tryptophan. These amino acids are some of the building blocks of protein synthesis and common precursors for plant N-containing compounds. In

plants, this group of compounds has been described to have a major role in plant defense. These metabolites exert their effects by acting as prevention to predators, toxicity, or precursors to physical defense systems.

Much work has been performed on several species of our major foodstuffs such as legumes, almonds, apricot, cherries, peaches, quinoa (Mazid et al. 2011; Lin et al. 2019; Haneklaus and Schnug 2004). Studies of species non-related to food include different species from which artemisinin, benzylisoquinoline, cannabinoids, ginsenosides, monoterpenoid indole alkaloids, withanolides, and taxol were isolated. These compounds have been the focus of exhaustive studies due to their pharmacologically significant bioactivities to human health. Besides being species-specific and organ-specific, the production of these compounds depends on many biotic and abiotic factors.

Four classes of N-containing secondary metabolites are commonly defined: alkaloids, cyanogenic glycosides, and non-protein amino acids. Some classifications include also glucosinolates because they are N- and sulfur-containing secondary metabolites. In this chapter, glucosinolates will be discussed in sulfur-containing secondary metabolites.

25.3.1 Alkaloids

One of the main groups of N-based compounds are the alkaloids. A common classification of alkaloids divides this wide group into three classes: the "true alkaloids," the "proto-alkaloids," and the "pseudo-alkaloids" based on their precursors (Chiocchio et al. 2021). The "true alkaloids" derive biosynthetically from amino acids and are heterocyclic N-containing compounds; the "proto-alkaloids" also derive biosynthetically from amino acids but are N-containing compounds where the nitrogen atom is not heterocyclic; and, the "pseudo-alkaloids" include compounds that do not originate from amino acids, but harbor a nitrogen atom inserted into the molecule. Upon this general classification and according to their basic chemical structure, more than 150 families of alkaloids are commonly described. Among these families the major ones include indoles (e.g., reserpine), isoquinolines (e.g., papaverine), piperidines (e.g., piperine), pyrrolidines (e.g., stachydrine), pyrrolizidines (e.g., heliotridine), quinolizidines (e.g., lupinine). Other common families are acridones, aromatics, bisindoles, ephedras, indolizidines, imidazoles, manzamines, oxindoles, phenylethylamines, phenylisoquinolines, purines, pyridines, pyrroloindoles, and tetrahydroisoquinolines (Tadeusz 2015).

In general, one or few amino acids are the precursors for the synthesis of alkaloids, namely lysine, tryptophan, or tyrosine, but the carbon skeleton of alkaloids might also contain other components such as cholesterol, that derive from the terpene pathway.

In plant kingdom, alkaloids are common in families of flowering vascular plants, e.g. *Apocynaceae*, *Leguminosae*, *Magnoliaceae*, *Ranunculaceae*, *Rubiaceae*, and *Solanaceae* (Tadeusz 2015). On the other hand, alkaloids are not common in lower

plants (Hussein and El-Anssary 2018). Alkaloids have a wide distribution in all organs such as leaves, flowers, fruits, bulbs, seeds, roots, stems, and bark. Due to their distribution in plants, many alkaloids occur in food and beverages. In the human diet, the most common food/plants that contain alkaloids include coffee (caffeine), cacao (theobromine), potatoes (solanine), tea leaves (theophylline, caffeine), and tomatoes (tomatine). In general, caffeine is the most worldwide consumed alkaloid. For some of these alkaloids known to occur in the human diet, the risks imminent from human intake are considered to be insignificant (Koleva et al. 2012). Nevertheless, governmental agencies are conscious of the potential risks and suitable regulatory actions are being considered for most alkaloids. These regulatory actions vary from try to state safe upper limits, set limits for the maximum concentration of a compound in feed, beverages, and foods, and advising the consumers to be cautious, among others.

It has been suggested that alkaloids have a function in protecting plants from damaging activities of some insect species and herbivorous organisms. In fact, alkaloids have reportedly a bitter taste, a common trait in many plant-based compounds to avoid intake by herbivorous organisms. In addition, several other activities have been reported in literature such as toxicity for vertebrates, cytotoxic activity, carcinogenic activity, antiviral, antifungal, and antibacterial. In fact, some alkaloids are so toxic to cause animals death if eaten. These lethal activities of some alkaloids are known for centuries; for example, the well-known strychnine (from *Strychnos* species).

The interest in the alkaloids rises from the diverse array of pharmacological actions on humans vastly demonstrated by these compounds. Among these actions are antineoplasic, analgesia, cardiac stimulation, hypertensive and hypotensive properties, local anesthesia, muscle relaxation and toxicity, respiratory stimulation and relaxation, vasoconstriction and neurotoxic effects. An extensive revision on these and other pharmacological actions has been recently reported (Dey et al. 2020). Other widely reported activities of alkaloids report them as antibacterial, antiviral, antifungal, and anticancer agents (Thawabteh et al. 2019).

In the pharmacological field, morphine is one of the most well-known alkaloids. Morphine is a potent narcotic used for medical purposes, namely to pain relief, but its utility is restricted because of addictive properties. On the other hand, codeine, the methyl ether derivative of morphine, is shown to be relatively nonaddictive while possessing an excellent analgesic activity. Both alkaloids act as cardiac or respiratory stimulants. Some other reported examples of worldwide-known alkaloids include atropine to treat bradycardia (low heart rate), ergonovine and ephedrine both acting as blood vessel constrictors, tubocurarine used in surgery as muscle relaxant, and the chemotherapeutic agent's vincristine and vinblastine used in the treatment of many cancer types. Quinine and quinidine are alkaloids from *Cinchona* species. Quinine is a potent antimalarial agent but is frequently replaced by synthetic drugs that are less toxic and more efficient. Quinidine is used for treatment of heart arrhythmias. Another common example is colchicine recognized for the treatment of severe gout attacks, existing in plants of *Liliaceae* family.

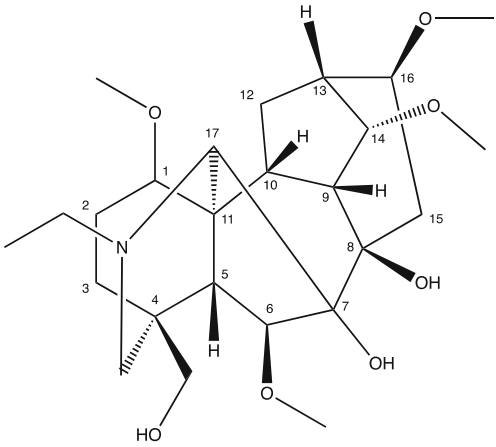

Fig. 25.7 Lycoctonine chemical structure

Nicotine is also a widely known alkaloid present in *Nicotiana tabacum*, the tobacco plant. It is the main ingredient in tobacco smoked cigarettes and is a powerful stimulant. This alkaloid is highly addictive.

Due to the high heterogeneity of the alkaloid group, it is practically impossible to have general structure–activity relationships. Nevertheless, some examples of structure–activity relationship within specific alkaloid families have been reported in literature and extensively revised. A study in which the comparative antiarrhythmic cardiac efficacies of diterpene alkaloids based on denudatine, heteratisine, lycoctonine, and napelline skeletons was studied, found some structure–activity relationships (Dzhakhangirov et al. 1997). On lycoctonine skeleton derivatives, the introduction into one of the C-1, C-4, C-6, and C-14 positions of an aromatic ester group (Fig. 25.7) leads to a sharp rise in the antiarrhythmic activity. In fact, this activity was typical both of amino alcohols and of their derivatives, rising as following: amino alcohols < amino alcohols monoacetyl derivatives < amino alcohols monoaromatic derivatives. A second group of extremely active antiarrhythmic compounds was formed by alkaloids bearing a benzoyl group at C-14. Furthermore, these authors attracted attention that a fine difference in the structure, as the occurrence of an anthranilic acid linked through a C18 methylene group, changes drastically the pharmacological effect, exhibiting no antiarrhythmic action. Another study summarized that the antibacterial, antifungal, and antiviral activity of isoquinoline alkaloids, namely N-methyltetrahydroprotoberberine, protoberberine, and benzophenanthridine, was increased by the presence of a quaternary nitrogen and the methylenedioxy at C-2 and C-3 (Qing et al. 2017). The antimalaria activity of an isoquinoline alkaloid (protoberberine) also seems to depend on the kind of quaternary structures, namely nitrogen with a positive charge in the aromatic ring, as well as on the type of substituent groups on rings A and D, and on the size and the nature of substituent groups at the C-13 position (Iwasa et al. 1998). The (anti)-carcinogenic activity of pyrrolizidine alkaloids by the biological activity of DNA-cross linking seems to depend on two structural determinants: the occurrence

of both an ά,β-unsaturated ester function and a macrocyclic necic acid ester (Hincks et al. 1991).

25.3.2 Cyanogenic Glucosides

Cyanogenic glucosides (CNglcs) consist of two regions, an aglycone (-α-hydroxynitrile-type) and a sugar moiety (Singh 2018). The most common sugar moieties in CNglcs are monoglycosides (mostly D-glucose) but di- and triglycosides also occur in some compounds. There are approximately 25 CNglcs dispersed in insects and in more than 2500 plant species including gymnosperms and angiosperms. Members of Compositae, Fabaceae, Leguminosae, Linaceae, and Rosaceae family have CNglcs. From these families a significant number of species are included in human diet such as apricots, apples, cherries, barley, beans, cassava, peaches, peas, plums, and sorghum (Singh 2018; Yulvianti and Zidorn 2021). Even though apples are rich sources of several bioactive compounds and vitamins, high levels of CNglcs are present in apple seeds, namely amygdalin (Bolarinwa et al. 2015). Moreover, high levels of CNglcs have been described to occur in many nutritious plants, which limits their use as food. Well-known examples are linamarin, lotaustralin, and amygdalin CNglcs present in cassava plant (*Manihot esculenta*) leaves, stem, and root. Taxiphyllin CNglcs in bamboo shoots is also an important problem. Despite its nutritional value, bamboo shoots hold lethal concentration of this CNglcs. Also, many forage plants have CNglcs, such as *Melilotus albus Medic, Lotus* spp., *Sorghum* spp., and *Trifolium* spp. (Sun et al. 2018b). Three most common CNglcs have been found in forage plants: dhurrin that is mostly found in sorghum and derives from tyrosine and; lotaustralin and linamarin that derive from isoleucine and valine, respectively, occur in white clover and lotus (Forslund et al. 2004). At the end, the most common occurrence pattern is that a specific CNglcs will occur in one or two plant families (Vetter 2000).

The precursors for most of the CNglcs are thought to be L-isoleucine, L-leucine, L-phenylalanine, L-tyrosine, L-valine, and cyclopentenyl-glycine, a non-protein amino acid. The general pathway of biosynthesis of CNglcs is given through a series of hydroxylation and glucosylation reactions of the precursor α-amino acid (Vetter 2000). Therefore, the variety of amino acid precursors dictates the diversity of CNglc structures. CNglcs are known to have significant roles in plant development, growth, and to promote resistance to abiotic and biotic stresses. CNglcs function in plant defense toward herbivores is mainly attributed to their capacity to release toxic hydrogen cyanide upon tissue disruption and enzymatic breakdown. CNglcs function in plant development is mainly due to their role as transporters of nitrogen and glucose as well as storage during specific developmental stages of plants in view of endogenous turnover pathways (Sun et al. 2018b).

The bottom line is that the presence of CNglcs in plants and crops can cause severe problems, for both animals and humans. The CNglcs hydrolysis occurs through the β-glucosidase enzyme, releasing the sugar moiety and a cyanohydrin

which decomposes spontaneously to hydrogen cyanide and an aldehyde or ketone. Alimentary exposure to high levels of some CNglcs might cause acute cyanide intoxication or an irreversible debilitating neurological condition. Examples of these neurological conditions are Konzo, an upper motor neuron disease, and tropical ataxic neuropathy whose symptoms include neuro sensory deafness, optical atrophy, sore tongue, and sensory gait ataxia (Bolarinwa et al. 2016). CNglcs consumption can also aggravate other health problems, namely goiter, cretinism, and growth retardation. There are no reported pharmacological applications for these compounds.

25.3.3 Non-protein Amino Acids

Amino acids are well-known as the building molecules of proteins. Their chemical structure has at least one basic amino group, one acidic carboxyl group, and a side chain. Unlike proteogenic amino acids that are used in proteins and that are naturally encoded by the genetic code, non-protein amino acids are not and are commonly named as non-coded amino acids (Ribas de Pouplana 2014). Non-protein amino acids are intermediates in metabolism or secondary metabolites, produced from natural protein amino acids, and some of them can be part of biological compounds. Examples include β-alanine, a building block of pantothenic acid (vitamin B5); and γ-amino butyric acid, a neurotransmitter in the brain of mammals, that is originated by decarboxylation of glutamic acid (Pizzarello 2015). Non-protein amino acids are found in numberless living systems and around 900 non-protein amino acids have been isolated from plants (Idrees et al. 2020) where they are abundantly found in legumes and seeds. In fact, around 250 of non-protein amino acids are found specifically in a small group of plant families such as the *Aceraceae*, *Cucurbitaceae*, *Leguminosae*, and *Hippocastanaceae* (Singh 2018). They have varied biological functions and are usually toxic in nature. In plants, non-protein amino acids have different roles including allelochemical, antimicrobial, and antiherbivory activity. These toxic effects have key ecological roles in the plants that synthesize them, for example, as antifeedant to predators or by preventing the growth of competing plant species (Bell 2003). Hypoglycine, canavanine, and mimosine are examples of non-protein amino acids (Yamane et al. 2010). Nevertheless, despite many non-protein amino acids have been reported, relatively little is known about their biosynthesis.

25.4 Sulphur-Containing Compounds

Sulphur-containing secondary metabolites in plants constitute a small group of low-molecular-weight natural products, which play a pivotal role in plant defense mainly present in the Asteraceae, Alliaceae, and Brassicaceae families. Their metabolism is

generally induced by biotic and abiotic stress and they are usually divided into phytoalexins, defensins, isothiocyanates, and cysteine sulfoxides. Their metabolism, including biosynthetic genes and pathways, their occurrence and structural diversity, and the promising pharmacological properties were researched over the following decades and will be summarized in this chapter.

25.4.1 Phytoalexins

Phytoalexins are secondary metabolites with highly recognized antimicrobial properties produced by plants in response to biotic and abiotic stressors. Indeed, the term phytoalexin was firstly introduced after the potato inoculation with *Phytophthora infestans*, which promoted the synthesis of a putative defense metabolite that provided resistance to a compatible race of the pathogen (Mueller et al. 1939). Phytoalexins can derive from the phenylpropanoid pathway or can be synthesized through the mevalonic acid pathway and/or the Tr pathway (Ahmed and Kovinich 2021).

Among the 58 families of transcription factors (TFs) in plant kingdom, 8 are involved in regulating phytoalexin biosynthesis; WRKY, MYB, NAC-no apical meristem (NAM), bHLH (basic helix-loop-helix), *Arabidopsis* transcription activation factor (ATAF1/2), cup-shaped cotyledon (CUC2)], AP2/ERF (APETALA2/ethylene responsive factor), and bZIP (basic leucine zipper); but the first 4 has been the most widely studied:

– *WRKY (WRKYGQK motif).* This family are formed by several transcription factors highlighting GaWRKY1 from cotton (*Gossypium arboreum L.*) elicited both spatially and temporally with gossypol biosynthesis genes by biotic (pathogen) and abiotic (inorganic) elicitors; WRKY33, which is activated by the fungal elicitor flg22 binding the biosynthesis gene phytoalexin deficient 3 (PAD3) by ChIP-PCR
– MYB (myeloblastosis-related), VvMYB14 and VvMYB15 (R2R3-type MYB TFs) from Chinese wild grape, *Vitis quinquangularis-Pingyi* and grapevine (*Vitis vinifera*) were co-induced with stilbene biosynthesis genes and could directly bind the promoter of stilbene synthase (STS) in transient promoter-reporter assays. ROS-responsive regions were reported on these TFs from *Vitis labrusca* "*Concord*" but the transacting factors that bind those promoter segments have not been reported yet. Besides, AtMYB34, AtMYB51, and AtMYB122 are Tfs from MYB family implicated in regulating camalexin biosynthesis in *Arabidopsis*
– NAC-no apical meristem (NAM) TF ANAC042 revealed that it was required for WT levels of camalexin elicitation by pathogens and heavy metals. Chromatin immunoprecipitation followed by high-throughput sequencing (ChIP-seq) found that ANAC042 directly binds key genes involved in gibberellin (GA) and brassinosteroid (BR) biosynthesis to suppress plant growth. Silencing the TFs

from NAC family, GmNAC42-1 in soybean hairy roots elicited with *P. sojae* WGE decreased the expressions of glyceollin gene transcripts and metabolites, demonstrating that it was essential for the elicitation of glyceollin biosynthesis

– bHLH (basic helix-loop-helix) regulates phytoalexin synthesis in rice, soybean, and *Medicago truncatula*. bHLHs are often required by R2R3-type MYBs for the MYBs to bind DNA in order to regulate various branches of phenylpropanoid metabolism

Since growing research has been made to understand the biosynthesis pathways and biosynthetic genes, how evolution resulted in conserved TFs regulating different phytoalexin biosynthetic pathways remains unclear. Cutting-edge technologies such as BLASTPs are being used but correlations between phytoalexin TFs from among different plant species were not identified in the top ten most similar proteins. This has led to the concept that phytoalexin TFs are as diverse as the biosynthetic pathways that they regulate. Indeed, while the upstream signaling pathways which regulate the process have been studied, further research is needed to understand the phytoalexin biosynthetic genes and the TFs that regulate them.

In a general overview, the plant immune system consists of two branches (Fig. 25.8). In the first branch, plant trans-membrane pattern recognition receptors (PRRs) recognize and trigger a response to highly conserved, slowly evolving, pathogen-associated molecular patterns (PAMPs). This activates PAMP-triggered immunity (PTI) that stimulates broadly the defense responses of plants with no pathogen-specific prioritization. The second branch, known as effector, triggered immunity (ETI), is triggered by effector proteins that are specific to certain species or races of a pathogen.

Briefly, several events typically flow in response to a biotic elicitor in plant cell receptors. These events usually includes a reversible phosphorylation–dephosphorylation reaction of plasma membrane and cytosolic proteins releasing Ca^{2+} and spiking of proton levels in the cytosol, reactive oxygen species (ROS) production, mitogen-activated protein kinase (MAPK) activation, defense gene expression, hormones such as ethylene/JA and oxylipin, or salicylic acid biosynthesis and signaling, and finally the activation or expression of TFs for defense gene expressions such as those for the biosynthesis of phytoalexins.

Overall, a systems-level study of the responses of a wide range of plant species to PAMP/effector combinations is needed to understand how these hormones signaling pathways function to effect phytoalexin elicitation.

25.4.2 Defensins

Plant defensins are small (c.a. 5 kDa), basic, cysteine-rich proteins with antimicrobial activities. Many of the defense peptides have a compact structure stabilized by disulphide bonds, which ensures their resistance to proteases, changes in temperature, or pH of the environment. They are ubiquitous in plants and form part of the

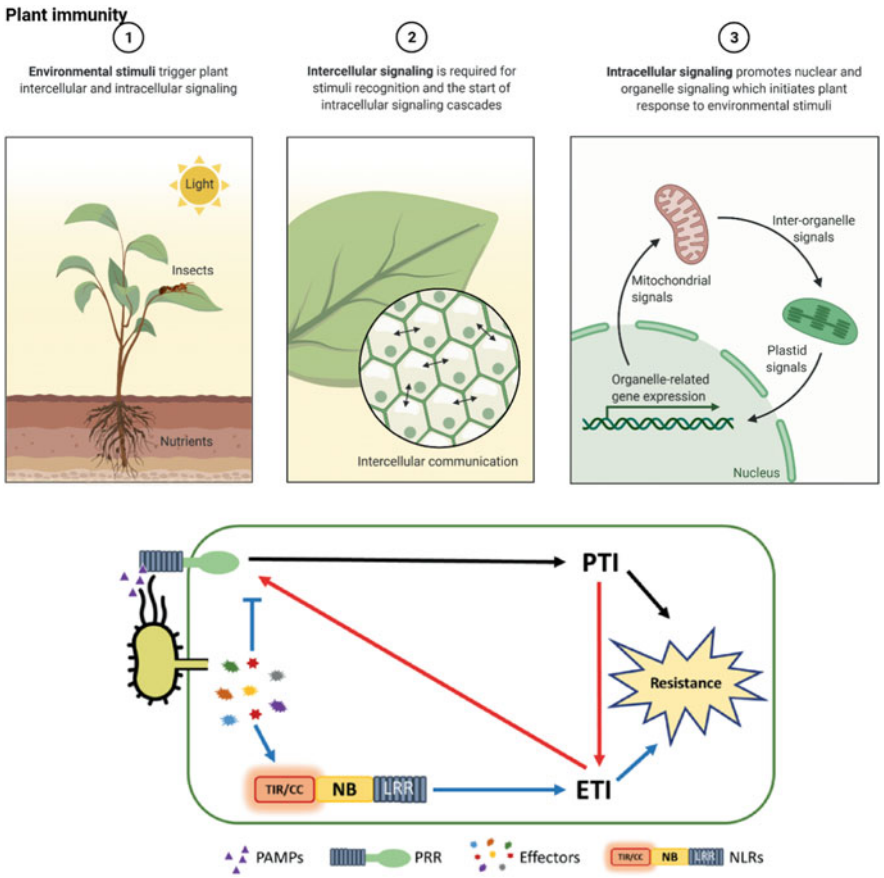

Fig. 25.8 Schematic representation of plant immune system. Adapted from Nguyen et al. (2021)

innate immunity arsenal. Peptides of the innate immune system of plants possess a wide range of biologic activity and participate in a whole complex of strategies aimed at the protection and survival of plants under stress conditions.

Plant defensins are encoded by small multigene families and are expressed in various plant tissues, but are best characterized in seeds. They are typically produced as pre-proteins; however, a small subset is produced as larger precursors with C-terminal pro-domains.

Most peptide factors of plant innate immunity are synthesized in the form of precursors containing a signaling N-terminal fragment of varying length, which provides their further secretion and extracellular localization. Many defense peptides are synthesized as preproproteins containing additional domains of various structure and function and implement their biological functions when located in plasma membrane or inside a plant cell. Some defense peptides are formed as a result of limited proteolysis of proteins or through other pathways of protein degradation.

The expression of several plant defensins is induced upon fungal infection of vegetative tissues but elevated levels of defensin expression were also verified after wounding. The defensins were identified in radish leaves, immature pea pods, sepals of tobacco following fungal infection mainly observed with *Alternaria brassicicola*.

The signaling pathways involve the pathogen-induced systemic activation of the *Arabidopsis defensin* PDF1.2 and the plant hormones salicylic acid, jasmonic acid (and its analog methyl jasmonate), and ethylene. In addition to pathogen-induction, the up-regulation of defensins can also be induced by environmental stress such as drought stress, salt (250 mM NaCl) stress, cold induction, and acclimation, suggesting higher pathogen resistance during winter (Lay and Anderson 2005).

25.4.3 Glucosinolates

Glucosinolates form the glucosinolate–myrosinase system, a relevant dual system on plant secondary metabolism. The glucosinolate–myrosinase system consists of glucosinolates and their hydrolytic enzymes, myrosinases, which catalyze the breakdown of the first into various bioactive compounds such as isothiocyanates as a response to tissue disruption or insect attack. In addition to isothiocyanates, thiocyanates and nitriles are considered as important breakdown products of glucosinolates, which played a pivotal role in plant defense against pathogenic attacks.

Glucosinolates are derived from amino acids and can thus be divided into three groups according to their amino acid precursor: aliphatic glucosinolates, derived from alanine, leucine, isoleucine, valine, and methionine; benzenic glucosinolates, derived from phenylalanine or tyrosine; and indolic glucosinolates, derived from tryptophan.

Biosynthesis proceeds through three independent stages: (1) chain elongation of selected precursor amino acids (only methionine and phenylalanine), (2) formation of the core glucosinolate structure, and (3) secondary modifications of the amino acid side chain (Sønderby et al. 2010).

25.4.4 Isothiocyanates

Isothiocyanates are compounds produced by several plants belonging to the families *Brassicaceae*, *Capparaceae*, and *Caricaceae* as a system of defense against pathogen attack, and they arise from the hydrolysis of glucosinolates by the enzyme myrosinase. Currently, however, their biosynthesis pathway is still not clear. Though most studies revealed that thio-glucoside biosynthesis of aglycone is derived from amino acids, recent studies found that *p*-coumaric acid is a more efficient precursor of *p*-hydroxybenzyl isothiocyanate than either phenylalanine or tyrosine.

Once hydrolyzed by the myrosinase enzymes that co-exist in the plant, they generate a range of bioactive compounds as well as a pungent aroma (Parker 2015).

25.4.5 Cysteine Sulfoxides

Cysteine sulfoxides are the flavor precursors of Allium species. Under this context, the biosynthesis of these secondary metabolites was mainly studied in garlic and onion plant or tissues. Experimental evidence for the synthesis of cysteine sulfoxides proceeding via an alk(en)ylated free cysteine and via S-alk(en)ylated glutathione.

All studies indicate that alliinase is present in Allium species tissue cultures, but the major flavor precursors are absent, or present at only low levels in undifferentiated callus. Furthermore, the different metabolites (methylated/ acylated allicin derived compounds) may differ in biosynthetic capacity among Allium species.

Synthesis of the full spectrum of flavor precursors may resume if the callus is allowed to re-differentiate or regain a capacity for phototrophic metabolism. Once garlic callus had re-differentiated into green shoots or roots the tissues contained allicin derivatives at levels comparable with intact plants. The resumption of flavor precursor synthesis after redifferentiation of onion callus into shoots and roots following removal of the auxin 2,4-dichlorophenoxyacetic acid, and the auxin 4-amino-3,5,6-trichloropicolinic acid (picloram) induced cysteine sulfoxides even at high levels that suppressed overt differentiation.

25.4.6 Natural Sources and Chemical Synthesis

Sulphur-containing metabolites are usually found in plants from *Asteraceae*, *Alliaceae*, and *Brassicaceae* families. Glucosinolates are present in cruciferous vegetables such as *Arabidopsis thaliana* and *Brassica* crop species, isothiocyanates are produced by several plants belonging to the families *Brassicaceae*, *Capparaceae*, and *Caricaceae*, cysteine sulphides in *Allium* species and phytoalexins and defensins usually appear in plants after biotic stressors. Since sulphur-containing metabolites are usually synthetized in plants under stress conditions in response to abiotic or biotic factors, the structural characterization is still a challenge. To date, 44 different structures have been described.

Organosulfur compounds (cysteine sulfoxides) are usually found in *Allium* species, where are synthesized by the enzyme alliinase, e.g. allicin is synthesized from non-proteinogenic amino acid alliin in crushed plant material.

Structural rearrangement of allicin leads to the formation of diallyl sulphoxides, dithiins and, ajoene. Furthermore, thiacremonone was also identified in garlic. Likewise, phytoalexins are only produced under stress conditions in response to biotic factors so the characterization is limited to infected plants. Some of the already

identified sulphur-derived metabolites are listed in Table 25.3 including the stressors promoting the synthesis in plant.

Despite cysteine sulphoxides and phytoalexins are barely characterized, more than 200 different glucosinolates structures have been identified in nature. As previously referred, glucosinolates form the essential component of the glucosinolate–myrosinase system, responsible for the formation of isothiocyanates after glucosinolates breakdown due to tissue disruption or insect attack. In addition to isothiocyanates, thiocyanates and nitriles are considered as important breakdown products of glucosinolates, which play a pivotal role in plant defense against various pathogenic microbes and herbivores.

25.4.7 Structure–Activity Relationships

The structural diversity of sulphur-containing natural products can be useful for the design of diferent drugs by mimicking this structural heterogenicity. Different sulphur bonds in molecules may have relevance for the medicinal effect of sulphur-containing natural products. Attention to disulphide bonds has increased given their ability to break into a reduced form of glutathione in a thioldisulphide exchange reaction, which are stable in human body, and have no physiological toxicity. Likewise, the antimicrobial potential of allium thiosulphinates is given by the presence of a disulphide bond, which reacts with the thiol groups of cellular proteins. Indeed, the sulfinyl group in ajoene has been found to be responsible for the antibacterial activity of allicin. Moreover, as reactive sulphur species, cysteine sulphoxides have oxidizing characteristics. This antioxidant ability induces oxidation of thiols within cells, including glutathione and cysteine residues in proteins, through disulphide bond formation. Consequently, redox-stimulated structural changes in proteins result in a net change of loss or gain function, which is known for the plant protein NPR1, a key protein in pathogen-triggered immunity.

Moreover, the conjugation of a double bond to a nonbonding electron on the sulphur in a ring system was described as relevant in cysteine sulphoxides reactivity. The vinyldithiins group with exo- and endo-double bonds in a ring system usually found in Allium species has demonstrated significantly higher antioxidative activity for human LDL, than aliphatic dialk(en)yl disulphides and trisulfides.

25.4.8 Pharmacological Applications. Active Principle and Targeted Diseases

The sulphur-containing secondary metabolites have been described as anti-inflammatory, antimicrobial, anticancer, and neuroprotective agents. In this regard, allicin and garlic preparations have been pharmacologically produced to prevent

Table 25.3 Main phytoalexins identified in nature (Abdalla and Mühling 2019)

Sulphur-derived metabolites	Vegetable	Biotic stress	Abiotic stress
Brassinin	Chinese Cabbage (*Brassica campestris*)	*Pseudomonas cichorii*	
1-Methoxybrassinin			
Cyclobrassinin			
Brassitin	Japanese Radish "Daikon"	*Pseudomonas cichorii*	
1-Methoxyspirobrassinol			
(2R,3R)-1-Methoxyspirobrassinol Methyl Ether			
4-Methoxybrassinin	*B. oleracea*	*Pseudomonas cichorii*	
Cyclobrassinin sulfoxide	*Brassica juncea*		
Sinalbin A	White mustard *(Sinapis alba)*		
Sinalbin B			
4-Methoxycyclobrassinin	Roots of Canola (*Brassica napus*)	*Plasmodiophora brassicae (clubroot)*	
Rutalexin			
Dehydrocyclobrassinin			
4-Methoxydehydrocyclobrassinin			
Spirobrassinin			
Brassicanate A			
Brassicanal A			
Caulilexin A			
Brassilexin			
1-Methoxybrassinin			
4-Methoxybrassinin			
1-Methoxybrassenins A	Cabbage Tissue *Brassica oleracea*	*Pseudomonas cichorii*	
1-Methoxybrassenins B			
Brassicanal C	Cauliflower (*Brassica oleracea*)		UV light
Isalexin			
Spirobrassinin			
1-Methoxybrassitin			
Caulilexin A			
Caulilexin B			
Wasalexin A	Wasabi		
Wasalexin B		*P. lingam, P. wasabiae*	CuCl2
Cyclobrassinone	Kohlrabi (*Brassica oleracea*)		UV light CuCl2
(R)-1-Methoxy-Spirobrassinin			
Spirobrassinin			
Erucalexin	Dog Mustard	*S. sclerotiorum*	CuCl2

(continued)

Table 25.3 (continued)

Sulphur-derived metabolites	Vegetable	Biotic stress	Abiotic stress
	Erucastrum gallicum		
Wasalexin A	Wasabi	*Thellungiella halophila*	UV radiation, NaCl irrigation, or CuCl2 spray
Wasalexin B			
Sinalexin	White mustard (*Sinapis alba*)	*Pseudomonas cichorii*	UV light
Brussalexin A	Brussels sprouts		UV light
Camalexin	*Camelina sativa* leaves	*Altenmia brassme*	
6-Methoxycamalexin			
1-Methyl-Camalexin	*Capsella bursa-pastoris*	*Alternaria brassicae*	
Rapalexin A	Canola plants *Brassica rapa*	*Albugo candida*	UV light
Rapalexin B			

stroke and arteriosclerosis (Abdalla and Mühling 2019). Recent in vitro investigation revealed that water-soluble organosulfur compounds of *S*-allyl cysteine (garlic extract) and diallyl-di-sulphide (garlic oil) are strong inhibitors of cholesterol synthesis (Subramanian et al. 2020). The clinical uses of garlic and its component extract on cardiovascular diseases have been widely studied. Indeed, according to clinical findings, the results obtained after using garlic extracts to control the blood pressure reach the same results than normal medication in approximately 29% of patients (Ried 2020). The mechanisms described this effect are studied in vitro verifying that the hydrogen sulphide content from the garlic helps relax the blood vessels by inhibiting the angiotensin II protein (Mansour et al. 2013). Moreover, allicin was revealed to induce macrophages that will degrade the LDL uptake, and it can modify the lipoprotein and reduces the lipid content in the blood vessels to stop the intracellular lipid aggregation playing a crucial role in antiatherosclerotic action.

A study has been reported that the sulphur compounds from garlic components are the effective inhibitors of bladder and colon cancer progression. The in vitro study has shown that the compounds can inhibit the arylamine N-acetyltransferase (NAT) activity to produce carcinogens from foreign substances, cell growth, and DNA adduct formation of bladder and colon tumor cells in a dose-dependent manner. The anticancer activity of allicin has been reported and several mechanisms have been described. Namely, allicin can induce the p53 mediated autophagic cell death in Hep G2 liver cancer cells; could regulate the mTOR signaling via AMPK activation and induce autophagic cell death in HepG2 cells; could induce apoptosis in SKOV3 ovarian cancer cells. Mechanistically, it was demonstrated that allicin stimulates the JNK pathway activation, which ultimately results in mitochondrial translocation of Bax and release of cytochrome C from mitochondria to cytosol, thus inducing the apoptosis of SKOV3 cells (Mansour et al. 2013; Lin et al. 2002).

In vitro experiments with purified sulphur-containing plant metabolites have demonstrated their therapeutic utility as antimicrobials against a range of clinically important bacteria and fungi. Most glucosinolates were described as potent as Vancomycin in the treatment of bacteria listed by the WHO as antibiotic-resistant "priority pathogens" and also act as anticancer agents through the induction of phase II antioxidant enzymes which inactivate potential carcinogens. Glucosinolates may be useful in the treatment of biofilms formed on medical implants and catheters by problematic pathogenic bacteria such as *Pseudomonas aeruginosa* and *Staphylococcus aureus* or *Helicobacter pylori* and are potent antimicrobials against a range of clinically important bacteria and fungi (Melrose 2019).

The isothiocyanate sulforaphane nitrile and phenylethyl isothiocyanate have the ability to block nuclear TFs, leading to a reduction in the secretion of inflammatory cytokines. In animal studies, indole-3-carbinol has been shown to reduce the number of individuals with developed forms of human papillomavirus (HPV) cervical cancer in a dose/dependent manner (Chen et al. 2001). Besides the use of natural extracts, the synthesis of sulphur-containing secondary metabolites was performed to mimic the natural plant defense system to human health applications.

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Chapter 26
New Trends from Fungi Secondary Metabolism in the Pharmaceutical Industry

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Abstract Fungi are eukaryotic organisms that can produce a wide range of secondary metabolites with a significant impact on society. Some metabolites are exploited for their activity as antioxidant, anti-inflammatory, antitumor, and anti-microbial agents, and in the production of cancer vaccines, among other pharmaceutical applications. Since the discovery of penicillin, the pharmaceutical industry has been greatly interested in fungi as sources of natural bioactive compounds, and fungi metabolites have made an indispensable contribution to improving human and animal health throughout the last decades. Starting with the development of antibiotics, the pharmaceutical industry has increasingly turned to these compounds for a variety of applications. The increase in the number of patents registered worldwide is a strong indicator that the market realizes the great potential of fungi secondary metabolites. In general, the pharmaceutical industry trend is centered on adopting different strategies to discover new drugs, and fungi secondary metabolites are

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viewed as having significant potential. This chapter explores the current pharmaceutical applications of secondary metabolites found in fungi. Initially, the most recent mushroom studies and their commercial pharmaceutical and cosmeceutical applications are explored. An overview of the different classes of fungi secondary metabolites with biologically relevant activities is then presented. Recently marine fungi were found to be a rich source of secondary metabolites. Due to the recent relevancy of marine fungi, an overview of marine fungi secondary metabolites with relevant pharmaceutical-related activities is also presented. Finally, the potential of fungi metabolites as a source of natural pigments and the methodologies used to characterize and explore fungi secondary metabolites are also analyzed.

26.1 Fungi as Sources of Secondary Metabolites

Fungi are heterotrophic, eukaryotic, and uni- or multicellular organisms grouped in the Fungi Kingdom. Fungi are the primary decomposers of organic matter and play a fundamental role in plant-pathogen symbiosis, essential to the terrestrial ecosystem function. They are also found in the form of mushrooms that are fruiting bodies of macroscopic filamentous fungi, with about 2000 species known in the world (edible and inedible). Mushrooms have been present in human food for over 2000 years, with several benefits for human health, including immunomodulatory, antitumor, cardiovascular, antiviral, antibacterial, antiparasitic, hepatoprotective, antioxidant, and antidiabetic activity, among others (Sabino Ferrari et al. 2021; Větrovský et al. 2020; Jacinto-Azevedo et al. 2021). Fungi present a high potential for biotechnological exploitation with several applications in the pharmaceutical industry and medicine. They can also be used in practices of crop optimization, forestry, food and beverage, sustainability, and strategies against human and plant diseases, as discussed by Hyde et al. (2019) in "50 Ways to exploit fungus industrially."

Resulting from the ground-breaking scientific findings proving the enormous potential of application of fungal secondary metabolites, activity in the intellectual property space is also increasing. As their value and potential becomes increasingly established and recognized, efforts to capture and control this value through the use of intellectual property rights have been growing, particularly at the level of patent registration (Singh et al. 2019).

Several applications using secondary metabolites of fungi have been registered in patents around the world, such as biomaterials for the treatment of wounds (EP3165233) or products that inhibit the growth of oral bacteria in humans (WO201965124) (Hüttner et al. 2020). Using databases such as Patent Lens, which allows the Search of patents from Espacenet, USPTO, WIPO, and IP Australia with coverage of more than 95 different jurisdictions in a universe of more than 100 million patents. Searching ("fungal secondary metabolites") AND ("drug discovery"), you can see that there are several patents registered around the world and that their number has increased, both patents granted and patent applications. Particularly in recent years, there has been a trend toward the registration of patents associated with the optimization of the production of secondary metabolites

of fungi, as well as their use as a potential for application in treatments of various pathologies.

For example: US US 10144719 B1 (2018), the creation of a screening program for fungal metabolites with anti-microbial activity, allowing the identification of new anti-microbial compounds against various targets such as ESKAPE pathogens, *Leishmania donovani, Mycobacterium tuberculosis, Clostridium difficile, Naegleria fowleri*, and cancer. Another example, registered in 2019, a patent directed to the prevention and treatment of Epilepsy using Pseurotins, a family of fungal secondary metabolites (WO 2019/043019 A1). In 2020, new compositions and methods for the treatment and prevention of malaria using a secondary metabolite are announced. Kozupeptins A and B isolated from Paracamarosporium sp. have been reported to be powerful antimalarial agents, particularly against chloroquine-sensitive and chloroquine-resistant strains of *P. falciparum*. Compounds such as antiamoebin, efrapeptins, and zervamycins derived from fungal species killed *P. falciparum* in culture (US US 2020/0115415 A1).

Thus, it is expected that this trend will lead to the consequent investment and increase in fungal drug discovery opportunities, which have successfully led to the discovery of fascinating molecules (Keller 2019).

26.1.1 Mushrooms Pharmaceutical Applications

There has been an expanding number of mushroom studies performed, especially in Europe (12,308) but also in Asia (9511) and North America (8708) (Fig. 26.1). As shown in the "global fungi" database search, 36,687 studies were performed (January 10, 2022). It is worth noting the presence of mushroom studies across the globe and the vast diversity of species explored (Větrovský et al. 2020).

It is worth noting that several studies demonstrated differences in chemical characteristics, and in antioxidant and microbial activities of mushrooms, according to different locations around the globe (Heleno et al. 2013) and even differences according to cultivation factors (Zawadzka et al. 2022). Table 26.1 presents several studies performed in recent years with mushrooms, presenting the respective bioactivity, part of the mushroom explored, and geographical origin.

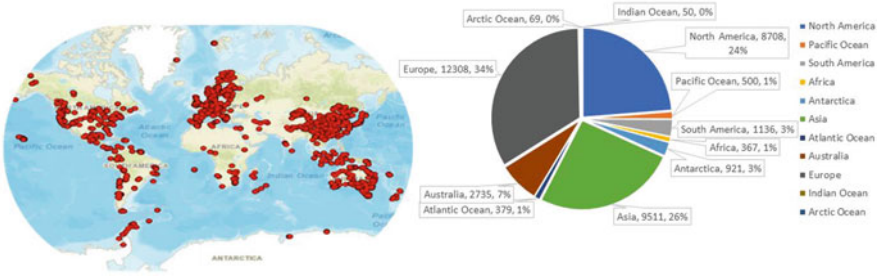


Fig. 26.1 Diversity of mushroom studies around the globe. Source: Větrovský et al. (2020) (https://globalfungi.com/, Accessed 7 January 2022)

Table 26.1 Examples of studies performed with mushroom extracts

Mushroom	Bioactivity	Part of the mushroom	Geographical origin	References
Boletus loyo	Growth inhibition of Gram-positive bacteria and antifungal activity	Fruitful bodies	Chile	Jacinto-Azevedo et al. (2021)
Cortinarius lebre	Growth inhibition of Gram-positive bacteria			
Lentinus edodes (Berk.) Pegler				
Morchella conica				
Ramaria flava	Antibacterial and antioxidant activity			
Morchella importuna	Antioxidant	Fruitful bodies	China	Juncai et al. (2021)
Bjerkandera adusta	Anti-microbial activity (*Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Micrococcus luteus*, *Streptococcus pneumoniae*, and *Candida albicans*)	Fruitful bodies	Egypt	Soliman and El-Sayed (2021)
Cyclocybe cylindracea				
Morchella esculenta (L.) Pers.	Radical scavenging activity, reducing power and antibacterial	Fruitful bodies	Portugal	Heleno et al. (2013)
	Lipid peroxidation inhibition and antibacterial		Serbia	
Agaricus subrufescens	Antioxidant	Fruitful bodies	Brazil	Sabino Ferrari et al. (2021)
			Japan	
			Belgic	
Auricularia auricula (Touro) Pat	Antibacterial	Fruitful bodies	Brazil	Volcão et al. (2021)
Russula xerampelina (Sch.) Pe. O. Kuntze				
Laccaria laccata (L.) Sf. Cinza				
Lactarius deliciosus (L.) Sf. Cinza				
Suillus granulatus (L.) Fr. O. Kuntze	Antioxidant and antibacterial		Brazil	

(continued)

Table 26.1 (continued)

Mushroom	Bioactivity	Part of the mushroom	Geographical origin	References
Lactarius piperatus	Antibacterial (*S. aureus* and E. Coli)	Fruitful bodies	Romania	Fogarasi et al. (2020)
Boletus edulis				
R. patagonica	Antioxidant	Fruitful bodies	Argentine	Toledo et al. (2016)
Fistulina hepatica	Antioxidant		Portugal	Vaz et al. (2011a)
C. odora	Antioxidant	Mycelial culture		Vaz et al. (2011b)
L. inversa	Antioxidant	Fruitful bodies		Vaz et al. (2010)
C. alexandri	Growth inhibitor of cancer cell lines: lung cancer (NCI-H460), breast cancer (MCF-7), colon cancer (HCT-15), and gastric cancer (AGS)	Fruitful bodies		
L. giganteus	Antioxidant	Fruitful bodies		Barros et al. (2007)
Agaricus silvaticus	Antioxidant	Fruitful bodies		Barros et al. (2008)
Boletus regius	Antioxidant	Fruitful bodies		Leal et al. (2013)
Fomitopsis pinicola	Antioxidant	Fruitful bodies		Reis et al. (2011)
Pleurotus ostreatus	Anti-inflammatory	Fruitful bodies		Taofiq et al. (2015)
Macrolepiota procera				
Boletus impolitus				
Agaricus bisporus				
Ganoderma lucidum	Antioxidant, antibacterial, and anti-tyrosinase activity	Fruitful bodies		Taofiq et al. (2017)
Ganoderma lucidum	Increased levels of LC3-II, p62 decreased levels, confirming autophagy in AGS cells	Fruitful bodies		Reis et al. (2015)
Ganoderma lucidum	Activity against *Aspergillus niger*	Fruitful bodies		Heleno et al. (2013)

The scientific evidence of all these studies combined allowed the pharmaceutical industry to evolve toward producing commercial mushroom-based products presented in the next section.

26.1.2 Commercial Pharmaceutical Mushroom-Based Products

Considering the potential biological properties of the mushroom extracts and derivatives, several products are already available in the cosmetics and cosmeceutical market.

Koltsovo Scientific Centre, Russia, developed the first mushroom cosmetic line worldwide, creating a line of creams with fly-agaric extract (*Amanita muscaria*) called the Mushroom Collection. In turn, under the brand Aveeno/Active Naturals, Johnson and Johnson advanced a new line of skin care products known as "Positively Ageless." This product is composed of active ingredients from *Ganoderma lucidum* and *Lentinula edodes*, with proven anti-aging products. Another example is the mushroom extract from *Tremella fuciformis,* which is very popular in China and Japan and is used as a replacement for sodium hyaluronic acid. In turn, Earthherbs® L.L.C. produces cosmeceuticals from the Siberian chaga mushroom (*Inonotus obliquus*), which contains mushroom cream that rejuvenates the skin. In addition, Murad's products use mushroom enzymes to promote gentle exfoliation. At the same time, the Murad's Age Diffusion Serum is an anti-aging serum for signs and symptoms of hormonal aging and contains *Lentinula edodes*. Table 26.2 summarizes the mushroom based-products available in the market.

Apart from commercial cosmetic products, there are also patents for using mushrooms in the cosmetic industry. The US Patent 6,843,995 uses common Truffle extracts in cosmetics formulations. Truffle extracts are obtained from mushroom species including *Choiromyces maeandriformis, Tuber uncinatum, Tuber melanosporum, Tuber magnatum, Tuber aestivum, and Tuber brumale* and mixtures thereof. These extracts stimulate the non-specific immune system and improve the body's antiviral defense mechanisms. They contain essential amino acids, vitamins B1, B2, and B3, and polysaccharides such as lentinan and eritadenine. Another example is the US Patent 6,645,502 B2: Revlon Consumer Products Corp., an anhydrous cosmetic composition comprising a water-insoluble polyporus extract. Applying this to the skin reduces the shiny appearance and skin imperfections.

26.2 Fungi Secondary Metabolite Families

More than 500,000 secondary metabolites have been described. About 350,000 of these are from plants, 100,000 from animals, and 70,000 from microorganisms (Bérdy 2005). Roughly 33,500 bioactive microbial metabolites have been described,

Table 26.2 List of products incorporating mushrooms available in the market

Product name	Mushroom	Properties
Aveeno Positively Ageless Daily Exfoliating Cleanser, US	*Lentinula edodes*	Moisturizing and anti-aging
One Love Organics Vitamin D Moisture Mist, UK	*Lentinula edodes*	Moisturizing
Osmia Organics Luz Facial Brightening Serum, US	*Lentinula edodes*	Moisturizing and whitening
Kat Burki Form Control Marine Collagen Gel, UK	*Ganoderma lucidum*	Moisturizing and anti-aging
Menard Embellir Refresh Massage, France	*Ganoderma lucidum*	Anti-aging
Yves Saint Laurent Temps Majeur Elixir De Nuit, France	*Ganoderma lucidum*	Anti-aging
Vitamega Facial Moisturizing Mask, Brazil	*Agaricus subrufescens*	Moisturizing and anti-aging
Kose Sekkisei Cream, Japan	*Cordyceps sinensis*	Moisturizing
Alqvimia Eternal Youth Cream Facial Máxima Regeneración, Spain	*Schizophyllum commune*	Anti-aging
Sulwhasoo Hydroaid, Korea	*Schizophyllum commune*	Moisturizing
La Prairie Advanced Marine Biology Night Solution, Switzerland	*Tremella fuciformis*	Moisturizing
BeautyDiy Aqua Circulation HydratingGel, Taiwan	*Tremella fuciformis*	Moisturizing
Hankook Sansim Firming Cream (Tan Ryuk SANG), Korea	*Ganoderma lucidum* and *Pleurotus ostreatus*	Anti-aging and whitening
Pureology NanoWorks Shineluxe, France	*Ganoderma lucidum, Lentinula edodes, and Mucor miehei*	Anti-aging
Snowberry Bright Defense Day Cream No. 1, New Zealand	Mushroom extract	Moisturizing
Murad Invisiblur Perfecting Shield, U.S.	Mushroom peptides	Anti-aging

Table adapted from Wu et al. (2016)

of which about 12.5% (4200) are metabolites of unicellular bacteria and cyanobacteria, 41% (13,700) are products of Actinomycete fermentations, and about 47% (15,600) are of fungal origin (Bérdy 2005).

Secondary metabolites provide an amazingly diversified spectrum of biological activities. Some have provided society life-changing benefits, while others are associated with severe, almost intractable problems (Bérdy 2005; Bills and Gloer 2017; Krause et al. 2018; Rohlfs and Churchill 2011) (Table 26.3).

Fungal metabolites with the most substantial negative impact include mammalian toxins known as mycotoxins. Up to 1000 fungal compounds have been reported with this label, comprising aflatoxins, trichothecenes, fumonisins, cytochalasins, and various indole-terpene tremorgenic compounds (Bräse et al. 2013).

The realization that fungi were a source of both harmful and beneficial compounds was brought to light on both sides by the Turkey X disease, an aflatoxin

Table 26.3 Bioactivity types of microbial metabolites

Anti-microbial activity (Hutchings et al. 2019)	Antibacterial (Gram-positive, Gram-negative, *Mycobacteria*) Antifungal (Yeasts, Phytopathogenic fungi, other fungi) Antiprotozoal
Chemotherapeutic activity (Barbero et al. 2018)	Antitumor (cytotoxic) Antiviral
Pharmacological activity (Zheng et al. 2021)	Enzyme inhibitor Immunological activity (suppressive, modulatory) Biochemical activity (DNS, tubulin, mitotic, etc.) Antagonistic, modulatory, anti-inflammatory activities.
Agricultural activity (Keswani et al. 2019)	Pesticide (antiparasitic, algicide, amoebicide, etc.) Herbicide (phytotoxic, plant growth, regulatory, etc.) Insecticide/Miticide/Larvicide/Deterrent Feed additive, preservative
Other activities (Rohlfs and Churchill 2011)	Microbial regulators (growth factors, microbial hormones, morphogens) Biophysical effects (surfactants, etc.)

Adapted from Bérdy (2005)

poisoning event, in the 1960s (Nesbitt et al. 1962), and by the discovery of penicillin, the first broad-spectrum antibiotic, considered the "wonder drug" of World War II (Quinn 2013).

Many essential pharmaceutical products have been discovered through the study of fungal chemistry. These products remain among the most valuable therapeutic agents in medicine, being notably important in developing powerful therapies for cancer, malaria, bacterial and fungal infections, neurological and cardiovascular diseases, and autoimmune disorders (Newman and Cragg 2016). Penicillins and cephalosporins are the most important class of beta (β) lactam antibiotics, accounting for 65% total antibiotic market. It is difficult to overstate these compounds' tremendous effect on global health due to their effectiveness against bacterial infections (Sawant and Vamkudoth 2022). The success of penicillins triggered the development of meaningful technological advances in microbiology, chemistry, biochemistry, and engineering and helped in countless ways to establish the modern pharmaceutical industry. There are many other fungi metabolites with known valuable activities, that have been developed into pharmaceutical, agrochemical, and cosmetic products (Bills and Gloer 2017)

Unlike primary metabolism, which forms compounds essential for the organism's development, secondary metabolites (popularly known as natural products) are not vital for fungus growth, energy generation, and cellular maintenance. Secondary metabolites are usually structurally diverse molecules of low molecular weight that perform functions that are not always known. The production of secondary metabolites is influenced by environmental and genetic factors and, in addition to playing a role in a variety of cellular processes (such as transcription, development, and cellular communication), they are fundamental for interactions with other organisms (Brakhage 2013; Calvo et al. 2002; Pusztahelyi et al. 2015; Keller 2019), as well as in drug development (Boruta 2018).

Many essential pharmaceutical products were discovered through the study of fungi chemistry. These products remain among the most valuable therapeutic agents in medicine, being notably important in developing powerful therapies for cancer, malaria, bacterial and fungal infections, neurological and cardiovascular diseases, and autoimmune disorders (Newman and Cragg 2016). Also, fungi metabolites were helpful in immune system stimulating therapy (Lange 2014), antiviral action, liver protection, and antioxidant or tumor modulator action (Ferreira et al. 2010). These metabolites were also proven to interact with cytokine networks, modulating the innate and adaptive immune system (Bertollo et al. 2022). Recent studies demonstrated that compounds present in the secondary metabolism of *Neosartorya* spp., *Penicillium* spp., *Aspergillus* spp., *Alternaria* sp., *A. terreus* were effective in cancer prevention (Noman et al. (2021). Also, in Japan, as mentioned by Mirończuk-Chodakowska et al. (2021), the consumption of Japanese shiitake (*Lentinula edodes* (Berk.) Pegler) was used for cancer prevention or to complement chemotherapy.

Among examples of known compounds present in secondary metabolism, penicillin was discovered in 1928 by Sir Alexander Fleming through the ability of the fungus *Penicillium* to eliminate bacteria (Castells et al. 2019). Penicillin presents bactericidal action by interfering in the bacterial cell wall synthesis due to its β-lactam ring scaffold (Sawant and Vamkudoth 2022). Penicillin is currently found in several forms (natural penicillin, penicillinase-stable penicillins, aminopenicillins, and extended-spectrum penicillins). Penicillin G (1) is an active form of natural penicillin presenting the 6-aminopenicillanic acid scaffold characteristic of Peninciline, but also with a thiazolidine ring and a benzyl side chain (Miller 2002; Bentley 2004; Bhattarai et al. 2021).

Many other fungi metabolites with known valuable activities have been developed in the pharmaceutical, agrochemical, and cosmetic industries (Bills and Gloer 2017). Cyclosporin was developed as an immunosuppressant (5) (Daley et al. 2017); lovastatin (2) as a cholesterol reducer (Brakhage 2013), mycophenolic acid (3) as an immunosuppressant (Keller 2019), and nominine (4) as a potential insecticidal fungal metabolite (Fig. 26.2).

Besides medicinal actions, compounds in fungi (terrestrial and marine) also demonstrate pigmenting capacity with potential as a source of pigments of natural origin. However, the importance of its application and toxicity has diminished. For example, yellow, orange, and purple pigments produced by the Monascus filamentous fungus were banned in Europe and the USA due to the presence of mycotoxins (Narsing Rao et al. 2017).

Fungi secondary metabolites are formed from precursors metabolites derived from the primary metabolism. These precursors include acetyl-CoA, several Krebs cycle metabolites, and amino acids. The fungi's secondary metabolites are classified in groups according to their structure scaffolds and include non-ribosomal peptides (NRPs); Polyketides (PKs); PKs-NRPs hybrids; Ribosomal synthesized and post-translationally modified peptides (RiPPs); Terpenes and Alkaloids (Bhattarai et al. 2021). The following sections describe the fungi's secondary metabolites classes and their bioactive potential.

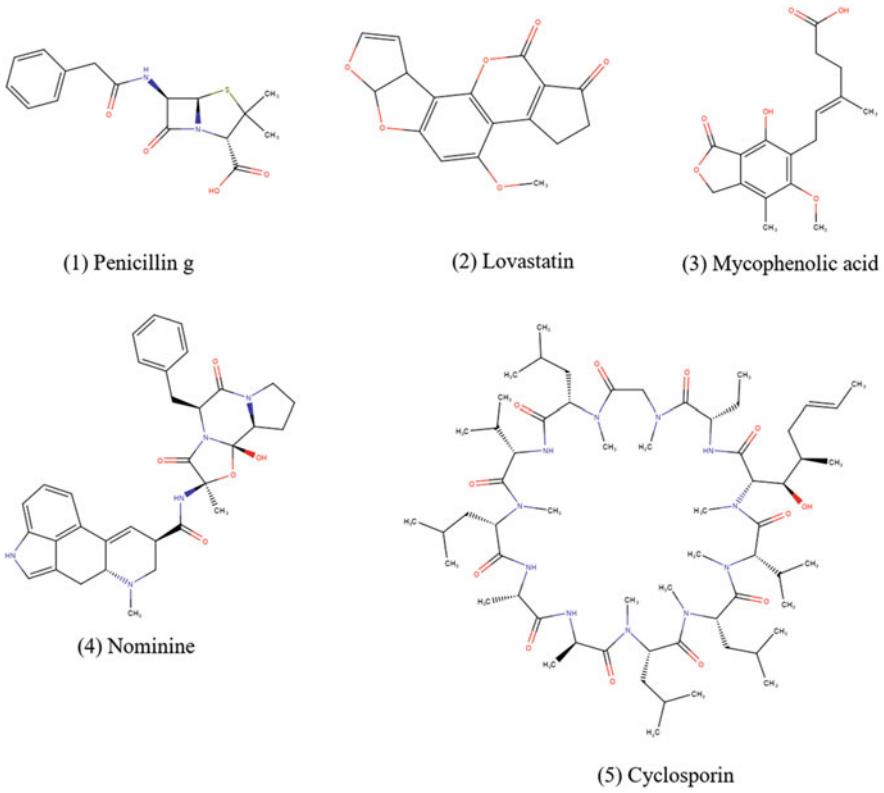
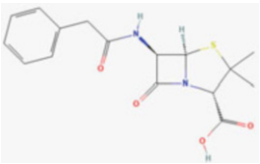
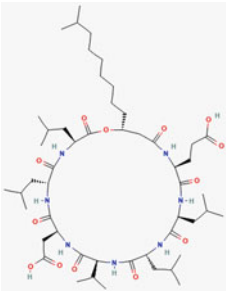
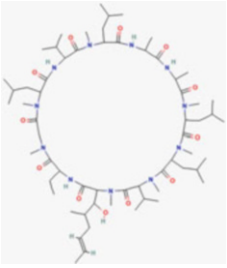
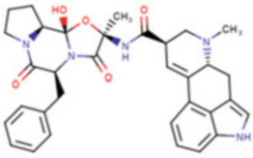
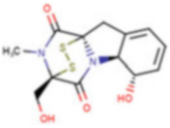


Fig. 26.2 Example of fungi secondary metabolites with medicinal activity

26.2.1 Non-ribosomal Peptides

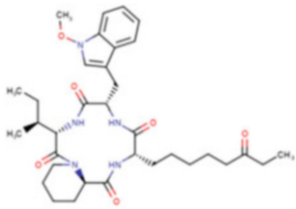
Non-ribosomal peptides (NRPs) are low molecular weight peptides (Brakhage 2013), derived from proteinogenic and non-proteinogenic amino acids, and are synthesized by multimodular NRP enzymes (Zeilinger et al. 2015). The NRP drug market contributes billions of dollars/euros to the chemical and pharmaceutical industry, with significant applications in antibacterial, topical, antitumor, and antifungal activities. For example, cyclosporin (5) is used for organ transplant patients, being produced by *Tolypocladium niveum*, and Penicillin G (1) presents peptidoglycan biosynthesis inhibition and antibiotic action. Besides the therapeutic area, NRPs also function as biosurfactants for oil extraction; for example, surfactin (6) is used in the remediation of oil-contaminated soils. Also NRPs are used in food and beverages, cosmetics and detergents (Martínez-Núñez and López 2016; Daley et al. 2017). Other NRP compounds include ergotamine (7), gliotoxin (8), and apicidin (9). Table 26.4 highlights some examples of NRPs compounds extracted from fungi with different bioactivities.

Table 26.4 Examples of non-ribosomal peptides (NRPs) present in fungi

Compound	Source	Bioactivity	Reference
Penicillin G (1) 	*Penicillium*	Inhibition of peptide-glycan biosynthesis and antibiotic	Martínez-Núñez and López (2016)
Surfactin (6) 	*B. subtilis*	Remediation of oil-contaminated soils	
Cyclosporine A (5) 	*Tolypocladium and Aspergillus*	Immunosuppressants	Brakhage (2013)
Ergotamine (7) 	*Claviceps*	Management and treatment of acute migraines	Avalos and Limón (2021); Ngo and Tadi (2022)
Gliotoxin (8) 	*Aspergillus*	Induces apoptosis and prevents the activation of nuclear factor-κB	

(continued)

Table 26.4 (continued)

Compound	Source	Bioactivity	Reference
Apicidin (9) 	*Fusarium*	Histone deacetylase inhibitor	

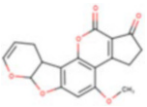
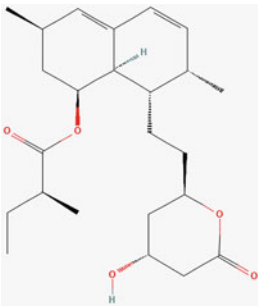
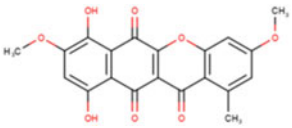
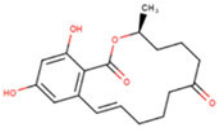
26.2.2 Polyketides (PKs)

Polyketides are a significant family of secondary metabolites produced mainly by Penicillium, Fusarium, and Alternaria genera species. They are synthesized by type I enzymes (polyketide synthases) from acetyl-CoA and malonyl-CoA primary metabolites. Polyketides comprise the mycotoxins class, including zearalenone (13) and anticholesteremic agents, including lovastatin (2) (Zeilinger et al. 2015; Avalos and Limón 2021). Other PKs include antioxidants such as resveratrol, antibiotics such as bikaverin (12), erythromycin, and tetracenomycin. Several PKs are used as chemotherapeutics, including doxorubicin, resistomycin, and mithramycin. Also, toxic compounds such as aflatoxins B1 (10) and B2 are PK compounds (Table 26.5) (Daley et al. 2017; Korman et al. 2010).

26.2.3 PKs-NRPs Hybrids

NRP/PKs hybrids are secondary metabolites obtained from the ensemble of NRPs and PKs. These hybrid molecules are composed of an N-terminal interactive PK module and a C-terminal NRP module (Avalos and Limón 2021). The hybridization process generates a combinatorial diversity of compounds that have immunosuppressor activity, including pseurotin A (17), obtained from the *Aspergillus* genus, and Emericellamide (18), obtained from the *Emericella* genus that presents anti-microbial activity against *Staphylococcus aureus* (Bhattarai et al. 2021). Other hybrid molecules synthesized by several fungi species include Tenuazonic acid (19), which inhibits protein biosynthesis (Yun et al. 2015), fusarin C (20), which is a mycotoxin found to be carcinogenetic in rodents, and Equisetin (21) an HIV integrase inhibitor (Avalos and Limón 2021). Table 26.6 presents several NRP/PKs with different biologic activities and respective fungi sources (Zeilinger et al. 2015).

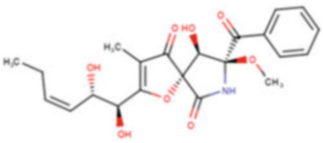
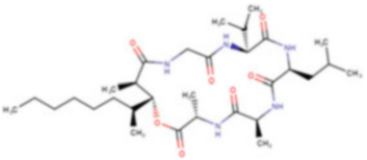
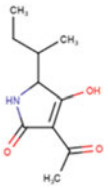
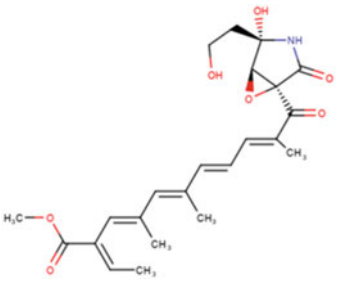
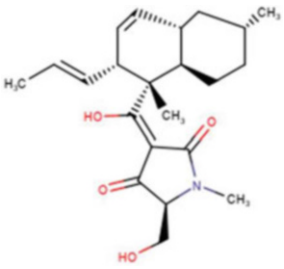
Table 26.5 Examples of polyketides (PKs) present in fungi

Compound	Source	Bioactivity	Reference
Aflatoxins B1 (10) 	*Aspergillus*	Adverse toxic activities	Brakhage (2013)
Lovastatin (2) 	*Tolypocladium* and *Aspergillus*	Cholesterol-reducing agent	
Bikaverin (12) 	*Fusarium*	Antibiotic (protozoa)	Avalos and Limón (2021)
Zearalenone (13) 	*Fusarium*	Mycotoxin	

26.2.4 *Ribosomally Synthesized and Post-translationally Modified Peptides (RiPPs)*

Ribosomally synthesized and post-translationally linked peptides (RiPPs) are a class of underexplored secondary metabolites, and only a few fungal RiPPs were characterized. RiPPs are essentially any peptide that presents a molecular weight below 10 kDa. Due to their bioactivities, these peptides have received significant attention in recent years (Kessler and Chooi 2022). The recent discovery of new classes of RiPPS shows how much still needs to be explored, a challenge that has been greatly assisted by genome mining (Kloosterman et al. 2021). So far, only four RiPP families of fungi origin have been reported, with few compounds discovered. The four families include cyclopeptides, borosins, dikaritins, and epichloëcyclins (Tsukui et al. 2015; Garcia et al. 2015; Vogt and Künzler 2019; Bhattarai et al. 2021; Kessler and Chooi 2022):

Table 26.6 Examples of NRP/PKs hybrids present in fungi

Compound	Source	Bioactivity	Reference
Pseurotin A (17) 	*Aspergillus*	Immunosuppressor	Bhattarai et al. (2021)
Emericellamide (18) 	*Emericella*	Anti-microbial activity against *Staphylococcus aureus*	
Tenuazonic acid (19) 	*Magnaporthe*	Inhibits protein biosynthesis on ribosomes, suppressing the release of new proteins	Yun et al. (2015)
Fusarin C (20) 	*Fusarium*	Mycotoxin (carcinogenesis in rodents)	Avalos and Limón (2021)
Equisetin (21) 	*Fusarium*	H.I.V. 1 integrase inhibitor	

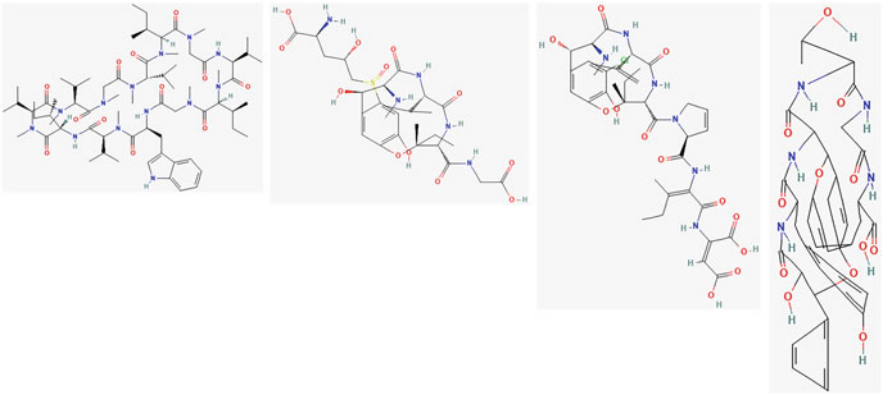


Fig. 26.3 Example of RiPPs present in fungi

Cyclopeptides are cyclic peptides that include amatoxins, a poisonous substance present in *Amanita* genus with high toxicology in insects, nematodes, and mammals. Amatoxins inhibit RNA II polymerase, interrupting mRNA transcription and leading to cell death. Also, phallotoxins are cyclopeptide examples, present in basidiomycete genus *Amanita*, *Galerina*, *Lepiota*, and *Conocybe*, and when injected into the bloodstream, cause severe liver damage. It is assumed that these compounds are present to protect the early stages of the fungi development.

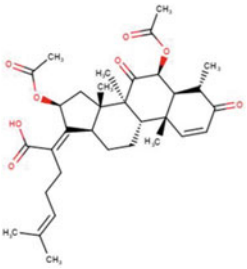
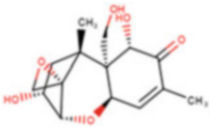
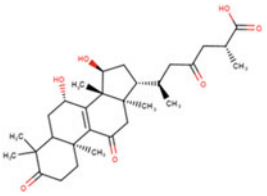
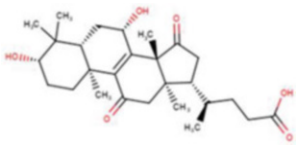
Borosins are RiPPS with N-methylations installed in the peptide backbone. Omphalotin A (23) is a Borosin example from *Omphalotus olearius* and is toxic to nematodes (Fig. 26.3). Dikaritins are present in species such as *Ustilaginoidea virens*, *Phomopsis leptostromiformis*, and *Aspergillus flavus*. An example of a dikaritin is ustiloxin (24), a four amino acid peptide found in *Ustilaginoidea virens* and presenting microtubule inhibitory activity. Ustiloxin causes phytotoxic effects on rice, wheat, and maize. Also, Phomopsin A (25) is a ustiloxin-related compound with a backbone of six amino acids as opposed to four (ustiloxins). Asperipin-2a (26) is another example of a dikaritin identified in the genome of *Aspergillus flavus*. Finally, the last family of fungi RiPPs are Epichloëcyclins, that are cyclic peptides with nine amino acids, produced by ascomycetes from the genus *Epichloë*.

26.2.5 Terpenes

Terpenes are secondary metabolic compounds from the mevalonate pathway that start by conjugating three Acetyl-CoA primary metabolites (Zeilinger et al. 2015). They are a large group of natural products in fungi, with applications such as flavoring agents, fragrances, pharmaceuticals (antibiotics, antitumor agents), phytohormones, and chemical biosynthesis to meet clinical needs and industrial production (Wang et al. 2021; Avalos and Limón 2021).

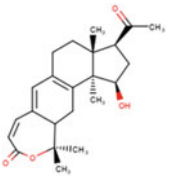
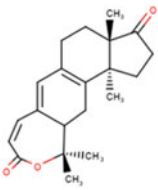
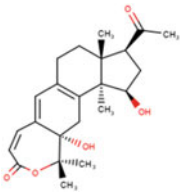
Several examples are presented in Table 26.7 and include helvolic acid (27) with antibiotic activity and deoxynivalenol (28), which acts as mycotoxin, provoking food toxicity. Also, Gibberellin (29) is a plant hormone produced by fungi. In plants, gibberellin stimulates growth and promotes the plant's developmental processes throughout its life cycle (seed germination, stem elongation, sexual expression,

Table 26.7 Examples of terpenes present in fungi

Compound	Source	Bioactivity	Reference
Helvolic acid (27) 	*Aspergillus* sp.	Antibiotic	Avalos and Limón (2021)
Deoxynivalenol (28) 	*Fusarium*	Mycotoxin (produces alimentary toxicity, acute gastroenteritis, and growth impairment, among other effects)	Avalos and Limón (2021)
Gibberellin (29) 	*Fusarium fujikuroi*	Plant hormones that are also produced by fungi	Brakhage (2013); Bömke and Tudzynski (2009)
Ganoderic acid (30) 	*Ganoderma*	Increase expression of T-helper type 1 cytokines, improving immune function	Weng and Yen (2010)
Lucidenic acid N (31) 	*Ganoderma*	Anti-invasive property in hepatoma (HepG2) cells due to reduction of collagenase activity	Weng and Yen (2010)

(continued)

Table 26.7 (continued)

Compound	Source	Bioactivity	Reference
Cattienoids A (32)  Cattienoids B (33)  Cattienoids C (34) 	*Tomophagus*	Compound (34) showed cytotoxicity against KB cells	Hien et al. (2013)

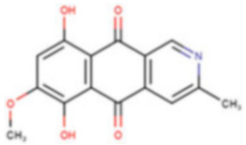
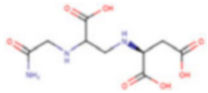
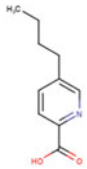
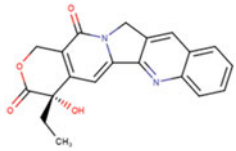
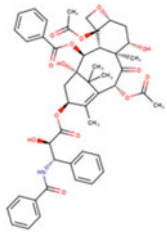
flowering, fruit formation, and senescence). Ganoderic acid (30) is another terpene that can increase the expression of T-helper type 1 cytokines (IL-2 and IFN-γ), improving immune function, and inhibiting tumor growth and lung metastasis. Lucidenic acid N (31) is a terpene that can stop the growth of HepG2 cells (hepatocarcinoma cell line) caused by reducing collagenase activity.

Cattienoids (32–34) are other terpenes isolated from the *Tomophagus* genus. Specifically, cattienoid C (34) presented cytotoxicity against tumoral KB cells (papilloma cell line).

26.2.6 Alkaloids

Alkaloids are secondary metabolites that contain at least one nitrogen atom in their structure. Usually, alkaloids are produced using amino acids as precursors. Alkaloids present different biomedical properties, including fungal, anticancer, and antiviral activities (Wieczorek et al. 2015; Zhang et al. 2012). Several relevant fungi alkaloids

Table 26.8 Examples of alkaloids present in fungi

Compound	Source	Bioactivity	Reference
Bostrycoidin (35) 	*F. bosteycoides*	Antibiotic	Bhattarai et al. (2021)
Lycomarasmine (36) 	*Fusarium*	Antibiotic	
Fusaric acid (37) 	*Fusarium*	Dopamine β-hydroxylase inhibitor	
Camptothecin (38) 	*Entrophospora infrequens*	Anticancer drug	Avalos and Limón (2021)
Taxol (39) 	*Taxomyces andreanae*		

are present in Table 26.8. For example, Bostrycoidin (37) is a fungus alkaloid with antibiotic activity against *Mycobacterium tuberculosis*. Also, Lycomarasmine (36) is used as an antibiotic, acting as an adjuvant to overcome clinical resistance caused by MBL-containing Gram-negative bacteria. Fusaric acid (37) is an alkaloid that presents dopamine β-hydroxylase inhibition activity. Other members of fungi alkaloids include Camptothecin (38) and Taxol (39), already used in medicine as an anticancer drug.

26.3 Secondary Metabolites from Marine Fungi

Half of the biodiversity in the world is marine, and marine fungi are a diverse group of microorganisms. Currently, at least 1112 species and 472 genera of marine fungi have been identified. These interact with the environment or host and can synthesize various compounds, including several secondary metabolites classes explored in Sect. 26.2. Marine fungi are less studied than the terrestrial fungi and can also present therapeutic activities, including anti-microbial, antioxidant, antitumor, anti-coagulant, and different enzyme inhibition activities (Balabanova et al. 2018; Deshmukh et al. 2018; Wang et al. 2021).

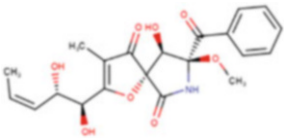
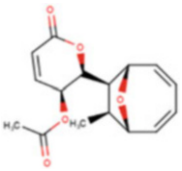
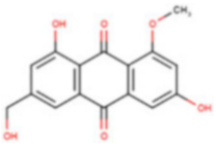
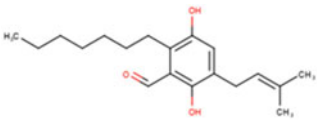
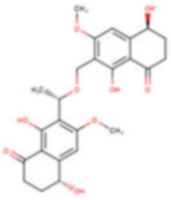
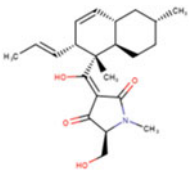
Researchers have recently been attracted to secondary metabolites obtained from marine fungi species. Recent investigations on fungi searching for biologically active secondary metabolites indicate their tremendous potential as a source of new medicines (Balabanova et al. 2018). Several secondary metabolites from marine fungi presented antibiotic, antitumor, and anti-inflammatory activity. Compound 14-norpseurotin A (40) is a terpenoid from *Aspergillus sydowii* that demonstrated anti-microbial activity (Table 26.9). Also, a large number of marine secondary metabolites belonging to the Polyketide family presented anti-inflammatory activity, including Mycoepoxydiene (41), Questinol (42), Flavoglaucin (43), and (4*R*, 10*S*, 4′ S)-leptothalenone B (44) (Xu et al. 2019).

Several marine fungi secondary metabolites have also presented anti-HIV activity, and at least 150 compounds with promising activities have already been identified (Tziveleka et al. 2003). These anti-HIV metabolites include Equisetin (45) and Integric acid (46). Also, other marine fungi metabolites presented Anti-Alzheimer's activity, including Penicillamine (47) and 3β,4α-dihidroxi-26-metoxiergosta-7, 24(28)-dien-6-ona 3β,4α-dihydroxy-26-methoxyergosta-7,24(28)-dien-6-one (48).

26.4 Fungi as a Source of Natural Pigments

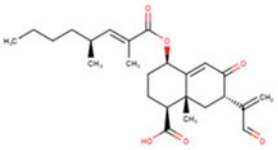
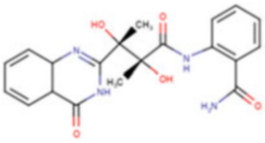
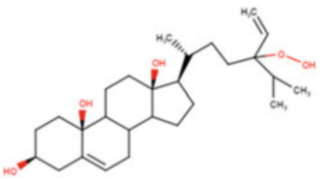
Regulators from the European Union (EU), the United States Food and Drug Administration (US-FDA), and the World Health Organization (WHO) have emitted warnings about the daily consumption of dyes. Since then, natural sources such as fungi have been considered an alternative to current pigments for industrial applications (food, medicine, cosmetics, and textiles), with advantages of sustainability (Urista et al. 2016; Kalra et al. 2020). For the production of carotenes alone, more than 200 fungal species are available. Fungi pigments of marine origin (*Eurotium rubrum*, *Halorosellinia Hortaea*, *Phaeotheca*, and *Trimmatostroma*) are also a great source of discusses compounds that present colors with pigmenting potential (Narsing Rao et al. 2017), Table 26.10.

Table 26.9 Examples of marine fungi secondary metabolites with medicinal biological activities

Compound	Family	Producer	Activity	Reference
14-norpseurotin A (40) 	Triterpenoid	*Aspergillus sydowii*	Anti-microbial	Devi and Jeyaseelan (2020)
Mycoepoxydiene (41) 	Polyketide	*Diaporthe* sp.	Anti-inflammatory	Xu et al. (2019)
Questinol (42) 	Polyketide	*E. amstelodami*		
Flavoglaucin (43) 	Polyketide	*Eurotium* sp. SF-5989		
(4*R*, 10*S*, 4′*S*)-leptothalenone B (44) 	Polyketide	*Gliomastix* sp.		
Equisetin (45) 	Sesquiterpenoid	*Fusarium heterosporum and a Phoma* sp.	Anti viral (HIV)	Tziveleka et al. (2003)

(continued)

Table 26.9 (continued)

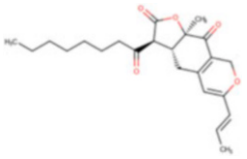
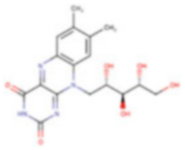
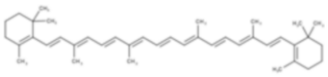
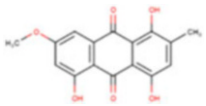
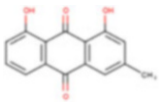
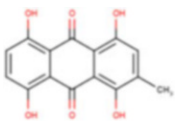
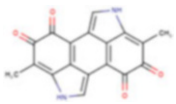
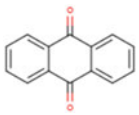
Compound	Family	Producer	Activity	Reference
Integric acid (46) 	Sesquiterpenoid	*Xylaria* sp.		
Penicillamine (47) 	Alkaloid	*Penicillium commune*	Anti-Alzheimer's disease	Hafez Ghoran and Kijjoa (2021)
3β,4α-dihydroxi-26-metoxiergosta-7, 24(28)-dien-6-ona (48) 	Steroid	*Aspergillus flavus*		

26.5 Characterization of Fungi Secondary Metabolites

The diversity of compounds present in fungi, as well as their morphological and molecular characteristics, is influenced by environmental factors and production practices. Several methods (sensory, instrumental, and molecular) are needed to evaluate sensory identification (morphological techniques, odor, and electronic nose technology), chemical composition (chromatography, mass spectrometry, and spectral technology), and molecular composition (molecular markers, sequencing, isothermal amplification, and endogenous reference gene) (Wei et al. 2022).

Table 26.11 presents examples of fungi chemical compound characterization and analysis techniques. GLC-MS (gas-liquid chromatography-mass spectroscopy) was used for compound identification, and NMR (Nuclear magnetic resonance) was used for structural analysis of heteroglycan polysaccharides (THPS), obtained from *Termitomyces heimii* (Maity et al. 2020). Pigments were isolated from oyster mushrooms (melanin) and identified by techniques such as UV-vis (UV visible spectroscopy analysis) to measure solubility. FTIR (Fourier Transform Infrared Spectroscopy) was used to identify functional groups and NMR for structural identification of fungus compounds. Elemental composition analysis and HPLC (High Precision Liquid Chromatography) were used to compare pigments,

Table 26.10 Fungi and their pigment molecules

Fungi	Color; pigment	Pigment	Reference
Monascus spp.	Yellow; Ankaflavin (42)		Narsing Rao et al. (2017)
Ashbya gossypii	Yellow; Riboflavin (43)		
Blakeslea trispora	Yellow-orange B-carotene (44)		
Eurotium amstelodami	Red; Erythroglaucin (45)		Kalra et al. (2020)
Curvularia lunata	Orange-red; Chrysophanol (46)		
Curvularia lunata	Bronze; Cynodontin (47)		
Aspergillus fumigatus B5233	Brown dark; Melanin (48)		Urista et al. (2016)
Curvularia lunata	Blue; Anthraquinone (49)		

concluding through the analysis of a pigment obtained from a mixture of eumelanin and pheomelanin (Zhang et al. 2022). Studies with the same oyster mushroom allowed the characterization of statin nanoparticles for medicinal purposes. This analysis was performed using DLS (Dynamic Light Scattering) technique to measure the size of the nanoparticles (Mehra et al. 2020). In another study, silver nanoparticles were synthesized and characterized using Reishi mushroom extracts. A microwave was used for nanoparticle synthesis, and X-ray transmission electron microscopy (TEM) was used for nanoparticles visualization. X-ray diffraction

Table 26.11 Examples of methods for chemical characterization of fungi products

Fungi	Genus	Characterisation method	Compounds	Comments	References
Termitomyces heimii	*Termitomyces*	GLC-MS and NMR	Heteroglycan (THPS.)	THPS (6:2:2:1) is glucose, galactose, mannose, and fucose	Maity et al. (2020)
Oyster	*Pleurotus*	UV-vis, FTIR, NMR and HPLC	Eumelanin and pheomelanin	Three different color pigments were obtained	Zhang et al. (2022)
Oyster	*Pleurotus sajor-caju*	DLS, UV-vis, FTIR, SEM and HPLC	Nano statins	Antioxidant activity	Mehra et al. (2020)
Reishi	*Ganoderma lucidum*	X-ray, TEM, UV-vis and FTIR	Silver nanoparticles (Ag NPs)	Antibacterial effect against Gram-positive and Gram-negative bacteria, antifungal effect	Aygün et al. (2020)

(XRD) was used to evaluate the crystal structure and UV-vis and (FTIR) (Aygün et al. 2020) for nanoparticle characterization.

Other methods for the classification and identification of fungi compounds were used, such as MALDI-TOF MS (Matrix-assisted laser desorption/ionization-time of flight MS), SSR (Simple sequence repeats); ISSR (Inter simple sequence repeats); RAPD (Random amplified polymorphic DNA) and RFLP (Restriction fragment length polymorphism) (Wei et al. 2022).

26.6 Conclusion

The ability of fungi to produce an infinity of different compounds with immense bioactive potential is clear, and their role in the pharmaceutical industry has intensified over the years. Therefore, we consider that the exploitation of the potential of secondary metabolites obtained from fungi should be intensified in the future in order to extract the greatest possible benefit, in particular for human health.

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Part VI
Other Industries

Chapter 27
Cosmetic Industry: Natural Secondary Metabolites for Beauty and Aging

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Abstract The skin is constantly exposed to exogenous and endogenous factors that accelerate its deterioration and the loss of its physiological properties. Maintaining the skin healthy and good-looking became a requirement for our society. So, the development of bioactive cosmetic formulations to minimize aging signs as well as improve skin beauty and health has been widely explored. The actual demand of consumers for skincare products with demonstrated efficacy has pushed the cosmetic industry and researchers to work on the metabolites characterization and prove clinical evidence regarding the beneficial properties of their products. Among different ingredients, the natural secondary metabolites from plants (medicinal and aromatic plants) and macro- and microalgae have been demonstrated to provide several benefits for the skin, such as hydration, antiaging, and whitening effects. The most significant secondary metabolites are polyphenols, terpenoids, alkaloids, lipids, amino acids, and polysaccharides. Herein we describe the potential of relevant plant and macro- and microalgae secondary metabolites for skincare, mainly focusing on their antiaging properties. In addition, growing trends are depicted in the field related to the use of natural skincare products based on nanotechnology and nutricosmetics that seem to boost antiaging product development.

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27.1 Skin Aging and Natural Products

Skin changes related to aging are inevitable and include skin thinning, sagging, wrinkling, and the appearance of age spots, broken blood vessels, and areas of dryness. Aging causes a gradual and progressive deterioration of the skin tissue integrity and is induced by various intrinsic and extrinsic factors. Intrinsic aging is a naturally occurring process that is genetically determined, resulting in slow tissue degeneration. It is accompanied by a decrease in the number of dermal fibroblasts, synthesis of collagen and elastin in the extracellular matrix (ECM), which promotes the formation of wrinkles and the loss of elasticity, making the skin more fragile. Moreover, cellular senescence also indicates tissue aging compromising cell renewal (Kammeyer and Luiten 2015; Kanaki et al. 2016; Gu et al. 2020).

In contrast, extrinsic aging is induced and accelerated by environmental factors exposure like ultraviolet (UV) radiation, smoking, air pollution, etc. (Wong and Chew 2021). This leads to the decline in the proliferation and renewal ability of keratinocytes and reduction of the number of epidermal stem cells, decreasing skin barrier function and the upregulation of metalloproteinases (MMP) expression that increases collagen and elastin degradation (Rittié and Fisher 2015). The different morphological and structural changes in skin aging are illustrated in Fig. 27.1.

Considering that the skin aging process is associated with the high activity of the enzymes responsible for ECM degradation, the inhibitors of elastase, hyaluronidase, tyrosinase, and matrix MMP enzymes are described as potential cosmetic ingredients for antiaging products since they seem to promote the re-establishment of skin elasticity, increase of moisture content, stimulate collagen synthesis, and provide a

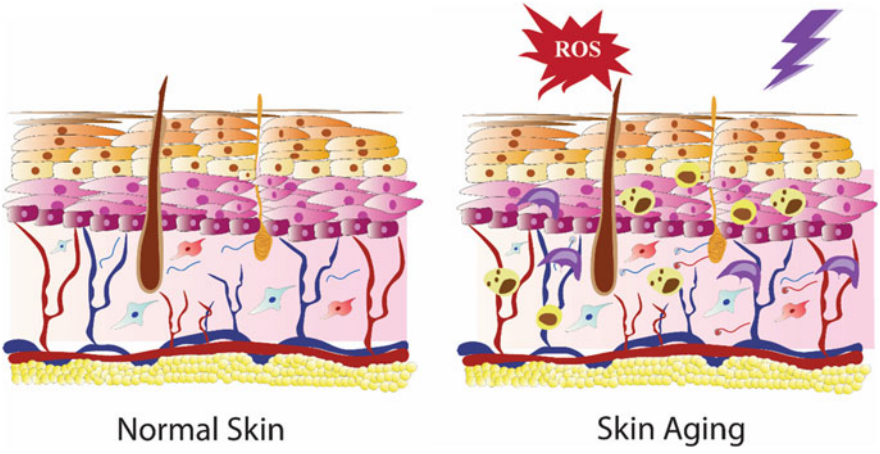

Fig. 27.1 Representation of the main changes associated with skin aging: increase of inflammatory cells activity, activation of enzymes (elastase, hyaluronidase, collagenases), which are induced by the skin exposition to UV radiation as well as other external agents that promote oxidative stress with the production of reactive oxygen species (ROS)

skin lightening effect (Afaq and Katiyar 2012; Lephart 2016; Zheng et al. 2019), and thereby minimizing the aging signals.

Considering skin needs and consumer requirements for natural ingredients that meet the same ethical requirements as their lifestyle, due to the awareness of the need for an alliance between health, sustainability, and environmental preservation, the cosmetic industry has been focused on the development of natural skincare products (ProFound 2005; Rossi et al. 2007; Vasiljević and Bojović 2018). In parallel, growing efforts are being made to study the mechanisms behind the effects of several natural products that have been used in traditional medicine and are currently available in cosmetics even without solid scientific support.

Thereby, cosmetic researchers are working on discovering novel natural and biodegradable ingredients that present relevant and effective properties and the vehiculation of natural bioactive compounds to boost their applicability and beneficial effects (de Morais et al. 2016; Nunes et al. 2019; Zhou et al. 2021a).

Plants synthesize different compounds (polyphenols, alkaloids, terpenoids, among others) in response to stress conditions (UV light, temperature, toxins) and external threats (virus, bacteria, fungi). These are known as secondary metabolites with antioxidant and anti-inflammatory properties that have been described to play essential roles in skincare. In addition, macro- and microalgae are a source of natural bioactive compounds (polyphenols, lipids, and polysaccharides (PS)) with beneficial potential for skincare (Miguel et al. 2021). Many attributes of plant extracts and essential oils (EOs), and micro- and macroalgae extracts used in cosmetics have been attributed to these compounds.

The following sections will describe the most relevant secondary metabolites from plants (extracts and essential oils) and macro- and microalgae used in skincare for antiaging properties.

27.2 Secondary Metabolites from Natural Products and Biological Activity for Cosmetics Development

27.2.1 Plants

Thousands of plant secondary metabolites have been identified so far in plant extracts and EOs such as (1) polyphenols, (2) alkaloids, (3) terpenes and terpenoids, among others. These compounds are synthesized by plants and are obtained from maceration with different solvents and distillation, depending on the solubility of the compounds of interest, with significant improvements registered for new extraction methods (Araujo et al. 2021). An infinite list of plant extracts-derived secondary metabolites and EOs are nowadays used in skincare products. The most relevant ones and their respective biological activities will be further described.

27.2.1.1 Polyphenols

Polyphenols constitute the major class of bioactive secondary metabolites especially known for their antioxidant activity. Due to their capacity to reduce oxidative damage, they are used to treat and prevent skin premature aging (Thring et al. 2009) mainly by providing photoprotection (Stevanato et al. 2014). Polyphenols can be divided into subclasses such as stilbenes, flavonoids, and hydroxycinnamic acids. These compounds protect from UV solar radiation, reducing oxidative damage and deleterious ROS generation (Stevanato et al. 2014), important features for antiaging formulations.

27.2.1.1.1 Stilbenes

One of the most widely known phenolic compounds is probably resveratrol due to its beneficial cardioprotective effects and its use in treating skin inflammation and other skin-related conditions. This stilbene is present in several plants, especially in *Vitis vinifera* L., *Polygonum cupsidatum* Siebold & Zucc. Nowadays, resveratrol can be found in several antioxidant and whitening cosmetic products of well-known brands like Estèe Lauder (Advanced Night Repair Intense Reset Concentrate), Caudalie (Resveratrol Lift Firming Serum), The Ordinary (Resveratrol 3% + Ferulic Acid 3%), SkinCeuticals (Resveratrol B E), Sesderma (Resveraderm Antiox Liposomal Serum) and Vichy (Slow Âge, Liftactiv). Moreover, the beneficial skin activities of resveratrol were demonstrated in a study comprising 20 patients with acne vulgaris by reducing the related lesion areas without causing side effects (Fabbrocini et al. 2011). Later on, it was also combined with baicalin and vitamin E, showing a clinical improvement in skin firmness and wrinkles appearance and the reduction of skin pigmentation after 12 weeks of topical application (Farris et al. 2014). Resveratrol also reduces skin hyperpigmentation induced by UV-light exposure, as demonstrated in an animal model (Lee et al. 2014). Its skin whitening effects are due to the capacity to downregulate the expression of proteins such as tyrosinase, tyrosinase-related proteins, and microphthalmia-associated transcription factor, which are involved in melanin synthesis. It was also shown to modulate nuclear factor erythroid 2-related factor 2-related mechanisms (Lee et al. 2014; Boo 2019).

Despite its remarkable properties, resveratrol presents limited stability. Different analogs such as resveratrate, resveratryl triacetate (RTA), and resveratryl triglycolate (RTG) have been developed to overcome this issue. As demonstrated in clinical trials, resveratrate protected the skin from sunburn and RTA, and RTG reduced skin UV-induced hyperpigmentation (Ryu et al. 2015; Jo et al. 2018).

27.2.1.1.2 Flavonoids

Amongst the different flavonoids synthesized by plants, quercetin is widely used in cosmetic formulations. Aside from its antioxidant activity, it also presents antimicrobial and anti-inflammatory properties. In a hairless mice model, quercetin topically administered helped to prevent UV-radiation deleterious effects on the skin, decreasing myeloperoxidase activity and glutathione levels and proteinase activity that leads to increased skin damage (Casagrande et al. 2006). More recently, in vitro and *ex vivo* (human abdominal skin) studies showed that this flavonoid inhibits cyclooxygenase-2 (COX-2), MMP1, and collagen degradation caused by UV-induced activation of nuclear factor-kappa B (NF-κB) and activator protein-1 (AP-1). It also inhibits extracellular signal-regulated kinase, c-Jun N terminal kinases (JNK), protein kinase B (Akt), and signal transducer and activator of transcription 3 signaling pathways. The same study also showed that quercetin binds to protein kinase C delta and Janus kinase 2 and inhibits their activity (Shin et al. 2019). Through these mechanisms, the role of quercetin is evident in skin protection from inflammation and premature aging. Regarding its whitening capacity, quercetin does not reduce skin hyperpigmentation, and only a few glycoside derivatives can show a concentration-dependent effect, according to a critical review (Choi and Shin 2016). Aside from its skin-related properties, quercetin has also demonstrated the capacity to stabilize UV filters used in sunscreen products (Scalia and Mezzena 2010).

As an antioxidant and anti-inflammatory, this compound is present in cosmetic products of Sesderma (Factor G Renew Serum, C-Vit Radiance), Paula's Choice (Resist Ultra-Light Super Antioxidant Concentrate Serum), Dr Dennis Gross (Ferulic + Retinol Serum Triple Correction Eye Serum, Alpha Beta® Pore Perfecting & Refining Serum), Epionce (Renewal Cream, Purifying Spot Gel), among others.

Another example of flavonoids is the anthocyanins subclass, which comprises different compounds responsible for different colors of plants and fruits and protects plants from UV radiation and other external factors (Abalos et al. 2016). In humans, anthocyanins present antioxidant and anti-inflammatory activities showing potential against skin photoaging. Anthocyanins extracted from black soybean seed coats showed both in vitro and in vivo protective effects against UVB radiation through NF-κB-dependent pathway and PI3 kinase/Akt pathway, inhibiting the synthesis of COX-2 and prostaglandin E2 (PGE2) (Tsoyi et al. 2008). Similar effects were observed for anthocyanins present in bog blueberry (cyanidin-, petunidin-, malvidin-, and delphinidin-3-glucosidase), blackberries, and red wine (Bae et al. 2009; Correia et al. 2021). Despite their relevant properties, anthocyanins seem to be used in cosmetics mainly as colorants. They can be found in products from brands such as Chemist Confessions (Gold Standard 30% Glycolic Acid Treatment), Missha (Extreme Renew Skin), Ta peau ton fruit (Wild Blueberry Serum), and Bybi (Babe balm).

Recently, isoorientin, an antioxidant of the sub-class of flavones, has attracted attention. This flavonoid can be found in plants as *Patrinia villosa* Juss., *Crataegus monogyna* Jacq., *Crataegus pentagyna* Waldst. & Kit. ex Willd and *Gentiana veitchiorum* Hemsl. with anti-inflammatory, anti-melanogenic, and antioxidant

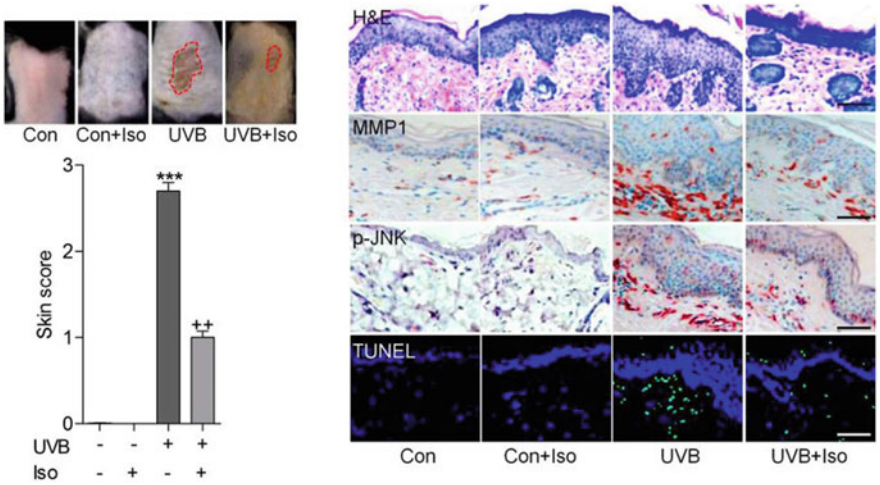

Fig. 27.2 Isoorientin topical treatment (25 mg/kg) attenuates UVB-induced skin damage in mouse skin. On the left side, representative images of the dorsal skin of mice after UVB irradiation and respective skin score. Data were shown as mean ± SEM. $^{***}P < 0.001$ versus the Control (Con) group and $^{++}P < 0.01$ versus the UVB group. Histological examinations (H & E) staining, immunohistochemical staining for MMP1 and p-JNK, and TUNEL (apoptosis assay) levels in skin tissue samples are on the right side. Reprinted from Biochemical and Biophysical Research Communications, vol. 514, Zheng et al., Isoorientin alleviates UVB-induced skin injury by regulating mitochondrial ROS and cellular autophagy 1133–1139. Copyright (2022), with permission from Elsevier

properties (Yuan et al. 2014; Wu et al. 2019). Other authors demonstrated that topical application of isoorientin in vivo (mouse model) could delay photoaging by inducing autophagy and reducing UVB-induced activation of JNK pathway, ROS production, MMP1 and MMP3 expression and apoptosis, as depicted in Fig. 27.2 (Zheng et al. 2019). Isoorientin is currently present in some commercial products such as Morechem Rooibos Extract (*Aspalathus linearis* Leaf) supplied by Morechem; Lespedeza Extract (Lespedeza Bicolor Bark) by Dermalab; Patrinia Extract-NS (*Patrinia villosa*) by The Garden of Natural Solution and Cosme-Phytami® Lemongrass (*Cymbopogon citratus* Leaf) by Alban Muller International, that are used in skincare formulations.

27.2.1.1.3 Hydroxycinnamic Acids

Among the hydroxycinnamic acid family, compounds such as coumaric acid and ferulic acid have been used in cosmetics. Para-coumaric acid (PCA), the most common coumaric acid isomer, is mainly found in cereals, fruits, and vegetables. It has been described that their anti-melanogenesis properties are responsible for inhibiting tyrosinase activity competitively and melanin synthesis more efficiently than Kojic acid and arbutin (An et al. 2010). PCA was also reported to protect human

epidermal keratinocytes against UV radiation by down-regulating stratifin expression and decreasing MMP1 expression (Seok and Boo 2015).

In humans, PCA could also reduce skin pigmentation and inflammation induced by UVB radiation exposure (Seo et al. 2011). Its UV-protective and anti-melanogenic effects make it an attractive compound for the cosmetic industry. It is supplied in different plant extracts by Dermalab (Dandelion, Ivy, Olive extracts), Repolar Pharmaceuticals (Resol®), The Garden of Natural Solution (Fumitory Extract-NS, Jeju Jori, Sweet Woodruff Extract-NS), Naturex (Blue Malva LG) with applications in skincare formulations. Moreover, ferulic acid is also used in cosmetic products. In vitro assays revealed that fibroblasts pretreated with ferulic acid and exposed to UVA radiation registered a reduction in ROS production and an increase in cell proliferation (Hahn et al. 2016). These beneficial effects were also confirmed in in vivo assays by oral administration of ferulic acid, resulting in the reduction of collagen degradation, epidermal hyperplasia and elastic fibers accumulation, typical histopathological signs of photoaging resultant from UVB exposure. According to the authors, this may be due to proteasome-mediated (posttranscriptional mechanisms) degradation of MMP2 and MMP9 (Staniforth et al. 2012). When topically applied in human volunteers, ferulic acid photoprotective effects were also observed by Saija et al. (2000). Moreover, Lin et al. (2005) described the potential of ferulic acid as a stabilizer for vitamins C and E as well as the photoprotection effect when included in cosmetic formulations composition.

Ferulic acid is thereby widely present in recent launched products from several brands such as Lancôme (Advanced Génifique Sensitive Antioxidant Serum), Eclat (Vitamin C Serum), Geek & Gorgeous (C-Glow 15% Vitamin C Serum), Facetheory (Resvera-f Serum), The Ordinary (Resveratrol 3% + Ferulic Acid 3%), SkinCeuticals (Phloretin Cf With Ferulic Acid, Prevent C E Ferulic® With 15% L-Ascorbic Acid), Medik8 (Super C30 + Intense), Dr Dennis Gross (Ferulic Acid + Retinol Brightening Solution), Paula's Choice (Resist Super Antioxidant Serum), Endocare (C-Ferulic Edafence), Sesderma (Ferulac Liposomal Serum), La Roche-Posay (Pigmentclar Serum) and many others.

Aside from the studies depicted above on plants derived secondary metabolites, there are other studies related to their phenolic content. Thring et al. (2009) studied 24 plant extracts (*Galium aparine* L., *Arctium lappa* L, *Fucus vesiculosus* L., *Illicium verum* Hook. F, among others), among which *Camellia sinensis* Kuntze (white tea) presented the highest anti-elastase, anti-collagenase, and antioxidant activities. The authors hypothesized that it was due to its higher phenolic content, especially gallic acid. This study shows that diverse plants' extracts (obtained from different plants and structures) have interesting antiaging properties related to their phenolics' content. Other extracts obtained from plants Eucalyptus globulus and Rosa damascena are also rich in phenolic compounds, namely gallic acid, responsible for photoprotective effects. In specific, Park et al. (2018), through in vivo assays, showed that *Eucalyptus globulus* extract topically applied inhibited MMPs by suppressing AP-1 and reversed collagen loss induced by the exposure to UVB radiation, demonstrating its photoprotective effect. On the other hand, both in vitro and in vivo studies, by oral administration of Rosa damascena extract in mice,

demonstrated its photoprotective effects against UVB radiation reversing collagen loss and skin thickening and reducing wrinkles appearance by inhibiting MMP, AP-1 and Smad7 and up-regulating Smad2/3 proteins (Park et al. 2017). Other products, namely EOs, can be obtained from these and other plants. The skin benefitial properties of these compounds are mainly attributed to terpens and terpenoids as will be further ilustrated in the following section.

27.2.1.2 Essential Oils as a Source of Terpenes and Terpenoids

More than 50,000 terpenoids (oxygen-containing terpenes) have been identified, primarily derived from plants. Terpenoids are recognized for having antibacterial, anti-inflammatory, antioxidant, immunomodulatory, and antiaging properties (Yang et al. 2020) and are generally recognized as safe skin penetration enhancers (Aqil et al. 2007; Chen et al. 2016).

Terpenoids are mostly volatile compounds mainly found in essential oils, highly concentrated products widely used for external application and oral administration. EOs are stored in plants' cells-glands, glandular hairs, oil ducts, or resin ducts of flowers, buds, leaves, seeds, and stems. Regardless of EOs complexity (usually 20–60 different components), these are mostly composed of terpenes and terpenoids but also aromatic and aliphatic compounds (alcohols, ethers, aldehydes, ketones, esters, amines, amides, phenols, and others) (Bowles 2003; Bakkali et al. 2008; Pandey et al. 2016; Dhifi et al. 2016) with important biological activities for skincare as recently outlined (Rodrigues et al. 2020; Araujo et al. 2021; Cunha et al. 2021; Coimbra et al. 2022).

Some examples of EOs commonly used in skincare products are obtained from *Lavandula angustifolia* Mill. (Lavender oil), *Helichrysum italicum* (Roth) G. Don fil (Immortelle oil), *Rosmarinus officinalis* L. (Rosemary oil), *Eucalyptus globulus* Labill. (Eucalyptus oil), *Melaleuca alternifolia* (Maiden & Betche), Cheel (Tea tree oil), *Mentha* × *piperita* L. (pro. sp.) (Peppermint oil), and *Rosa damascena* mill L. (Damask rose oil). These EOs are included as active ingredients in several cosmetic products. Brands such as L' Occitane (Immortelle Divine Serum; Le Petit Remède, Créme Harmione Divine), Lancôme (Absolue The Serum), Caudalie (Vinopure Blemish Control, Vinopure Skin Perfecting Mattifying Fluid), Garnier (Organic Lavandin Eye Cream, Lavandin Anti Age Face Cream), L'Oréal Paris (Extraordinary Facial Oil), Naturavia (Crema Rosa Mosqueta), Lush (Vanishing Cream), are some examples that have included at least one or a combination of EOs.

27.2.1.2.1 *Lavandula angustifolia* Mill.

It is commonly known as lavender oil and is extensively used in the perfume and cosmetic industries to treat minor health problems (Carrasco et al. 2016; Pokajewicz et al. 2021). This EO is employed in a broad type of personal care products (soaps, lotions, tonics, creams, shampoos, conditioners, and shower gels) (Sabara and Kunicka-Styczyńska 2009; Białoń et al. 2019). The main constituents reported

were linalyl acetate and linalool (Miastkowska et al. 2021) but could contain more than 100 components, including terpinen-4-ol and lavandulol lavandulyl acetate, 1,8-cineole, limonene, *cis*- and *trans*-*β*-ocimene (Pokajewicz et al. 2021). It is recognized for its anti-inflammatory and antimicrobial effects (Cavanagh and Wilkinson 2005), improving wound healing (Mori et al. 2016), and controlling psoriasis symptoms (Cavanagh and Wilkinson 2002; Rai et al. 2020). In addition, de Andrade et al. (2021) showed that *L. angustifolia* EO (5% in a carrier oil) applied in human skin penetrates only the most superficial layers and that it reinforces the skin barrier capacity and improves hydration, demonstrating the usefulness of including this EO in cosmetic formulations.

Regarding the antimicrobial potential of EOs, this can be of great use in the field of natural preservatives for the cosmetic industry since the stability and safety of the cosmetic products are compromised by different microorganisms. Michalek et al. (2019) analyzed the records of the Rapex (European Union Rapid Information System for dangerous non-food products) database on cosmetics from 2005 to 2018. They reported that the majority of the contamination cases were caused by Gram-negative bacteria (59.6% mainly *Pseudomonas* spp. and *Enterobacte*r spp.), but also by Gram-positive bacteria (6.7%, mainly *Staphylococcus aureus*), fungi (1.9% *Candida albicans*), mould (1.9%), yeast (0.9%), and other unspecified microorganisms. *Lavandula angustifolia* extracts and EOs, revealed antimicrobial effect against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Candida albicans,* among other microorganisms, which was attributed to the phenolic compounds (caffeic, rosemeric, and the 4-hydroxybenzoic acids) (Turgut et al. 2017), suggesting the usefulness of its application directly or incorporated into the cosmetics formulations as natural preservatives.

27.2.1.2.2 *Helichrysum italicum* (Roth) G. Don fil

Helichrysum italicum EO is a pale yellow to red oily liquid with a strong aroma honey-like and is usually called "Immortelle" (immortal) oil. According to several studies, the composition of *H. italicum* EO varies according to geographical regions, with ten chemotypes described. Hydrocarbon monoterpenes, specially α-pinene and limonene, have a relevant presence. Nowadays, this EO is one of the most popular in cosmetics as it stimulates blood circulation, has a potent skin regenerating capacity, anti-inflammatory and antioxidant properties, and reduces the appearance of fine lines and wrinkles (Fraternale et al. 2019; Genčić et al. 2021). *H. italicum* EO effect on collagenase and elastase enzyme activities inhibition was attributed to limonene and α-pinene compounds (Fraternale et al. 2019). On the other hand, Genčić et al. (2021) hypothesized that the antimicrobial and anti-inflammatory activities of this EO can be attributed to the synergy of neryl esters, α-pinene, γ- and *ar*-curcumenes, and β-diketones but also to some minor constituents.

27.2.1.2.3 *Rosmarinus officinalis* L.

Rosemary EO is important in medicinal and aromatic areas. The predominant components of this EO are camphene, limonene, α-pinene, camphor, borneol, linalool, 1,8-cineole, (E)-caryophyllene, verbenone, and bornyl acetate, which also depends on the geographical location and season (Pintore et al. 2002; Celiktas et al. 2007; Afshar et al. 2021). Besides other clinical applications (Nieto et al. 2018; Labib et al. 2019), this EO has also shown wound healing potential, which was demonstrated using an excision wound animal model after the application of topical formulations (Labib et al. 2019), antimicrobial activity against diverse bacteria and fungi (Celiktas et al. 2007; Bozin et al. 2007) and also antioxidant effects (Bozin et al. 2007; Wang et al. 2018). In a study conducted in the United States, Fiume et al. (2018) observed that rosemary is present in 729 formulations, namely fragrances and skin conditioners. Through this work, the Cosmetic Ingredient Review Expert Panel assessed the safety of ten *Rosmarinus officinalis* (rosemary)-derived ingredients and verified that these ingredients are safe to be used in cosmetics.

27.2.1.2.4 *Eucalyptus globulus* Labill.

Eucalyptus globulus EO consists mainly of 1,8-cineole (eucalyptol) and α-pinene (Kumar et al. 2012; Vieira et al. 2017; Vivekanandhan et al. 2020), the main responsible for its characteristic odor (Kesharwani et al. 2018). The terpenoid eucalyptol has also demonstrated to function as a skin penetration enhancer, which could be usefull for specific topical treatments (Williams et al. 1990 DOI: 10.1023/A:1015813803205). Aside from its well-known anti-inflammatory and decongestant properties (Vecchio et al. 2016; Abelan et al. 2021), it also shows antioxidant, antimicrobial, antihistaminic, and antitumoral activities (Vecchio et al. 2016; Kesharwani et al. 2018). Its antibacterial effects against Gram-positive and Gram-negative bacteria (Salehi et al. 2019) demonstrate the potential to be used as a preservative in cosmetic formulations.

27.2.1.2.5 *Melaleuca alternifolia* (Maiden & Betche) Cheel

The tea tree, or melaleuca EO, possesses a sharp camphoraceous odor followed by a menthol-like cooling sensation. It contains terpinen-4-ol and delta-terpinene, as main components, but also α-terpinene, 1,8-cineole, p-cymene, terpinolene, and a-terpineol (Larson and Jacob 2012). The antimicrobial properties of *M. alternifolia* EO were intensively investigated, mainly ascribed to terpinen-4-ol as reviewed by Sharifi-Rad et al. (2017). Besides that, this major component also exhibits anti-inflammatory and antioxidant properties. Furthermore, several studies suggested the use of this EO in acne vulgaris and seborrheic dermatitis and improvement of wound healing (Pazyar et al. 2013; Capetti et al. 2020). Considering its beneficial effects, Bassett et al. (1990) demonstrated a marked improvement in

volunteers with acne vulgaris after tea tree oil at 5% topical application for 3 months, which was also observed in another study (Enshaieh et al. 2007). Moreover, Oliveira et al. (2016a) combined this EO with resveratrol as a sunscreen formulation for acne skin, reducing *Propionibacterium acnes* and skin oiliness, improving skin hydration, and showing a moderate photoprotection against UVA and UVB radiation.

27.2.1.2.6 *Mentha* × *piperita* L.

Peppermint EO, the so-called *Mentha piperita*, possesses a fresh sharp menthol odor and a pungent taste followed by a cooling sensation (Herro and Jacob 2010). This EO main components are menthol, menthone, menthyl acetate, neomenthol, 1,8-cineole, isomenthone and menthofuran (Güntert et al. 2001). Topical preparations of peppermint EO have been used to calm pruritus, relieve skin irritation and inflammation (Herro and Jacob 2010) and promote the wound healing process in vivo (Modarresi et al. 2019). In addition, it shows antimicrobial activity against *P. aeruginosa*, *S. aureus*, *C. albicans*, among other microorganisms (Marjanović-Balaban et al. 2018; Benzaid et al. 2019).

27.2.1.2.7 *Rosa damascena mill* L.

Damask rose, or Rose oil, is mainly composed of rose oxide, linalool, geraniol, citronellol, and nerol. Citronellol and geraniol provide the rose-like odor characteristic of this plant. It is also known for its antimicrobial, antioxidant, and anti-inflammatory activities (Nayebi et al. 2017; Alizadeh and Fattahi 2021), which are important properties when looking for an anti-aging effect.

This luxurious and expensive EO is used in different cosmetic products, for its skin beneficial properties as well as for its indistinguishable fragrance, such as Glimmer Of Hope™ Shimmering Facial Oil (Clarity Rx), Plantscription Powerful Lifting Concentrate (Origins), Pure Color Envy Nighttime Rescue Lip Oil-Serum (Estée Lauder), and Absolue The Serum (Lancôme).

27.2.1.3 Alkaloids

Alkaloids include a diverse group of secondary metabolites found in hundreds of plants, such as caffeine, nicotine, and cocaine (Othman et al. 2019). Among these examples, caffeine, which is present mainly in coffee beans, tea leaves, and cocoa, is widely used in cosmetics for its anti-cellulitis and antiaging properties. The antioxidant, anti-inflammatory, and UV-radiation protectant properties have also been described in several works (Devasagayam et al. 1996; Lu et al. 2007; Herman and Herman 2013). Recently, Rosado et al. (2019) demonstrated both in vitro and in vivo assays that formulations including caffeine (2.5%, w/w) provided a higher sun protection factor (SPF) and improved by 25% the protection of the skin against UVB radiation, demonstrating its added value as UV filter adjuvant.

Table 27.1 Examples of products containing plant-derived essential oils and extracts used in preparing skincare products, corresponding suppliers, and their biological activities

	Name	Supplier	Biological activities
Plant extracts	Resve	Minasolve	Antioxidant, anti-inflammatory, antiaging, whitening, anti-sebum and antimicrobial, used in skincare products
	Borealine Protect	Alban Muller International	Antioxidant, anti-inflammatory, anti-wrinkles, used in sun and skincare products
	NLT QueSphere 2.0™	Biospectrum	Antioxidant, anti-wrinkle used in skincare products
	Creeping saxifrage extract	Dermalab	Antioxidant, antimicrobial, and moisturizing, used in skincare products
	Red Cabbage Anthocyanin A-50 water soluble powder, E 163	Omya	Pigments for cosmetics
	Kohlrabi Extract-NS	The Garden of Natural Solution	Antioxidant and anti-inflammatory used in cosmetic products
	Morechem Rooibos Extract	Morechem	Antioxidant and UV filter used in cosmetic products
	Lespedeza Extract	Dermalab	Antioxidant, anti-inflammatory, and anti-atopy, used in skincare products
	Patrinia Extract-NS	The Garden of Natural Solution	Anti-inflammatory, antibacterial, anti-viral, and antioxidant used in cosmetic products
	Jeju Jori	The Garden of Natural Solution	Antioxidant, anti-inflammatory, and brightening, used in cosmetic products
	Ivy extract	Dermalab	Anti-inflammatory and enhances blood circulation, used in skincare products
	Resol®	Repolar Pharmaceuticals	Antimicrobial, anti-inflammatory, improves skin regeneration, used in nail and skincare
	Natural Ferulic Acid	MakingCosmetics	Antioxidant, antiaging, used in sun, skincare and antiperspirant products
	Freesia extract	The Garden of Natural Solution	Anti-inflammatory, antioxidant, and brightening used in cosmetic products
Plant essential oils	Lavender Essential Oil	Ayali Group	Anti-inflammatory and perfuming, used in toiletries, antiperspirants, and perfumes
	Lavandula Angustifolia (Lavender) Oil	Koei Kogyo	Fragrance enhancer and conditioning, used in perfumes and cosmetic products
	Helichrysum Italicum Essential Oil	Ayali Group	Antioxidant, antimicrobial, antibacterial, and anti-inflammatory,

(continued)

Table 27.1 (continued)

	Name	Supplier	Biological activities
			used in perfumes and cosmetic products
	Helichrysum Organic Essential Oil	New Directions Aromatics	Fragrance enhancer used in perfumes and cosmetic products
	BioNaturoil® Rosemary	BioOrganic Concepts	Antioxidant used in hair and skincare products
	BioScent® Rosemary Oil	BioOrganic Concepts	Antibacterial, antioxidant, antiseptic and rubefacient, used in hair and skincare products
	BioNaturoil® Eucalyptus Globulas Refined	BioOrganic Concepts	Antimicrobial, soothing, used in hair and skincare products
	Eucalyptus Leaf Oil	Green Angel	Antimicrobial and healing, used in skincare products
	Tea Tree Oil, Organic	SanaBio	Antioxidant and fragrance used in perfumes, hair and skincare products
	Tea Tree Oil	Provital	Antimicrobial, anti-dandruff, and antiseptic used in hair and skincare products
	BioNaturoil® Tea Tree	BioOrganic Concepts	Broad-spectrum antimicrobial and anti-inflammatory, used in skincare products
	BioScent® Peppermint Oil	BioOrganic Concepts	Anti-inflammatory and antimicrobial, used in skincare products
	Mint Piperita	Chemir	Analgesic, antimicrobial and astringent, used in perfumes and cosmetic products
	BioScent® Peppermint Oil	BioOrganic Concepts	Anti-inflammatory and antimicrobial, used in skincare products
	Rose Essential Oil	Ayali Group	Moisturizing, regenerating, fragrance used in perfumes and skin care products
	Bulgarian Rose Essential Oil	Esperis	Anti-wrinkle and astringent, used in skincare products

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These above-described properties make caffeine an attractive antiaging active component and is present in different skincare products from The Ordinary (Caffeine Solution 5% + EGCG), L'Oréal (Revitalift Derm Intensives Hyaluronic Acid + Caffeine Eye Serum), Vichy (Minéral 89 Eyes Hyaluronic Acid Eye Fortifier), La Roche-Posay (Pigmentclar eye cream), Kiehl's (Facial Fuel Energizing Moisture), SkinCeuticals (A.G.E. Eye Complex), Clinique (All About Eyes™ Serum De-Puffing Eye Massage, Id Active Cartridge For Uneven Skin Tone).

Table 27.1 describes commercial products containing plant-derived EOs and extracts used for cosmetic formulations, with the identification of their suppliers and main characteristics and biological activities. The increasing demand by the

cosmetics and personal care industry and food and aromatherapy is expected to lead the EOs market to grow 7.5% annually from 2020 to 2027 (Grand View Research 2020).

27.2.2 Macroalgae

In the global and actual challenge for the search of alternative and natural sources of active compounds for cosmetic formulations to meet consumer demands, algae-based products offer several advantages, as they are environmentally friendly, less toxic, non-carcinogenic, and have lesser side effects, as reviewed by Kalasariya et al. (2021). In the last decade, algae biotechnological relevance as a source of compounds has received increasing attention due to the variety of rheological and biological properties that confer to cosmetic products (Romano et al. 2017; Berthon et al. 2017).

Macroalgae are an important source of bioactive compounds (amino acids, lipids, polyphenols, terpenoids), which provide a multitude of benefits, including reducing redness and blemishes, improving brightening, hydrating, re-mineralizing, reducing sun damage, and firming skin (Thomas and Kim 2013; Pereira 2018).

The chemical composition of macroalgae depends on the environmental conditions (light intensity, growth habitat, seawater salinity, temperature) and genetic differences among species (Škrovánková 2011; Mæhre et al. 2014) and is reflected on the biological activities relevant for different industries, namely cosmetics.

Some recent works highlighted the potential of macroalgae extracts as anti-inflammatory and cell proliferation promoters. The methanolic extracts from *Phorphyra dentate* demonstrated an anti-inflammatory effect in mouse RAW 264.7 macrophages cell line (Kazłowska et al. 2010). In contrast, those extracted from *Caulerpa Mexicana* promoted a decrease in xylene-induced ear edema (Bitencourt et al. 2011). In turn, the ethanolic extracts from *Dictyopteris prolifera*, *Grateloupia lanceolata*, and *Grateloupia filicina* reduced the lipopolysaccharide-induced PGE2 production and the expression of inducible nitric oxide synthase (NOS) and COX-2 (Ahn et al. 2015), and the brown macroalgae *Sargassum glaucescens* extract was tested in the *stratum basale* and dermis layer UVA radiation burned skin and promoted cell proliferation (Li et al. 2019).

Considering macroalgae extracts' attractive properties, these have been widely explored in the cosmetic industry to promote skin beauty and reduce antiaging signals (through antioxidant and anti-inflammatory activities), as represented in Fig. 27.3.

Moreover, the cosmetics market launched different antiaging products such as Harmonie Divine Serum (L'Occitane), Absolue Premium Bx Night (Lancôme Paris), BlueTherapy (Biotherm), Pionnière XMF (Phytomer) containing *Jania rubens*, *Padina pavonica*, *Laminaria ochroleuca*, and *Laminaria digitata* extracts, respectively. Macroalgae extract products, like Codivelante® including Codium tomentosum extract, and Tonikelp®, Ocea Health®, and Firm'Act from Gelyma

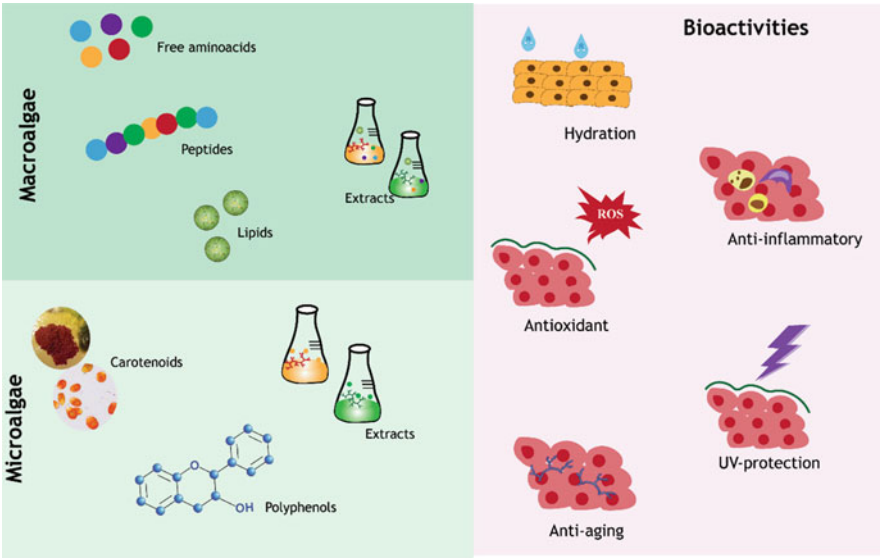

Fig. 27.3 Identification of the main metabolites derived from macroalgae and microalgae and their respective biological activities with potential for cosmetic applications

containing *Macrocystis pyrifera*, *Padina pavonica thallus*, and *Himanthalia elongata* and *Fucus vesiculosus* extracts, respectively, are also being used in the development of cosmetic formulations. Other examples of macroalgae-derived extract products are listed in Table 27.2.

Apart from macroalgae extracts, some researchers have been dedicated to extract and characterize secondary metabolites such as polyphenols, terpenoids, lipids, and mycosporine-like aminoacids (MAAs) and their respective biological activities. The following subsection will describe the application of these metabolites in cosmetics formulations and their antioxidant and antiaging effects.

27.2.2.1 Polyphenols

As for plants, macroalgae polyphenols present antioxidant properties with relevant activities on skin antiaging. For instance, phlorotannins (polyphenolic compounds more common in the brown algae) extracted from *Eisenia bicyclis,* and *Ecklonia kurome* demonstrated a strong hyaluronidase inhibitory effect, promoting the ECM functions' recovery (Shibata et al. 2002). Besides this, these bioactive compounds can also promote the synthesis of fibronectin, elastin, and cell-cell adhesion, which will reduce the appearance of wrinkles, as was demonstrated later on by Zappelli et al. (2016).

Further, the mixture of hydro-stable phlorotannins from brown algae *Ecklonia cava*, sulfated fucoidan from *Undaria pinnatifida* and saccharide glycosaminoglycan from sea squirt revealed the capacity to inhibit melanin synthesis, with an

Table 27.2 Examples of products containing macroalgae and microalgae extracts used to prepare skincare products, corresponding suppliers, and their biological activities

	Commercial Name	Supplier	Biological activities
Macroalgae	DermalRx® KBGA	Biocogent	Nourishing and antiaging
	Biogründl Marine Collagen	Biogründl	Provides nutrition, regeneration, protection, and moisturizing properties, used in hydrating, anti-wrinkle, eye creams, and other cutaneous preparations
	Mediterranean Algae Cocktail		Antiaging used in preparations for the treatment of eye bags and double chin and anti-cellulitis
	Laminaria (algae) extract	BotanicalsPlus	Cooling and healing agent recommended for the body, eye, face, hair, and skin care applications
	Red alga gel	Georges Walther	Moisturizing and softening agent, suitable for personal care products
	NMF-26	Onlystar Biotechnology	Moisturizer, used in hair- and skincare products
	Algae Oil	OQEMA	Emollient, carrier, moisturizing, and softening
	Codiavelante® BG	Seppic	Moisturizer, used in skincare products
	Herbal Extract Algae EG	Peter Jarvis	Firmness, soothing, and toning, used for hair and skincare products
	Fucomer®	Sensient Cosmetic Technologies	Moisturizer, restructuring, and firming used in skincare products
	Algae Polysaccharide	Spec-Chem Industry	Moisturizer, emolliate. Can form a transparent and air-permeable membrane on skin and hair surface
	Lipoplastidine laminaria	Vevy	Emollient, used in skincare products
Microalgae	Akomarine® Gum Complex	Akott	Hydrating agent. Used in the preparation of films with high controlled viscosity degree
	BiEau® Actif Green Algae	Active Concepts	Moisturizer, decreases transepidermal water loss, improves skin hydration and appearance used in skincare products
	Spirulina B	Bionest	Regenerating, whitening, photoprotective, smoothing and antiaging, used in sun-care and skincare products
	Sprinulina L		Regenerating, whitening, smoothing, antiaging and photoprotector, used in the sun- and skincare products
	Spirulina Extract	Carrubba	Antiaging, anti-wrinkle (improves collagen synthesis), antioxidant and nourishing, used for hair and skincare
	Spirulina Platensis	C.E. Roeper	Colorant for cosmetic products

(continued)

Table 27.2 (continued)

	Commercial Name	Supplier	Biological activities
	Spirulina	ieS LABO	Smoothing agent used in cosmetic products
	Spirulina	Parnika	Softening and shining agent, recommended for use in skin and hair care formulations
	Blue algae extract	BotanicalsPlus	Antiaging, suitable for body-, face-, and hair care applications
	Tego® Stemlastin	Evonik	Antiaging used sun- and skincare formulations

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excellent whitening effect (Wang et al. 2019). Similarly, the phenolic compounds extracted from brown algae *Padina boryana* were able to suppress tyrosinase and melanin expression in the B16F10 murine cell model, which is crucial to prevent hyperpigmentation (Jayawardena et al. 2020), as well as *Fucus spiralis* and *Cystoseira stricta* phenolics exhibited a pronounced response against tyrosinase activity and reduced the effect of free radicals (Grina et al. 2020).

27.2.2.2 Terpenoids

As previously described for plants, algae also synthesize terpenoids with the potential for skincare. For instance, fucoxanthin, one of the brown algae's most abundant and characteristic pigments, can also be found in red and green algae and microalgae and exhibits anti-melanogenic, antiaging and antioxidant properties. The skin protective effects of fucoxanthin have been recently reviewed by Lourenço-Lopes et al. (2021), namely regarding the prevention of wrinkle formation, epidermal hypertrophy, and other signs of photoaging and oxidative damage.

Also, the anti-melanogenic effect of fucoxanthin isolated from *Laminaria japonic*a was elucidated by reducing tyrosinase activity, suppressing PGE2 synthesis and melanogenic stimulant receptors in in vivo assays both for oral and topical treatments (Shimoda et al. 2010; Azam et al. 2017). *Laminaria japonica* extracts rich in fucoxanthin and PS are commercially available from Nuwen, offering skin-whitening effects.

27.2.2.3 Lipids and Fatty Acids

The lipid content in macroalgae species is relatively low, possessing less than 5%. Such fraction also contains glycolipids and phospholipids. Among them, palmitic acid has been used in cosmetics development as emulsifiers, where the derivated ascorbyl palmitate is an antioxidant with effective antiaging and anti-wrinkle effects (Yarnpakdee et al. 2018). As an example, the ω-6 polyunsaturated fatty acids, namely

the C-18 fatty acids obtained from seaweeds as *Laminaria* sp. have been used in cosmetics to prevent skin water loss (Guillerme et al. 2017; Alparslan et al. 2018).

In a study conducted by Otero et al. (2019) the lipid molecules extracted from brown macroalgae *Laminaria ochroleuca* (linoleic acid, oleic acid, linolenic acid, and palmitoleic acid) have been suggested as responsible for maintaining skin integrity. On the other hand, Undaria pinnatifida is rich in polyunsaturated fatty acids revealed to be relevant in reinforcing the skin barrier. Among them, linoleic acid is one of the most effective ω-6 polyunsaturated fatty acids that restore transepidermal water loss, as reviewed by Couteau and Coiffard (2016).

27.2.2.4 Mycosporine-Like Amino Acids

The mycosporine-like amino acids are low-molecular-weight secondary metabolites, colorless, and uncharged, considered to be the strongest UVA-absorbing compounds in nature. Hence, these metabolites have been identified as promising to be used as natural skin protection ingredients in photoprotective formulations.

The MAAs features are associated with their ability to dissipate the absorbed harmful UV radiation as heat energy into surroundings without forming reactive photoproducts or ROS, making them an ideal candidate for commercial sunscreens (Sen and Mallick 2021). In fact, red macroalga *Porphyra umbilicalis* extract rich in MAAs are currently used in sunscreens products as Helioguard™365 and Noriguard nc by Mibelle Biochemistry Group, or Helionori® by Gelyma. Other examples are Ronacare® RenouMer based on Polysipohonia elongate extracts and the sunscreen Alga Maris® from Laboratoires Biarritz containing Gelidium corneum extracts. In addition, two novel types of MAAs extracted from green algae *Klebsormidium* and *Interfilum*, also demonstrated the ability to act as a natural sun blocker (Gharib et al. 2020).

The anti-photoaging activity of MAAs seems to be related to the reduction of lipid peroxidation. These compounds can exert antioxidant and anti-inflammatory activities by regulating the expression of COX-2. Moreover, MAAs also inhibit the UV-enhanced activity of elastase and matrix metalloproteinases, which leads to the control of the decomposition of elastin and the formation of wrinkles, inhibiting the protein glycation and collagenase activity as recently was reviewed by Singh et al. (2021). These compounds also display antiaging effects, namely in wrinkle and fine line reduction, skin firming, and skin whitening (Vega et al. 2021). As for the relevance of MAAs, databases on their distribution in marine and freshwater organisms have been developed (Sinha et al. 2007; Wada et al. 2015; Sun et al. 2020; Geraldes and Pinto 2021).

27.2.3 Microalgae

Microalgae are photosynthetic microorganisms that produce valuable metabolites when exposed to adverse environmental conditions. Their metabolism can be modulated by manipulating culture conditions, namely temperature, salinity, irradiation,

etc. The main secondary metabolites derived from microalgae are terpenoids, polyphenols, fatty acids, and PS. Their respective biological activities are especially promising for the cosmetic industry to be used as antiaging, sunscreen, and skin-whitening products. Some examples of microalgae extracts used in cosmetics are Starting Over ™ by Origins and Pionnière XMF by Phytomer, based on *Chlorella vulgaris*.

27.2.3.1 Polyphenols

Commonly found in microalgae and cyanobacteria, phenolic compounds are also described as antioxidants (Guedes et al. 2013; Wollina et al. 2018; Barkia et al. 2019), which can act as skin-whitening agents. Hämäläinen et al. (2007) reported that up to 36 microalgal flavonoids acted as inhibitors of NF-κB activity, which consequently prevented the expression of NOS and the production of NO in macrophages which is of utmost relevance for anti-inflammatory activity. *Nannochloropsis* sp. G1-5 extract is rich in phenolic compounds as carotenoids and flavonoids, and fatty acids, known to have antiaging photoprotection, anti-inflammatory, antioxidant, anti-melanogenesis, and moisturizing properties through the: (1) decrease of melanin content and inhibition of the tyrosinase activity; (2) enhancement of hyaluronan synthetase (HAS-2) expression; (3) suppression of MMP1 expression and (4) increase procollagen synthesis (Kim et al. 2021). Another example is *Spirulina platensis* extract, which possesses a considerable amount of phenolic compounds, which could produce a high concentration of sun protection factors, inhibiting tyrosinase activity. On the other hand, the photochemoprotective effects exhibited by phenolic compounds of *S. platensis* were crucial to protect against UBV radiation's effect and maintain optimal moisture content (Mapoung et al. 2020).

Other microalgae have also been used in the sun- and hair care products: a protein-rich extract from *Arthrospira* that repairs the signs of early skin aging, exerting a tightening effect and preventing stria formation (Protulines, Exsymol S. A.M., Monaco); and an extract from *Chlorella vulgaris* that stimulates collagen synthesis, supporting tissue regeneration and wrinkles reduction (Dermochlorella, Codif, St. Malo, France). In turn, Pentapharm (Basel, Switzerland) launched an ingredient from *Nannochloropsis oculata* with excellent skin-tightening properties (Pepha-Tight) and another one from *Dunaliella salina*, which shows the ability to stimulate cell proliferation and turnover (Pepha-Ctive) (Stolz and Obermayer 2005).

27.2.3.2 Terpenoids

The carotenoids derived from microalgae with potential interest in the cosmetic industry, show antioxidant, skin regenerative, anti-blemish, antiaging, and anti-wrinkling properties (de Morais et al. 2015; Michalak and Chojnacka 2015;

Sathasivam and Ki 2018), as well as photoprotection, anti-inflammatory and improve collagen synthesis (Xu et al. 2017), as illustrated in Fig. 27.3.

The main carotenoids encountered in microalgae are β-carotene, lutein, zeaxanthin, astaxanthin, neoxanthin, fucoxanthin, and violaxanthin. β-carotene extracted from several microalgae and cyanobacteria has been described to regulate UVA-induced gene expression in human keratinocytes (Lisby et al. 2005), to modulate various biological molecules involved in skin barrier function after oral administration (Takahashi et al. 2019; Kake et al. 2019) as well as to improve photoprotection and reduce skin aging signs after both topical application and oral administration (Balić and Mokos 2019), in mice models.

Astaxanthin, a carotenoid commonly found in microalgae, protects against oxidative damage through various mechanisms. Astaxanthin from natural origin, namely the green microalga *Haematococcus pluviallis*, is industrially produced and currently included as INCI in products such as Solasta™ Astaxanthin (Solix Algredients), and PromaCare® ATT (Uniproma Chemical). Astaxanthin has been described as a promising bioactive with an important role in skin homeostasis, photoprotection, and age-related skin diseases, based on antioxidant and anti-inflammatory effects as reviewed by Davinelli et al. (2018), which results in the acceleration of skin regeneration and the reduction of wrinkles, related with radical scavenging and downregulation of the inducible NOS, COX-2, and tumor necrosis factor-α, interleukin IL-1β and IL-6 (Sathasivam and Ki 2018; Lopes et al. 2020). Moreover, their positive effects on the human skin were registered for oral supplementation and topical application with improvements in skin elasticity, texture, and moisture content and decreased skin dryness, wrinkles, and transepidermal water loss (Singh et al. 2020; Zhou et al. 2021b).

Thereby, there is a continuous effort to identify feasible natural sources of carotenoids for cosmetics development.

A high content of carotenoids was identified in ethanolic extracts from seven cyanobacteria strains (picoplanktonic *Cyanobium* sp. LEGE 06113, *Cyanobium* sp. LEGE 07175, *Synechocystis salina* LEGE 06099, *Synechocystis salina* LEGE 06155, the filamentous *Phormidium* sp. LEGE 05292, *Nodosilinea nodulosa* LEGE 06102 and *Tychonema* sp. LEGE 07196). It was observed that these compounds were able to scavenge deleterious free radicals implicated in skin aging, with the highest scavenging ability observed for *S. salina* LEGE 06099, the richest strain in total carotenoids, zeaxanthin and lutein. Moreover, a significant increase in fibroblasts and endothelial cells' viability was observed for this strain. In general, these microalgae extracts showed potential for dermal matrix fill and consequent wrinkle fading and for delaying hyaluronic acid degradation through hyaluronidase inhibition (Morone et al. 2020).

27.2.3.3 Alkenoates and Alkenones

Alkenoates and alkenones are polyunsaturated long-chain esters and ketones biosynthesized by haptophyte microalgae, namely from the genus *Emiliania*,

Gephyrocapsa, *Isochrysis*, and *Chrysotila*, with potential applications in different sectors, as reviewed by Blasio and Balzano (Blasio and Balzano 2021). Alkenones were stated as a sustainable alternative to commercially available waxes used in cosmetics, namely for sunscreens formulations, due to their ability to improve photoprotection, without increasing the apparent viscosity of these products (Huynh et al. 2019), and as structuring agents in lipsticks (Huynh et al. 2020).

27.2.3.4 Polysaccharides

Microalgal PS, namely sulfated PS, as antioxidant, moisturizer, and humectant agents have also been used in the cosmetic industry for the development of lotions and creams (Raposo et al. 2013; Amna Kashif et al. 2018). A few cyanobacterial polymers have also been considered for their bioactive capacities with interest for skincare. The genus *Nostoc* is one of the most productive cyanobacteria for sulfated PS, with specific biochemical features (Otero and Vincenzini 2004), but the application in biomedicine and cosmetics is still scarce. Recently, it was reported that PS-rich extract of *Nostoc commune* was able to inhibit IL-6, β-hexosaminidase, and promote collagen I secretion, which plays a vital role in the control of inflammation, degranulation inhibitory activity, and skin aging prevention and wound healing, respectively (Tseng et al. 2021). In addition, *Nostoc* sp. PCC 7913 and PCC 7936 are efficient producers of anionic exopolysaccharides with shear-thinning fluid behavior and significant capacity to stimulate wound healing (Alvarez et al. 2021). These findings provide useful information for the cosmetics industry and present an important step in evaluating and identifying the potential effects of PS produced by the *Nostoc* for commercial applications.

Another PS with unique physical characteristics and exceptional moisture retention capacity, skin barrier functions, and anti-inflammatory properties is sacran (Okeyoshi et al. 2021). This giant PS (molecular weight $> 10^7$ g/mol) is extracted from *Aphanothece sacrum* and has been described as an anti-inflammatory against skin edema and atopic dermatitis (Fukushima et al. 2016; Ngatu et al. 2017; Motoyama et al. 2018) through the inhibition of Th2 cells and reduction of IgE release (Ren et al. 2022).

Also, as reviewed by Puluhulawa et al. (2021), sacran can inhibit the excessive evaporation of water from the skin and provide optimal conditions for keratinocyte differentiation. In addition, due to its high viscoelasticity and water retention (higher than hyaluronic acid), sacran is currently used as an additive in some cosmetics formulations.

On the other hand, cyanoflan, an extracellular sulfated carbohydrate polymer, is produced by the cyanobacterium *Cyanothece* sp. CCY 0110, exhibits high apparent viscosity, typical of a non-Newtonian fluid with pseudoplastic behavior and high emulsifying activity, comparable to xanthan gum. Therefore, this polymer can be useful in different industrial applications, namely as emulsifying/thickening agent (Mota et al. 2020).

Other PS isolated from cyanobacteria and microalgae like *Porphyridium cruentum* (Sun et al. 2009) *Rhodella reticulata* (Chen et al. 2010) *Schizochytrium* sp. (S.Y. Wang, Y. Jiang, C. Meng, Y.H. OuYang 2011), *Tetraselmis* spp. (Sansone et al. 2017) and *Rhodosorus* sp. SCSIO-45730 (Wang et al. 2021), diatom *Navicula* sp. (Fimbres-Olivarria et al. 2018), and *Graesiella* sp. (Trabelsi et al. 2016), have been described as antioxidants. The sulfated PS extracted from *Porphyridium* sp have also shown anti-inflammatory properties in vivo and the capacity to inhibit the development of erythema in human subjects (Matsui et al. 2003).

The mechanisms associated with the anti-inflammatory effect of red microalgae PS are related to the decrease of inflammatory cytokines release and the induction of NO, reducing blood vessel permeability and extravasation. In addition, it was also noticed a reduction in the recruitment and adhesion of polymorphonuclear leukocytes involved in the secretion of pro-inflammatory agents for *Coccomyx agloeobotrydiformis*, *Porphyridium* sp. (Matsui et al. 2003; Levy-Ontman et al. 2017; Dai et al. 2018). Besides, microalgal PS could have an important role in the modulation of the release of MMPs (such as collagenase and elastase), immune cells and in the control of the inflammation process, consequently impacting skin aging (Favas et al. 2021).

Table 27.2 summarizes micro- and macroalgae extracts of bioactive compounds that emerged in the market to develop cosmetic products.

27.3 New Trends in Natural Skincare

27.3.1 Nanotechnology

Cosmetic researchers have been searching for novel active ingredients (AIs) with promising properties for common skin issues (acne, aging, dark spots, among others). However, despite the significant efforts, the results were not as effective as expected, mainly due to the inability of these components to surpass skin barriers and reach the target layer or cells and the chemical instability. In this way, nanotechnology arises as a promising approach with nanodevices incorporating AIs and improving their stability and controlled delivery. According to the European Union legislation, a nanomaterial refers to an "insoluble or bio-persistent and intentionally manufactured material" presenting a scale of 1–100 nm (www.ec.europa.eu). In fact, since the 1970s, when L'Oréal developed niosomes, which were later introduced in the cosmetics market by Lancôme (Niosôme), a countless number of cosmetics containing nanocarriers have been launched (www.loreal.com). The enormous success rate and acceptance of these products are due to the great benefits that nanocarriers provide, namely: (1) improve AIs' solubility, stability (photo and thermal degradation), and controlled release; (2) enhance AIs retention or penetration through the skin; (3) decrease AIs skin irritation, as reviewed by Araujo et al. (2015) and herein represented in Fig. 27.4.

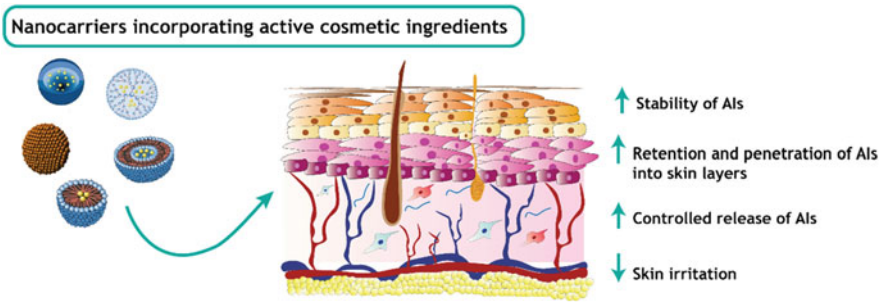

Fig. 27.4 Representation of nanocarriers incorporating active cosmetic ingredients (AIs) used in skincare and their benefits

For instance, different studies have reported the encapsulation of β-carotene in lipid nanoparticles (solid lipid nanoparticles, nanostructured lipid carriers, and liposomes) (Oliveira et al. 2016b; Pezeshki et al. 2019; Schjoerring-Thyssen et al. 2019; Maretti et al. 2021) and resveratrol in polymeric, lipidic and metallic nanoparticles as well as dendrimers (Soleymani et al. 2019; Szulc-Musioł and Sarecka-Hujar 2021) to improve their stability and penetration through the skin.

Regarding the cosmetic and dermatological applications of astaxanthin, several delivery systems have been developed to improve the skin application, as recently outlined by Lima et al. (2021), pointing out that nanoparticles and nanoemulsions seem to be the most developed and robust (Lima et al. 2021). Also, nanosystems have been proposed for other bioactive matrices, namely EOs. Rosemary EO was incorporated in a lipid nanocarrier, reducing wrinkles' depth in in vivo studies of photoprotection (Kamel et al. 2017) and into terpolymeric capsules, as an antifungal component in cosmetics (Neves et al. 2019). Carbone et al. (2018) also used nanostructured lipid carriers to encapsulate *Rosmarinus officinalis* L., *Lavandula* × *intermedia* "Sumian," *Origanum vulgare*, and *Thymus capitatus* EOs, enhancing their biocompatibility and biological (antioxidant, antimicrobial) activities.

As for the relevance and promising potential of nanotechnology applied to encapsulate natural compounds for the cosmetic industry, several recent reviews outlined the emergent solutions (Aziz et al. 2019; Salvioni et al. 2021; Zhou et al. 2021a).

In Table 27.3 are presented several antiaging cosmetic products (creams, serums), including liposomes and nanoemulsions in their composition. Sesderma, a Spanish company, is one of the several brands that use liposomes either to encapsulate hydrophilic (vitamin C, ferulic acid) and lipophilic (Vitamin E, retinoids) compounds in several of their product lines (moisturizing, antiaging, or whitening). However, most of the brands do not provide the composition or the AIs encapsulated in the nanosystems. Aside from cosmetics, other companies are specialized in developing nanocarriers for AIs encapsulation and efficient skin delivery, such as Basf, Lipotec, Istituto Ricerche Applicate, Active Concepts, Nanovec, Lipomize, among others.

Table 27.3 Antiaging cosmetic products (moisturizing, anti-wrinkle, whitening) containing nanocarriers, their respective main active ingredients, and manufacturer

Product	Active ingredient	Manufacturer
Liposomes		
Lumessence	Oat protein, rye seed extract	Aubrey Organics
Liposome (eye cream)	Polyglutamic acid	Cosme Decorte
Clinicians Complex Liposome	Vitamin E, hyaluronic acid, superoxide dismutase	Clinicians Complex
Derma Nature Bft	Ceramide, hyaluronic acid	Celimax
Cream Nanorepair Q10	Coenzyme Q10, polypeptides	Dr. Rimpler GmbH
ACC Vitamin C	Vitamin C, niacinamide, Stay C®	Dermafix
Melacure serum	Ascorbic acid	Dermabien
Serum Night Repair	Hyaluronic acid, Multi recovery complex	Estée Lauder
Hyaluronic Peptide Serum	Peptides, vitamin C, E, B, retinol	Ebanel
The Originals Proteos Liposome	Vitamin C, E, proteoglycans	Martiderm
Lifting Me	Ceramide, Lepidium Meyenii root extract	Make p:rem
Melatonin Liposome	Melatonin, hyaluronic acid	Maxclinic
Stemfactor - Growth Factor Serum	Growth factors, peptides	Osmosis StemFactor
Active Antiaging Eye Cream	Vitamin C	Perris Swiss Laboratory
Longevity cream	Vitamin C, cooper	Setarè
Eternalist A.G.E.	Retinyl palmitate	Sensilis
Snail Brightening	Snail secretion filtrate, alpha-arbutin	Sidmool
Revilatizing	Resveratrol	SanDaWha
Liposome Softener	Alpha-bisabolol	SanDaWha
Azelac	Azelaic acid	Sesderma
Resveraderm	Resveratrol	
Fillderma nano	Hyaluronic acid	
Ferulac	Ferulic acid	
Reti Age	Retinaldehyde, retinol	
Hydraderm	Hyaluronic acid, retinal, ceramide	
C-Vit	Vitamin C, gingko biloba extract	
Daeses Lifting	Dimethylaminoethanol (DMAE)	
Acglicolic	Glycolic acid	
Nanoemulsions		
NanoVital	Adenosine, niacinamide, herb extracts	Vitacos Cosmetics
Bepanthol Ultra facial cream	Vitamin E, B5, B3, ceramide	Bayer
Nano emulsion	Peptides, squalene, hyaluronic acid, mushroom extract	Hanacure

27.3.2 Nutricosmetics

As described above, secondary metabolites' beneficial effects were not only observed with topical application. As such, consumers are combining both topical (cosmetics) and oral (nutricosmetics or supplements) products to enhance their beneficial effects for healthy, young, and good-looking skin. The booming nutraceutical field also known as "Beauty from within" or SkinFood reached a market of $7 million in 2020, and due to its increasing demand, it is estimated to continue to rise (www.mordorintelligence.com) to about $17 billion by 2023 (Perbal and Gabaron 2021).

According to the Mordor Intelligence analysis, the nutricosmetics market's most prominent players are Amway and Herbalife International Inc., followed by Pfizer Limited, Lonza Group Ltd., and Reckitt Benckiser in Europe and General Nutrition Centers Inc, Beiersdorf AG and Shiseido Co Ltd in the USA (www.mordorintelligence.com).

A wide variety of compounds such as polyphenols, vitamins, carotenoids and fatty acids from plants, fruits and macro- and microalgae extracts, and minerals, peptides, proteins, and PS are available nowadays in nutricosmetics in the form of beverages, powders, or tablets. As outlined above, according to different in vitro and in vivo studies, secondary metabolites are highly versatile compounds that can be topically applied and orally administered. They also provide many beneficial effects to the skin, such as reducing skin inflammation, skin wrinkles and hyperpigmentation, and improving skin hydration and elasticity, mainly related to their antioxidant, anti-inflammatory, and photoprotective properties. In addition, as recently reviewed by Vollmer et al. (2018), these can also greatly impact gut and skin microbiome.

Among the plant, macro- and microalgae derived secondary metabolites, carotenoids and polyphenols are the most relevant. Carotenoids (β-carotene, lycopene, lutein, zeaxanthin, astaxanthin) are important antioxidant and photoprotective compounds especially relevant in the protection against exposure to UV radiation, that is responsible for fastening skin aging and premature formation of wrinkles and dark spots (Vollmer et al. 2018; Pereira et al. 2021). NuSkin developed G3, with carotenoids extracted from *Momordica cochinchinensis* (Lour.) Spreng. aimed for healthy skin. On the other hand, polyphenols such as resveratrol, isoflavones, and anthocyanins are important antioxidant and anti-inflammatory agents that reduce skin aging signals upon dietary intake (Luo et al. 2021). As such, Elixir, developed by Dekos, and Innéov firmeza 45+ developed by Innéov, containing resveratrol from *Vitis vinnífera* spp and soy isoflavones, respectively, are used for preventing and reducing skin aging signals.

In order to understand the effects of the consumption of these compounds through supplements, aside from their daily dietary intake, to prevent skin premature aging and reverse photoaging signs, different studies are being conducted in humans under controlled conditions (clinical studies), as shown in Table 27.4. Interestingly, many of the clinical trials included only female participants. This could be due to the

Table 27.4 Supplements tested in clinical trials for skin aging, acne, and general skin health (https://clinicaltrial.gov)

Supplement	Skin indication	Participants (number, age)	Dose, duration	Identifier
Isoflavone & Astaxantthin	Photoaging	$n = 90$ (female); > 45 years old	1 tablet/day; 24 weeks	NCT02373111
Beauty Image (collagen, peptides, phosphatidylserine, *Saussurea involucrate*, lutein)	Aging	$n = 80$; 28–55 years old	1.97 g twice/day; 8 weeks	NCT04733755
Astaxanthin & Licopene & D-L-alpha-Tocopherol	Aging	$n = 10$; 30–60 years old	Once/day; 12 weeks	NCT03460860
Nutrakos® (Amino Acids mixture)	Photoaging	$n = 12$ (female); 35–70 years old	2 stick packs/day; 4 weeks	NCT03801343
Revival Soy	Aging	$n = 40$ (female); 25–45 years old	20 g soy protein/day; 6 months	NCT00352157
LifePak Nano (multinutrient)	Aging	$n = 37$ (female); >50 years old	Daily use; 12 weeks	NCT00541931
Pre-Hyaluron 465 Innēov (glucosamine, manganese)	Moderate-severe aging	$n = 66$ (female); 35–60 years old	1 tablet/day; 4 months	NCT03274154
Quvital syrup with Q10vital® (coenzyme Q10)	Aging	$n = 33$ (female); 45–60 years old	1 tablet/day; 12 weeks	NCT02604641
Crucera-SGS (glucoraphanin); Meriva 500-SF (curcumin)	Photoaging, inflammation	$n = 25$; 18–70 years old	9 or 2 capsules/day; 8 weeks;	NCT03289832
Chenopodium Formosanum and Fagopyrum Esculentum Extrac drink	Aging	$n = 50$; 20–65 years old	30 mL/day; 56 days	NCT04237818
Imedeen	Photoaging	$n = 194$ (female); 35–65 years old	2 tablets/day; 6 months	NCT01787461
Anthocyanin	Skin health	$n = 62$ (female); <70 years old	500 mg/day; 12 weeks	NCT00574574
Cocoa flavanol beverage	Photoaging	$n = 64$ (female);	320 mg flavonol/day; 24 weeks	NCT02060097

(continued)

Table 27.4 (continued)

Supplement	Skin indication	Participants (number, age)	Dose, duration	Identifier
		>40 years old		
Pomegranate extract or juice	Photoaging, inflammation	$n = 75$ (female); 30–45 years old	100 mg extract or 8 oz juice/ day 12 weeks	NCT02258776
Retinol Gummy	Aging, adult acne	$n = 35$; 18–45 years old	1 gummy/day 12 weeks	NCT04884516
Vegetarian Collagen; keratin: Ceramides; Astaxanthin	Aging, acne	$n = 75$; >26 years old	Daily use; 3 months	NCT04249128

higher demand of women for antiaging solutions but also to women's skin's faster aging caused by its thinner epidermis and menopause-related hormonal dysregulation (Farage et al. 2012; Wong and Chew 2021). Overall, these studies show that nutricosmetics present beneficial effects for skin care. For instance, the consumption of antioxidants as flavonoids through cocoa supplements was studied in a blind, randomized, controlled trial (NCT02060097) in women presenting moderate skin photoaging. During 24 weeks, Korean women (over 40 years old) took daily a drink containing fat-reduced cocoa powder, rich in flavanols (4 g cocoa, 320 mg flavonols). This supplement showed an improvement in skin elasticity (after 12 weeks) and wrinkles appearance (after 24 weeks), but no significant changes in skin hydration levels nor its barrier integrity were observed (Yoon et al. 2016).

Another example is pomegranate supplement. This fruit is also rich in polyphenols (tannins, anthocyanins, ellagic acid), and its consumption has been reported to reduce aging caused by UVB exposure and inflammation. In a randomized trial, the daily consumption of pomegranate extract (1 g) and juice (240 mL) for 12 weeks was demonstrated to increase skin protection against UVB radiation and a slight decrease in melanin synthesis (Henning et al. 2019).

Despite the motivating effects observed in humans, corroborating the in vitro, in vivo, and clinical studies, their comparison is difficult due to major differences among them, namely type of population (gender, ethnicity, age), number of participants, type and amount of supplement, duration of the study, among others that have been shown to influence significantly the outcomes of these studies (Corrêa et al. 2018). A major shortcoming in published studies is that the existing scientific investigations were carried out with a small number of subjects, mainly female volunteers. Indeed, good clinical practice is often lacking in the protocols for cosmetic and nutricosmetics products. This is a major challenge for industry and academy to cooperate in this field and generate more data on safety and efficacy to improve the benefit for consumers. These studies would support the claims for activities and justify high market prices for cosmetic products evaluated and characterized following standards required in biomedical research.

27.4 Conclusions and Future Prospects

Skincare is important from an aesthetic and medical point of view. As for the increasing demand for natural, sustainable, and effective products, cosmetic researchers have been pushed to study these metabolites' mechanisms and find new ones. The diverse secondary metabolites synthesized by plants, macro- and microalgae share remarkable features as antioxidant and anti-inflammatory and provide outstanding effects against skin aging. Polyphenols and carotenoids seem to be the most promising for skincare, reducing wrinkles and hyperpigmentation, and enhancing photoprotection against UV radiation. The use of these bioactive metabolites as a cosmeceutical ingredient needs to overcome some challenges such as extraction methods, chemical stability, and quality assurance and regulations. Recently, the development of nanocarriers for the controlled and targeted release of these bioactives emerged as an important tool in cosmetics development.

On the other hand, the combination of topical cosmetics with nutricosmetics demonstrates potentiating secondary metabolites' beneficial properties on the skin. Besides, the emergent relevance of gut-skin microbiome might modulate how we currently treat the skin, with more research focused on active ingredients targeting skin microbiota and assessing their action mode. Plant-, algal-, and other natural sources-based active ingredients positively affect maintaining skin microbiota balance. In addition, the ingestion of probiotics, widely described for the beneficial modulation of the gut microbiota, also provides beneficial effects to the skin. In this way, a new skincare field will be the interaction of the gut-skin microbiota as a determinant for the approach of "Beauty from within" and for cosmetic innovation. In this sense, the combination of the afore-described secondary metabolites with pre-, pro-, and postbiotics, as topical and oral products, could benefit different areas of skin care and should be considered one of the most promising and innovative research areas in the future.

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Chapter 28
Biodyes: A Sustainable Approach for Textile Dyeing

Nuno Belino, Jesus Rodilla, and Roshan Paul

Abstract This chapter highlights the use of plant secondary metabolites, as natural colourants, aiming at reducing the environmental impact of the traditional dyeing process with synthetic dyes and other harmful chemicals. A comprehensive state of the art in the field of textile colouration technologies, their principles and application methodologies are presented. Authors also introduce fundamental knowledge about the use of natural dyes over time, examine the pros and cons of the different type of colourants and offer detailed information on the classification of natural dyes. All issues related to the natural dyeing process, their different stages and requirements are addressed and some standard recipes and dyeing parameters are provided. Finally, the use of *Reseda luteola* extract, as a biocolourant, and the natural dyeing of 100% wool fabric are fully described.

28.1 Dyeing Process

Dyeing is the complex and delicate art of colouring different textile raw materials in a manner that the attained colour can endure daily life conditions, namely: domestic washings, friction, perspiration, and sunlight. Dyeing may be considered as a form of art, through which we embellish the textile materials, reshaping their aesthetic and appealing value, and therefore, increasing their economic worthiness.

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Dyeing is a technical complex process with many variants and key parameters that must be firmly controlled. The objectives of such process may be described as follows:

1. Achieving the colouration of textile materials in accordance with volition or with a customer demand.
2. Attaining the colouration of textile materials with an adjusted level of dye penetration in the fibre.
3. Getting an even colour with good fastness properties.
4. Performing the dyeing process with a minimum of fibre degradation and loss of hand feel.
5. Accomplishing all the above goals in the most economic manner.

Dyed textiles are materials in which colourant is uniformly distributed and retained inside the amorphous areas of the fibre or held onto the surface. In this process a solid textile material is placed in close contact with a solution or dispersion of the dyes, absorbing it, and showing some resistance to return it into the dyebath. This resistance is the consequence of the energy of newly formed links, which depend upon the complex relation between the molecular structures of the fibre and of the dye, and upon the conditions in which the dyeing process took place.

An even colouration of the textile requires more than the superficial deposition of colouring materials. A long-lasting colouration entails their penetration inside the fibre structure and posterior fixation through a wide plethora of different chemical bonds. The packaging of the fibres into the yarn structure and the arrangement of yarns into the fabric structure play a key role in the colouration process.

The interpretation of the dye phenomena is not straightforward and has been the object of countless discussions and theories concerning the chemical and physical aspects of the process. Nowadays, the predominant opinion is that both classes of forces are present and conjugated in such a way that we do not have a clear perception of the limit and prevalence of each phenomenon. Thus, to have a better grasp of the dyeing mechanism, we must understand it as the expected behaviour of a colloidal solution in touch with the solids, which is precisely what occurs between the dyes and fibres within the dyebath (Cegarra et al. 1981; Wardman 2018a).

A normal dyeing process encompasses four stages, which are figuratively represented in Fig. 28.1, and can be described as follows:

1. The dye aggregated molecules are dissolved or dispersed in the dyebath and move throughout the liquid phase towards the fibre. This movement is known as diffusion of the dyestuff within the dyebath.
2. Afterwards, the dye molecules, spontaneously, go from the liquid phase to the solid phase (fibre phase) in a movement characterized by the adsorption of the dye molecules onto the fibre surface.
3. Subsequentially, the dye molecules migrate from the fibre surface into the fibre interior. This stage is identified as dye diffusion throughout the fibre.
4. The final stage comprehends the establishment of new bonds between the dye and the fibre, which is commonly known as dye fixation

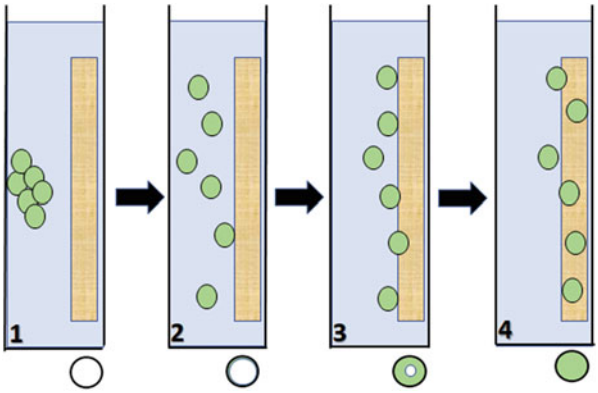


Fig. 28.1 Stages of the dyeing process

The colouration of textiles is the process of adding colour to fibres. It can be carried out with dyes or with pigments. Thus, it is of paramount importance to differentiate between them. Both classes are a type of colourant. Dyes, or dyestuff, are soluble in water or, at least, can be made soluble for their application, whereas pigments are insoluble and have no affinity for the textile fibre, demanding other sort of application technique. It is noteworthy to mention that dyes and pigments differ by physical characteristics, dyes are much smaller than pigments (1–2 μ), but not by chemical characteristics (Burkinshaw 2016).

Dyes are organic chemical substances with selective absorbance and reflectance of different wavelengths of light within the visible range of the electromagnetic spectrum.

A dye molecule must have a conjugated system, videlicet, alternated double and single covalent bonds, between the atoms that form their structure, which is comprised by specific groups named chromophores, which provides colour and auxochromes that intensify, deepen and increase solubility in water.

Dyes are retained in the fibre by primary forces (ionic or covalent bonds) and by secondary forces (hydrogen bonds) whilst pigments are held by mechanical entrapment and the use of a resin (Wardman 2018b).

The economic and technical viability of dyes is strictly related to the fulfilment of certain requirements, namely:

1. Providing a suitable colour with high tinctorial strength.
2. Being lightfast.
3. Having good substantivity for the textile fibre.
4. Having good fastness properties when adsorbed by the fibre.

Different classes of dyes are applied to different textile substrates due to their specific substantivity. Table 28.1 depicts this relationship, for the most representative dye groups and for the most used textile fibres.

There are two main distinct dyeing processes: Discontinuous, usually referred to as exhaustion or batch process, and continuous, commonly known by padding and fixation techniques (Mohsin 2017). The discontinuous process is characterized by

Table 28.1 Relationship between dye and textile fibre

Dye	Wool	Cotton	Polyester	Polyamide	Acrylic
Acid	Yes			Yes	
Azo		Yes	Yes	Yes	Yes
Basic					Yes
Direct	Yes	Yes		Yes	
Disperse			Yes	Yes	Yes
Metallic Complex	Yes			Yes	
Reactive	Yes	Yes		Yes	
Sulphur		Yes			
Vat	Yes	Yes			
Mordant	Yes		Yes		

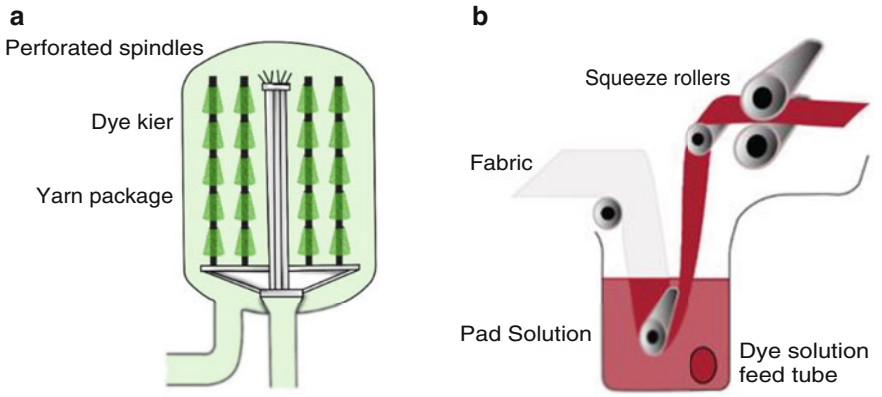


Fig. 28.2 Schematic view of an exhaustion dyeing apparatus for yarn (**a**) and padding apparatus (**b**)

the dyeing of a certain amount or length of raw material, namely: fibre, yarn, fabric. Figure 28.2a exemplifies this method for yarn dyeing.

In the continuous process, remarkably high lengths of fabric are dyed. This process can be subdivided into two other subclasses according to the dye fixation method: Continuous (Pad-steam, Pad-dry and Thermosol process) and semi-continuous (Pad-Batch, Pad-jig and Pad-roll process). Those methods are very suitable for cotton and cotton blends. Figure 28.2b illustrates the padding technique.

Dyeing is a very flexible and versatile procedure which, by economical or production criterion, can be performed in distinct stages of the textile manufacturing process (Mahapatra 2016). Hence, the main industrial dyeing methods are as follows:

1. Producer dyeing—Placed in the very beginning of the textile process, it can only be performed for manufactured fibres. It consists in the addition of colourants to the polymer solution during the extrusion process.

2. Gel dyeing—Similar to the above technique, it is carried out by the addition of colourants to wet-spun fibres whilst they are in the gel state, videlicet, not totally crystalized, and oriented.
3. Tow-Dyeing—Only applicable to manufactured fibres, takes place soon after the extrusion process. It comprehends the addition of dye to long and heavy cables of filaments, termed as tow, which will undergo a conversion process (cutting or bursting) to form staple fibre.
4. Stock Dyeing—Performed in the begging of the textile transformation process, it entails the addition of dye to loose fibres. This technique provides particularly good evenness colour distribution to fabrics.
5. Yarn Dyeing—It befalls the knitting and weaving operations and is achieved by the addition of dye to the yarn.
6. Piece dyeing—Also named fabric dyeing, is performed whilst the textile substrate, a fabric or knit, in piece, is immersed in the dyebath.
7. Garment Dyeing—Is the ultimate possible stage for the dyeing process, it occurs in the fully fabricated product, by the addition of dye to garments to attain a solid colour.

28.2 Natural Dyeing

28.2.1 Historic Perspective of Natural Dyeing

Since the beginning of time, Humanity has been attracted by the colours that were observed in Nature. Ancient civilizations began to use various materials to reproduce the colours of nature. The use of the different materials that were found has served to prepare the different colours and shades. The primitive peoples began to use minerals, vegetables, insects, animals to prepare the dyes that were used to make their expressions on the walls of the caves, to paint their bodies in their ceremonies, in the preparation of food, in the fibres used, in prepared textiles, in the use of leather and in everyday objects.

In this way the different cultures began to develop the techniques for dyeing fibres, textiles from natural dyes that are extracted from plants, invertebrates, minerals, etc. The most important natural dyes used in the different primitive cultures are of vegetable origin, such as: roots, bark, berries, leaves, and wood, there are cases in which fungi were also used.

We currently have data and information from archaeological studies carried out all over the world, of fibres and dyed textiles that correspond to Neolithic times. In Chinese culture, the knowledge of the use of plants, barks, insects was more advanced, the use of natural dyes can be traced back more than 5000 years, there are writings of the Chinese culture of the use of natural dyes that come from 2600 years ago.

With the discovery of the New World and other ancient cultures, we know that they used other materials to produce natural dyes, such as cochineal, used in dyeing

textiles and in paints in areas of Mexico and Peru. In the Bronze Age in Chinese culture, natural dyes such as alizarin, glitter and indigo were used (Adrosko 1971). In Europe alizarin and glitter were also used by ancient settlers; there were other areas where the ancient settlers painted their bodies with pastel (which corresponds to the same chemical compound as indigo) at the time of the Roman conquests.

Logically, the ancient settlers, in the different areas, used the materials that they had available in their localities, there were other rarer natural dyes that presented permanent and brilliant colours, obtained from invertebrates, such as Tyrian purple and Kermes crimson, which were highly prized luxury items in the Middle Ages.

At the beginning of the Middle Ages, the main natural dyes were produced from plants such as pastel (Isatis tinctoria), indigo, saffron, and madder, beginning the cultivation of these plants to increase the production of natural dyes giving a development to the economies of countries in Africa, Asia and Europe.

This dyeing industry with natural dyes for fibres and textiles was developed until the nineteenth century, at this time the first synthetic dyes began to appear and began to displace natural dyes. The exchange of natural dyes between the New World and the Old World was carried out with the colonization carried out in the new territories, dyes such as cochineal and campeche (Haematoxylum campechianum) were introduced in Europe.

With the subsequent industrial development, new synthetic dyes were easier to produce and cheaper, for this reason the use of natural dyes was reduced, currently representing one per cent of all dyes marketed. Today, we have a considerable number of colourants for different industries around the world (Agarwal and Tiwari 1989).

We know and it is documented that many countries have a strong tradition in the use of natural dyes in the dyeing of fibres and textiles, such as India, Turkey, Morocco, Mexico or African countries. About written documents on dyeing techniques and colourants begin to appear in Europe from the year 1429 in Italy, this information is documented in the work published by Hana Křížová: "Natural dyes: their past, present, future and sustainability" (Křížová 2015).

Although the use of synthetic dyes currently predominates in the dyeing industries, home dyeing has been maintained and developed at a particular level as a hobby, these tasks are documented and published in some books in the USA (Buchanan 1995; Buchanan 1987). This has allowed the resurgence of dyeing techniques with plants and natural dyes from the year 1970.

In Turkey, a project for the Research and Development of Natural Dyes (DOBAG) was launched with the collaboration of Germany in 1981, showing that textiles and fibres treated with natural dyes had better quality and greater durability than fibres and textiles dyed with synthetic dyes (Křížová 2015). This DOBAG project has succeeded in relaunching the traditional Turkish carpet industry and has revived dyeing with natural dyes in other areas (Chadramouli 1993; Chairat et al. 2008; Chattopadhyay et al. 2013).

Currently, great efforts have been made by researchers and some organizations to carry out the exchange of research work in Seminars, Symposiums, Workshops and in Research articles to show and disseminate the various sources of obtaining natural

colourants, all this information is available in the literature (Deveoglu 2013; Grierson et al. 1985; Grifoni et al. 2011; Gupta et al. 2004, 2013). There is no doubt that today there is a greater public interest in the use of natural products, natural colourants already present a greater variety of important applications. They are used in textile dyeing, functional finishes (antimicrobial, anti-food, deodorants, UV protectors), they can be used as food dyes, in cosmetics, additives, pH indicators and other different uses (Gulrajani and Gupta 1992).

28.2.2 Classification of Natural Dyes

The term "natural dye" is commonly used to refer to coloured matter extracted and made available from a natural resource. The classification of these dyes has been evolving over time in close relation with the advances of science. The earliest attempt to produce a systematic classification was made by botanists, and it was based upon the alphabetical order of the plant or by their botanical name.

Since they do not have a universal classification, they are sorted in many ways. Thus, it is a common procedure to group them in accordance with some specific characteristics, namely: origin, chemical constitution, application method, hue, etc. (Gupta 2020; Adeel et al. 2020).

One way to classify natural dyes is based upon their natural affinity to the fibre. Those colorants which are inherently substantive to textile materials dye the fibre directly, and therefore do not require the use of a mordant, and adjectives, which is the large majority and demand the use of a mordant to affix the dye into textile material.

Grouping in monogenetic dyes, which are natural dyes that produce only one colour, regardless of the used mordant, and polygenetic dyes which produce different colour depending upon the chosen mordant.

According to their origin, natural dyes are subdivided into three main categories. Table 28.2 illustrates the classification of natural dyes according to origin (Saxena and Raja 2014). Besides these bigger groups, it is possible to add other categories: Fungi, Mushrooms, Lichens and Microbial sources.

Table 28.2 Classification of natural dyes according to their origin

Origin	Natural sources	
Vegetable	Root	Turmeric, Madder, Onion, Beetroot, Dolu
	Bark/Branches	Eucalyptus, Sappan wood, Sandalwood, Jack Fruit tree,
	Leaf	Indigo, Henna, Eucalyptus, Teak, Tea, Cardamom
	Flowers	Marigold, Dahlia, Flame of the forest, Gulmohar
	Fruits/Seeds	Harda, Pomegranate, Beetle nut, Saffron, Kamala
Animal	Cochineal, Lac, kermes, Dactylopius coccus, Sea mollusc *Murex*	
Mineral	Manganese oxide, Cinnabar, Red ochre, Malachite, Prussian blue	

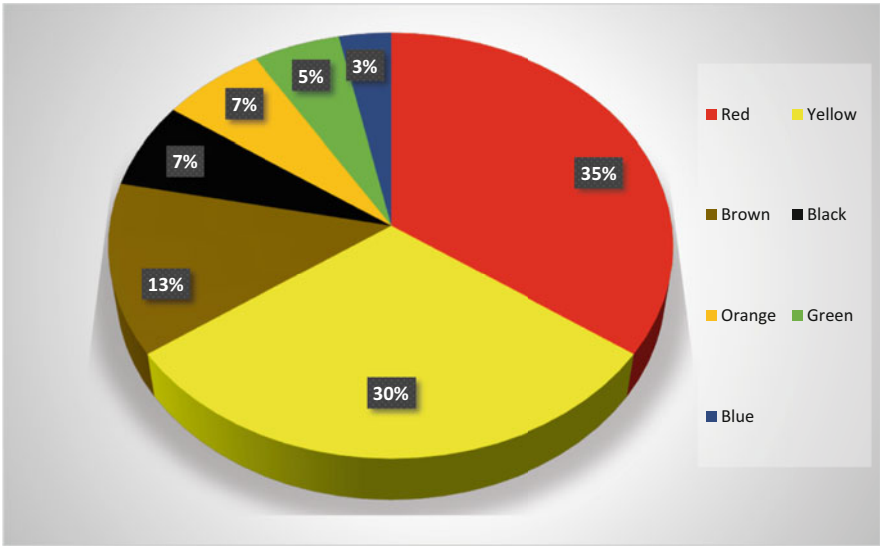


Fig. 28.3 Percentage of natural dyes according to their hue. Source: CI colour index

Table 28.3 Classification of natural dyes according to their chemical constitution

Chemical class	Natural sources
Indigoid	Indigofera, Pala indigo, Woad (Isatis), privet berries
Anthraquinone	Madder, Lac, Cochineal, kermes, Morinda
Naphthoquinones/Benzoquinones	Henna, Black walnut, Taigu, Ratanjot
Flavonoids	Weld, Marigold, onion, oak galls,
Dihydropyrans	Logwood, Sappan wood, Brazil-wood
Anthocyanidins	Bignonia chica, Beetroot, Grapes, Blackberries
Carotenoids	Carrots, Pumpkin, Bixin, Norbixin, Saffron

Taking into account the standardized classification of the colour index, natural dyes are subdivided according to their hue, videlicet, their predominant colour. Figure 28.3 shows their number as per the colour index.

Based on their chemical constitution, natural dyes may be categorized as depicted in Table 28.3.

To what concern to the application method, natural dyes are subdivided into six major categories as described in Fig. 28.4.

28.2.3 Pros and Cons of Natural Dyes

Natural dyes are deemed to be an eco-friendlier alternative to synthetic dyes. Their usage enables a considerable number of advantages but also imposes drawbacks

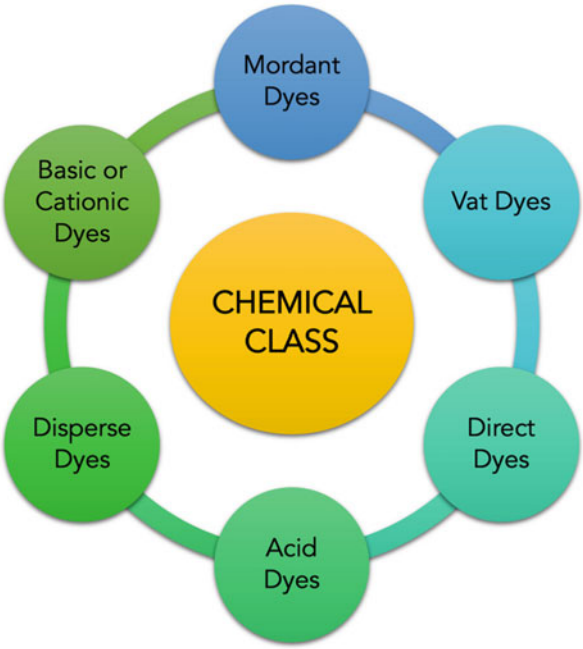


Fig. 28.4 Classification of natural dyes according to their application method

(Gupta 2020; Adeel et al. 2020; Saxena and Raja 2014; Viana et al. 2015; Prabhu and Bhute 2012). A comparative analysis for both, natural and synthetic dyes, is presented in Table 28.4.

28.2.4 Development of the Dyeing Process with Natural Dyes

28.2.4.1 Extraction

– **Extraction**

Natural colourants can be isolated from distinct parts of plants, lichens, animals, and others. They can be extracted from the starting material fresh (green) or dry and can be conducted with different solvents, methanol, ethanol, water or different mixtures of water and ethanol. In certain cases, these solvents can be accompanied by acids (HCl) or bases (NaOH).

These natural colourants correspond to products that can be classified as: phenols, flavonoids, anthraquinones and tannins mainly.

The raw material for the extraction of natural colourants contains lesser amounts of colourants and these are associated with other plant and animal components such as carbohydrates, fibres, proteins, chlorophylls and tannins. Natural colourants must

Table 28.4 Pros and cons of natural and synthetic dyes

Natural dyes advantages	Natural dyes disadvantages
They produce soft dyed shades	Difficulty in colour/shade reproduction
	Reduced colour gamut
Obtained from renewable resources	Low colour yield
Organic extraction wastes and by-products may be used for other applications	Requires more severe dyeing conditions (more time and energy consumption)
They value some disregarded land fields	Standardization of the colouration process is more difficult
They create job opportunities	Requires the usage of toxic mordants and higher dyes concentration
Possibility to earn carbon credits/carbon fixation	Lower fastness properties
Many natural dyes inherently anti-allergic and/or present an anti-bacterial behaviour	Requires skilled workers and more intensive labour
Some of them are insect repellents mothproof	Obtained from non-renewable resources
Biodegradable	There is not enough quantity available for industrial massification
Some of them are UV-blockers	The exploration of new natural dye sources requires more scientific background and safety studies
Some of them possess curative and healing properties	Non-biodegradable
Easy to treat water effluents	Some forms of natural dyes are not suitable for industrial application
Certain baths may be reused upon their correction	Purified extract of natural dyes is expensive
	Logistics and maintenance are difficult

Synthetic dyes advantages	Synthetic dyes disadvantages
Good dye repeatability	Non-renewable
Better standardization features	Non-biodegradable
More economical	Bigger water waste
Brilliant colours	Bigger energy loss
Wide range of colour shade	Highly water pollutant
Deeper colour strength	Health hazards
Better fastness properties	Poses environmental risks
Greater fastness properties	
Large availability	
Easier to manage and to maintain	
Industrial feasibility	
Enlarged application fields	

be extracted, not only to prepare pure natural colourants, but also in the use of other preparations.

– **Aqueous Extraction**

The starting material, plant or animal is chopped or ground to facilitate the entry of the solvent into the material used. Extraction with these solvents can be done cold, or we can normally use temperatures which can range from 60 to 100 °C, if we use water. The ratio between the extraction material and the solvent used must be in a ratio of 5–15 g of material per 100 ml of solvents.

If the extraction of some material is conducted at room temperature, the extraction time is longer, we can use times of 24–48 h; at higher temperatures, the extraction time is shorter, for example, for temperatures of 70–100 °C, it can be carried out between 1 and 2 h.

To conduct the extraction of the starting material, it is placed in a container directly with the solvent, we can provide some type of agitation and when the extraction time is over, it is filtered to separate the dye solution from the rest of the starting material. Different methods can be used for the filtration process.

We have different extraction methods that we can apply for natural colourants, in addition to the one indicated above. We can do the extraction of the starting material in green or dry, using a Soxhlet apparatus at the boiling temperature of the solvent. This system allows the extraction of the dyes, directly, in this case a solution of the dye free from the plant material is obtained. We can use any solvent or solvent mixtures.

– **Acid and Alkali Extraction Process**

There are many natural dyes that appear in the form of glycosides, in this case the extraction is conducted under diluted acidic or basic conditions. The addition of the acid or the base facilitates the hydrolysis of the glycosides, producing a better extraction and a higher yield of the natural dyes. Dilute acid solutions allow the extraction of flavones (dyes) as they prevent oxidative degradation.

Alkaline extractions are suitable for dyes that have phenolic groups, since these are soluble in bases and improve extraction yield. Subsequently, the dyes are precipitated using an acid medium. The colourants of the annatto seeds are extracted by this method. This technique is also used to extract the lac dye from the secretions of lac insects and the red dye from the petals of the safflower flower (Mohanty et al. 1984).

The disadvantage that this method presents is that some colouring materials can be destroyed in basic conditions, we must consider the fact that some of the natural colourants are sensitive to the pH of the solutions.

Natural colourants are normally a mixture of different chemical compounds, changing the pH of the extraction medium by adding acid or base can lead to the extraction of different components of the colourant, which can impart different shades in subsequent dyeing and differences in colour fastness properties.

A good many of researchers have studied the extraction of natural dyes under various pH conditions and have compared the colour and fastness properties of dyed

fabric to discover the optimal extraction conditions for natural dyes, and more information continues to be added to the scientific literature every year.

– **Ultrasonic and Microwave Extraction**

They are the latest and most innovative extraction techniques. They are extraction processes assisted by microwaves and ultrasounds in which an increase in extraction efficiency has been observed due to the use of ultrasounds or microwaves. In these new methods, the amount of solvent necessary, the time and temperature of extraction must be considered. When plant material (or raw material) containing natural colourants is treated with water or any other solvent in the presence of ultrasound, exceedingly small bubbles or cavitation are formed in the liquid and begin to increase in size, and upon reaching a certain size can no longer retain their shape. When this happens, the cavity collapses or the bubbles burst creating elevated temperature and pressure. Millions of these bubbles are formed and collapse every second. The creation of extremely hot temperatures and pressures during the extraction increases the efficiency of the extraction in a brief time. Also, this process can be performed at a lower temperature and therefore the extraction of heat-sensitive dye molecules is better. The use of these new techniques has already been applied recently in different investigations of dye extractions (Liu et al. 2009; Mishra et al. 2012; Pradeep et al. 2012; Rahman et al. 2013).

In microwave extraction, natural material is treated with a minimal amount of solvent in the presence of a microwave energy source. The microwave works by increasing the speed of the processes so that the extraction can be completed in a shorter time with better yield. Sinha et al. (2013) have reported on the extraction of annatto dye with microwave energy. Previously, his research group had reported on the microwave-assisted extraction of blue dye from butterfly pea (Sinha et al. 2012). These new methods of microwave and ultrasonic extractions can be considered as ecological processes due to the reduction of extraction temperature, solvent use, and time, which results in lower energy consumption.

– **Fermentation**

It is a method that uses the enzymes produced by different microorganisms that are found in natural environments to help in the extraction processes.

The most common example of this type of extraction is the extraction of indigo. The freshly picked leaves and green parts of the plant are soaked in lukewarm water (around 32 °C). Fermentation begins and the colourless indigo bound to the glycoside unit found in the plant material is hydrolysed into the glucose molecule and the indoxyl molecule by the enzyme indimulsin present in the plant leaves. The fermentation is completed in about 10–15 h and the yellow solution containing the indoxyl molecule is transferred to shaker containers where the indoxyl molecule is oxidized with the air to the blue insoluble indigotine molecule that is deposited in the bottom. The solid is collected, washed and, after removing excess water, pressed into cakes.

The extraction of indigo from other plants that contain this colourant (for example, annatto) is also conducted with fermentation. This process can also be used with other colourants. In this method, the microorganisms naturally disintegrate the

binding substances of the colouring matter. The longer extraction time, the need to extract the colourants immediately after collecting the plant material, the bad odour caused by microbial action, etc. are some of the disadvantages of this method.

– **Enzymatic Extraction**

Studies have been conducted by different researchers to soften the extraction conditions, since plant tissues contain cellulose, starches and pectins as binding materials. In the researchers' studies, they used commercial enzymes such as cellulases, amylases and pectinases to remove the enveloping material, allowing the dye molecules to be extracted under milder conditions. This method is beneficial and facilitates the extraction of colourants from hard plant materials such as bark, roots and the like.

– **Solvent Extraction**

Depending on the polar nature of the natural dye molecules, they can be extracted with different organic solvents such as acetone, petroleum ether, chloroform, ethanol, methanol or use mixtures of these solvents such as mixtures of ethanol and methanol, or mixtures of water with booze etc. The extraction method with a mixture of water/alcohol can extract compounds from plant resources, both soluble and insoluble in water. For this reason, the extraction yield is higher, compared to the aqueous method, as more chemicals and dyes can be extracted. Acids or bases can also be added to the alcoholic solvents (or mixtures) to facilitate the hydrolysis of the glycosides and allow the release of the dye molecules. The purification of the extracted dyes is easier since the solvents used can be easily removed by distillation and reused. The extraction is carried out at a lower temperature, so the possibilities of degradation of the dyes diminish. The two most important drawbacks of the method are the presence of traces of solvents that are toxic and the greenhouse effect that they can cause. Another disadvantage of this method is that the extracted colouring material is not readily soluble in water and the subsequent dyeing technique must be conducted in an aqueous medium. The co-extraction of substances such as chlorophylls and waxy materials also create interference and problems.

– **Supercritical Fluid Extraction**

Supercritical fluid extraction is a very recent and emerging method in the extraction and purification of natural products.

A gas behaves like a supercritical fluid above its critical values of temperature and pressure. This fluid has physical properties between those of a liquid and those of a gas. They can spread across a surface more easily than a true liquid because they have a much lower surface tension than liquids. As they have a low viscosity, they have exceptionally good diffusivity and therefore a better interaction with the substrate. In turn, a supercritical fluid can dissolve many substances like a liquid, since the solubility of a substance in any solvent is greater at higher pressure and temperature, and these conditions are necessary to maintain a gas in a supercritical state. In the extraction with supercritical fluids, such as carbon dioxide (CO_2), it is a viable alternative to solvent extraction, since this technique is non-toxic, cheap,

readily available and leaves no residue. The critical values of temperature and pressure for the use of carbon dioxide are 31.4 °C and 1070 pounds per square inch (psi) or 73.8 bar, respectively. The supercritical CO_2 extraction method typically operates at temperatures between 32 and 49 °C and pressures between 1070 and 3500 psi. Since CO_2 is a nonpolar molecule, in the supercritical state, it behaves like a nonpolar organic solvent. A cosolvent or modifier may be added to the method to improve the solubility of slightly polar solutes. The advantage of the method is that the extract produced is free from trace residual solvents and heavy metals and is light in colour due to the absence of polar polymerizing substances, thus the process has gained popularity in the extraction of purified natural products for food and pharmaceutical applications. The disadvantage of the method is the high cost of the equipment and the low extraction of the most polar substances.

28.2.4.2 Pre-treatment

Before the dyeing process itself, grey textile materials are submitted to some preliminary operations that envision the removal of impurities such as: grease, fats, waxes, oily matter from the manufacturing process, dirt, dust, coloured materials and other contaminants, thus improving the wettability of the material, raising the dyeing yield, preventing stains, and fostering dyeing evenness (Singh and Bharati 2014; Jordeva et al. 2020; Barani and Rahimpour 2014).

The type and the severity of these preliminary operations depend upon the state of the material and their final end-use. Normally, all grey textile materials are subjected to a preliminary washing procedure. In case of wool and cotton material, a deepen cleansing operation is achieved by scouring. When white colour or pale shades are required, bleaching is required as well.

28.2.4.3 Mordanting

Poor fastness properties along low yield and colour limitation triggered the search for an auxiliary that could improve the dye uptake and fixation by textile materials. Therefore, to assure a certain degree of affinity a mordant must be added to the dyeing process, traditionally, a chemical compound in the form of a metallic salt.

The final appearance and imparted colour of the dyed material depend not only on the used dye and fibre, but also on the selected mordant and their application process. Mordants function as bridge between the textile materials and dyes, and for this, they must have some kind of affinity for the dye and for the textile fibre.

Mordants increase the rub fastness, washing fastness and light fastness of dyed goods and are traditionally classified into three main groups: natural and biomordants; metallic salts and oils mordants (Saxena and Raja 2014; Erdem İşmal and Yıldırım 2019). Figure 28.5 shows the most used mordants for each category.

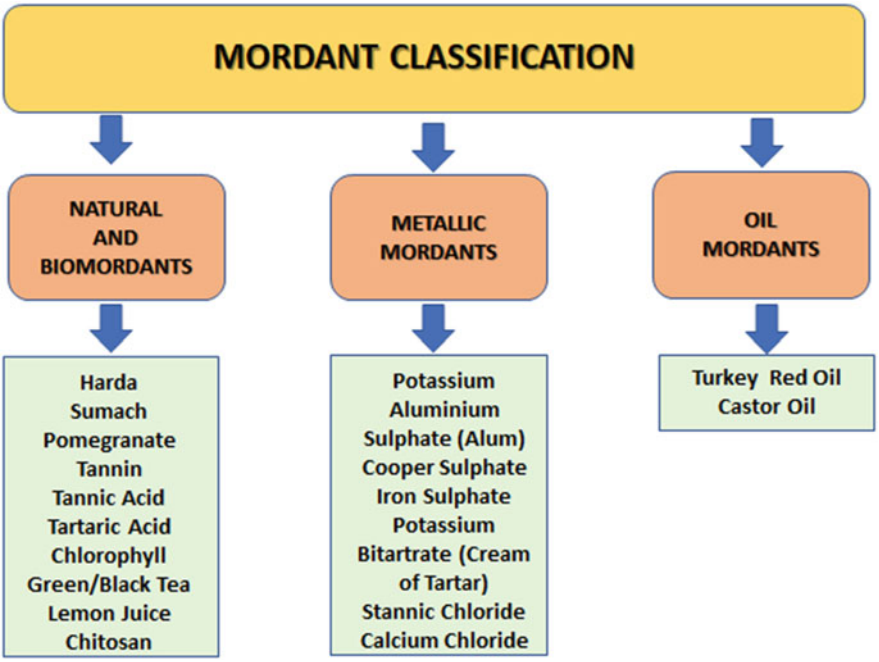


Fig. 28.5 Classification of mordants and a few examples thereof

Nowadays, a wider range of mordants options are available, including enzymes, food, wood and agricultural wastes and other organic by-products (Guesmi et al. 2013; Hosseinnezhad et al. 2020; Mathur and Gupta 2003; Pinheiro et al. 2019).

The mordanting process is not standardized and depends on key factors like the used textile material, chosen mordant and intended final colour. Notwithstanding, a compilation of most prevalent mordanting conditions, found in published papers, shows that mordant concentration varies from a minimum of 0.5–30 g/L; mordanting temperatures range from room temperature to boiling temperature; pH goes from acidic (3) to alkaline (10) whereas mordant time revolves around half-hour to one hour.

The application of mordants is commonly, known as mordanting, can be performed in four diverse ways:

1. Mordanting prior dyeing named pre-mordanting.
2. Mordanting after dyeing, called post-mordanting.
3. Simultaneous mordanting and dyeing known as meta-mordanting.
4. A combination of pre-mordanting and post-mordanting.

28.2.4.4 Dyeing

Natural dyes are most used in the colouration of natural fibres such as cotton, linen, wool and silk. Normally, a combination of one single colourant along with a mordant agent is applied, to achieve a predetermined colour under a strict set of tinctorial conditions due to their reproducibility issues (Mathur and Gupta 2003; Pinheiro et al. 2019; Ali and El-Mohamedy 2011; Belino et al. 2021).

Nonetheless, the usage of natural dyes in the colouration of synthetic fibres is possible and is more frequent with polyamide and polyester. Beside the primary colours, secondary colours may be obtained with judicious control of natural colourant and mordant or by a careful mixture of other two or three different compatible colourants. This last option is destinated to attain a specific colour that otherwise would not be possible.

Dyeing with natural colourants can be performed at any stage of the textile manufacturing process, similarly as their synthetic counterpart. Dyeing in an exceedingly early stage as fleece enhances colour evenness due to the subsequent blending operations and is particularly suitable for semi-industrial scale. Notwithstanding, most dyers prefer the colouration phase in a more advanced processing stage, specifically, yarn or fabric. Yarn hank dyeing is reserved for high valued raw materials considering that this process preserves better fibre orientation and placement in the yarn and minimizes the material damaging.

The traditional process of natural dyeing, carried out by artisans or small entrepreneurs, more oriented towards the handcraft market, is carried out in a manual process with large vessels made of aluminium, stainless steel, iron and cooper, whereas in industrial environment the process is done with bigger lots and performed in the jigger, winch or jet machines for fabrics and package dyeing machine for yarn dyeing. In industrial scale natural dyes can be found in the extracted form or in more user-friendly purified form. In this latter form the dyeing process takes place in the same manner as with synthetic dyes.

The amount of natural dye is usually expressed in % owf, meaning the necessary amount of dye, in grammes, required for the dyeing of one hundred grammes of textile material. The dye's concentration for purified dyes ranges from 2 to 5% whereas, when using coloured extracts, it can go up to 40%.

Considering that natural dyes possess different chemical constitutions and, textile fibres also have different chemical compositions, the standardization of a unique dyeing procedure is not feasible (Samanta and Konar 2011; Hu 2008; Giacomini et al. 2015). Hence, we should optimize the overall tinctorial conditions on a per case basis, notably, by adjusting the dye and mordant concentration, the pH, dyebath temperature, dyeing time, and used machine for each textile material/recipe.

According to Bechtold and Mussak (2009), an interesting and noteworthy attempt to scale up natural dyeing is being conducted by the Research Group of Natural Dyes at the University of Innsbruck along with some major Austrian textile mills.

28.3 Secondary Metabolites and Biocolouration

The biological systems that we know and use for the most varied applications present some ways to synthesize and produce products such as: carbohydrates, lipids, proteins and nucleic acids, which are the same in all of them, taking into account small variations.

These metabolic processes that are necessary for the functioning of biological systems are designated as primary metabolism and the products involved in them are the primary metabolites.

The designation of secondary metabolites corresponds to the products that have a more limited distribution in nature. Secondary metabolites are only found in certain organisms or groups of organisms and correspond to an expression of the individuality of the species.

The role of secondary metabolites in the producing organism, in most cases, is not yet known. In other cases, they are produced as protection systems for the organisms that produce them, such as: protection against being eaten by animals (herbivores), defence toxins against predators, repellence systems against other external organisms and so on.

Secondly, metabolites come from the primary metabolic system. The general process of obtaining primary and secondary metabolites begins with photosynthesis: the process of reacting CO_2 with H_2O to produce glucose and other carbohydrates.

D-glucose is the main starting product used by the metabolic process to produce all the primary and secondary metabolites they need.

Figure 28.6 shows a General Scheme of the main biosynthetic pathways.

In this metabolic system, nature uses an extremely limited number of precursors to produce many compounds and, above all, the interconnection between the products of primary and secondary metabolism is important.

The most important key precursors that nature uses in the biosynthesis of secondary metabolites are the following compounds: acetyl-CoA, this intermediate occupies a principal place in the metabolic system; shikimic acid (or shikimate, in the ionic form) and mevalonic acid (or mevalonate), which originate the three main routes in the biosynthesis of secondary metabolites.

From the main intermediary, acetyl-CoA, the polyketides are produced by condensation, which originate by subsequent reduction processes the fatty acids that participate in the primary metabolism, if the polyketides carry out a cyclization process or subsequent cyclization processes, they can produce the phenolic compounds, anthraquinones and other derivatives of the phenolic compounds that would be in the secondary metabolism.

If the metabolic route uses the shikimate intermediate, this intermediate is produced from carbohydrates, producing aromatic compounds such as cinnamic acids, coumarins, lignins or aromatic amino acids that will be the direct precursors of alkaloids as secondary metabolites.

The other important intermediate for producing secondary metabolites is mevalonate, which is produced in this pathway by the condensation of three

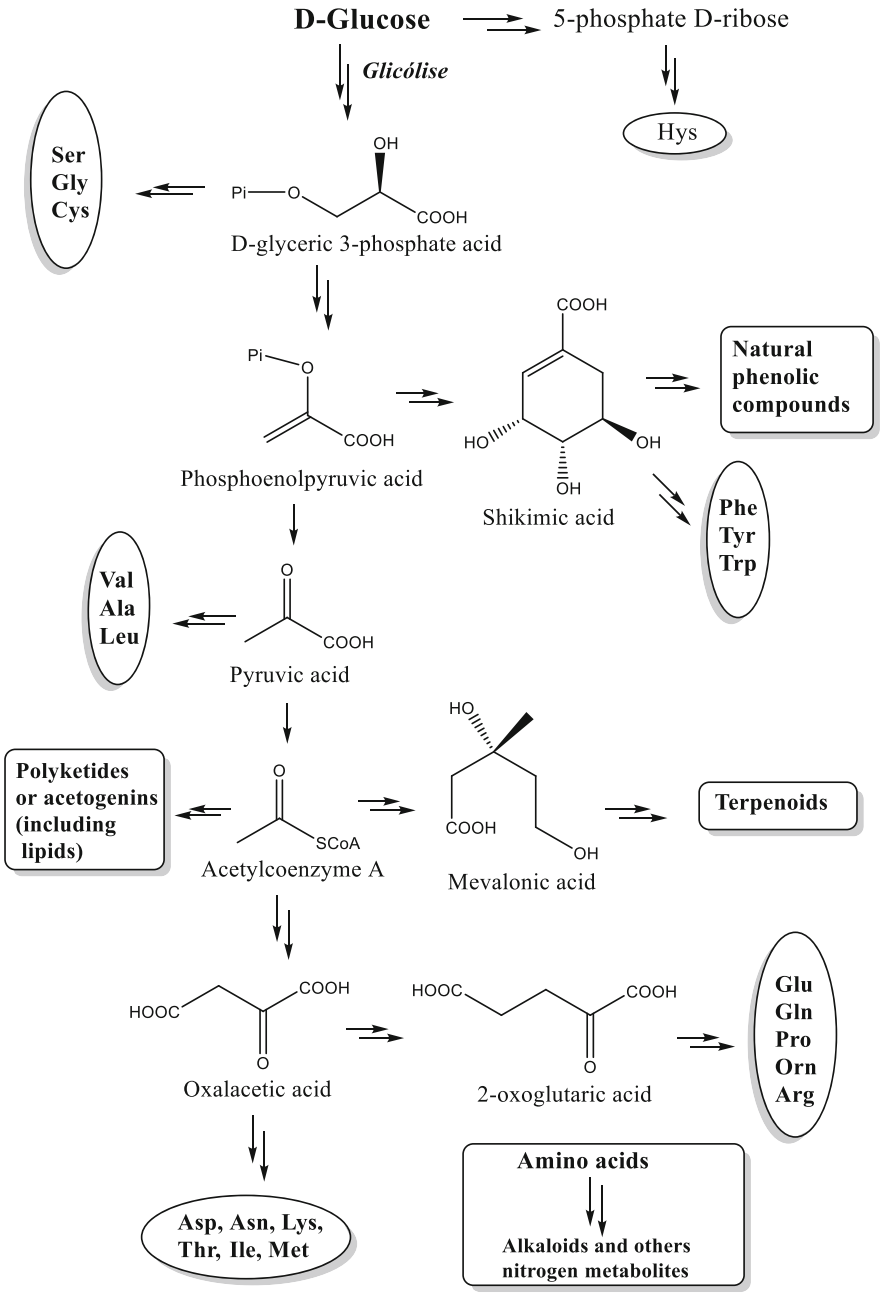


Fig. 28.6 General scheme of the main biosynthetic pathways

acetyl-CoA molecules. The mevalonate biosynthetic pathway will produce a series of different compounds other than the acetate biosynthetic pathway, in this case they will produce terpenoids and steroids.

On the other hand, we are also going to find secondary metabolites that have a mixed biogenesis, elements from different metabolic pathways will participate in their synthesis. This is the case of flavonoids where part of the molecule comes from a polyketone chain (polyketide, formed in the acetate metabolic pathway) and another part of the molecule comes from cinnamic acid (formed in the shikimate metabolic pathway).

– **Flavonoids**

Flavonoids are one of the most common groups of secondary metabolites, which are widely distributed in nature, and are only characteristic (of secondary metabolites) of plants. These compounds contribute to the colour of flowers and fruits, among others, in nature.

The name of flavonol comes from the Latin term *flavus* which means yellow, flavonols normally give the colour yellow or orange; anthocyanins are red, blue or violet, these compounds can be almost any colour of the rainbow except green.

Flavonoids are oxygen heterocycles and are secondary metabolites that are restricted to higher plants and ferns. Mosses normally have a certain type of flavonoid that can be isolated, but these products cannot be found in algae, fungi or bacteria.

Flavonoids are biologically important compounds because they play a significant role in pollination and feeding of insects in plants, certain molecules have a bitter taste that is used to repel certain caterpillars, which prevents them from eating the leaves.

Humans ingest substantial amounts of flavonoids in the plant diet, which constitutes an especially important benefit, due to their polyphenolic nature, they act as antioxidant agents trapping free radicals that are harmful to biological systems.

– **Classification**

The base of the chemical structure of the flavonoid molecules is a C_6-C_3-C_6 group, formed by two aromatic rings, **A** and **B**, functionalized with hydroxyl groups that are joined by a fragment of three carbon atoms, as shown in Fig. 28.7.

The biosynthetic origin of the two aromatic rings is different, aromatic ring **A** is produced in the acetate biosynthetic pathway (via acetyl-CoA) and aromatic ring **B** is from the shikimic acid biosynthetic pathway. Depending on the oxidation state of the C_3 unit (central heterocycle), the flavonoid subgroups are classified as follows:

1. Flavanones.
2. Flavones.
3. Isoflavones.
4. Flavonols.
5. Anthocyanidins.

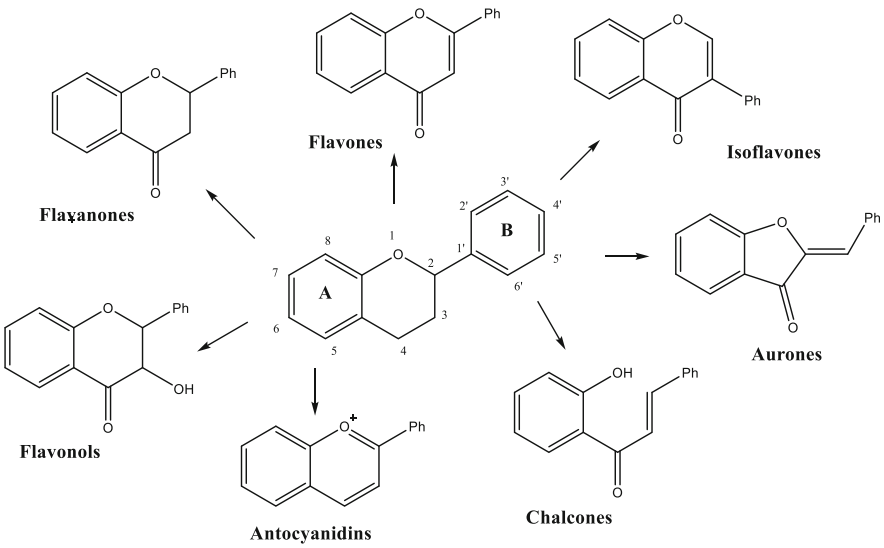


Fig. 28.7 Classification of flavonoids

6. Chalcones
7. Aurones.

28.4 Bicolouration with Plant Extract

Plants produce natural colourants as their secondary metabolites. Two representative examples of textile colouration with these secondary metabolites are followingly demonstrated.

28.4.1 Reseda Luteola

The weld is an herbaceous biannual plant, whose height may reach 1 m, from the family *Resedaceae* and *Reseda luteola* species. It is a weedy species that is used from ancient times as source of brilliant yellow dye. Originally from the Middle East and Mediterranean area, the plant spread throughout Europe. Most of the colourant matter is contained in the seeds and their most significant secondary metabolite for textile colouration is luteolin, whose chemical structure is displayed in Fig. 28.8.

The coloured matter of *Reseda luteola* was extracted by a solution of 50%water and 50%ethanol. Next, the extract was lyophilized and microencapsulated with maltodextrin. This material functioned as the natural dye used to carry out the

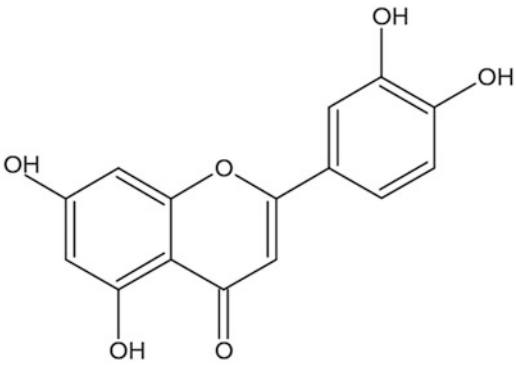


Fig. 28.8 Luteolin chemical structure

Table 28.5 Fabric main characteristics

Composition	100% Wool
Warp yarn linear density	240.38 tex
Weft yarn linear density	198.81 tex
Ends/cm	15
Picks/cm	12
GSM	654 g/m^2
Weave design	2/2 Twill

dyeing of a 100% wool fabric, whose main characteristics are displayed in Table 28.5.

The 100% wool fabric, bought from a local company, was previously scoured with a 3 g/L non-ionic detergent and 1 g/L sodium carbonate at 70 °C for 30 min. Then, it was thoroughly rinsed and air dried at ambient temperature. Prior to dyeing, the fabric underwent a wetting operation with Invadine PBN with a concentration of 10 g/L for 15 min. Afterwards, the fabrics were immersed in a bath containing a mordant, copper (II) sulphate pentahydrate with a concentration of 10 g/L, and the dye extract with various concentrations 5, 10, 20 and 30 g/L at 60 °C and in the presence of an acrylic resin, AC40. The pH was adjusted to 4 with glacial acetic acid or with sodium bicarbonate and padded on a two-bowl padding mangle at 80% pick up.

The padding process took place under two different techniques: a semi-continuous process, pad-batch, in which the padded fabric was rolled on a metallic rod with a black plastic sheet wrapped around, at room temperature for 24 h, as illustrated in Fig. 28.9.

And by a continuous process, pad-dry, in which padded fabric was dried and curated at 90 °C for 15 min, as illustrated in Fig. 28.10. After the dyeing process the fabrics were washed with a non-ionic detergent Nekanil 907 at 40 °C for 15 min, rinsed and dried at room temperature.

The colour strength and colour depth of mordanted and dyed samples were determined calorimetrically (colour space: CIELab (1976)/D65) by light reflectance

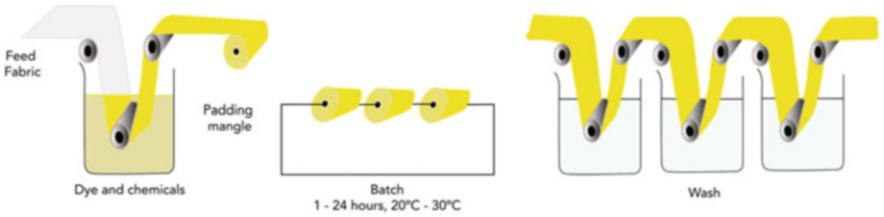


Fig. 28.9 Schematic view of the pad-batch dyeing process

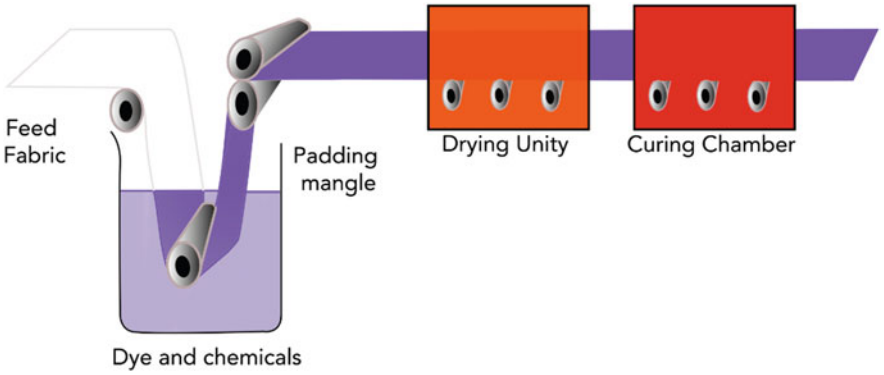


Fig. 28.10 Schematic view of the pad-dry dyeing process

technique. The colour strength (*K*/*S*) of all the samples was assessed by the Kubelka-Munk equation:

$$K/S = \frac{(1-R)^2}{2R}$$

With *R* being the observed reflectance at the wavelength of maximum absorption (λ_{max}= 420 nm) of the dyed wool samples, *K* is the absorption coefficient and *S* the light scattering coefficient. The colours are given in CIELab coordinates (*L**, *a**, *b**), which refer to the three axes of the colour space, in which *L** indicates the perceived brightness (the scale runs from 0 = black to 100 = white), whereas *a** denotes the red (+*a**) and green (–*a**) value and *b** specifies the yellow (+*b**) and (–*b**) coordinate. Other values normally measured are *C**, which stands for the saturation value calculated from the reflectance data and h° which is the hue angle. The attained results are shown in Tables 28.6, 28.7 and 28.8.

Washing fastness properties were evaluated in accordance with ISO 105 C06: 2010 standard method. The washing operation was conducted in the Linitest apparatus at 60 °C for 30 min. Upon washing, samples were rinsed with tap water and air dried at room temperature. We repeated the procedure for 5, 10, 15, 20 and 25 cycles. The results are shown in Tables 28.9, 28.10 and 28.11.

Table 28.6 Visual aspect of unmordanted dyed samples

Concentration g/L	Unmordanted			
	Pad dry		Pad batch	
	With resin	Without resin	With resin	Without resin
5				
10				
20				
30				

Table 28.7 Visual aspect of mordanted dyed samples

Concentration g/L	Mordanted			
	Pad dry		Pad batch	
	With resin	Without resin	With resin	Without resin
5				
10				
20				
30				

The colour fastness to rubbing was conducted as defined in ISO 105-X12:2001, and the fastness rating is showed in Table 28.12.

A visual analysis of dyed samples clearly shows that all dyed samples present a uniform and a quite well-affixed colour, with darker beige-brownish shades in mordanted samples and brighter shades in unmordanted samples.

Table 28.8 *K/S* and CIELab values for the dyed samples

	Pad dry								Pad batch							
	With resin				Without resin				With resin				Without resin			
	K/S	*L**	*a**	*b**	*K/S*	*L**	*a**	*b**	*K/S*	*L**	*a**	*b**	*K/S*	*L**	*a**	*b**
Unmordanted																
5	1.68	71.95	2.37	18.47	1.52	72.62	2.16	17.34	1.75	71.88	2.93	19.43	1.72	71.89	2.44	18.30
10	1.83	72.22	1.96	19.43	1.77	71.66	2.03	17.39	1.91	70.84	2.71	19.30	1.89	71.04	2.77	18.56
20	2.09	70.97	2.04	20.32	1.86	72.53	1.64	17.61	2.02	71.41	2.40	20.01	1.93	72.26	1.65	19.47
30	2.11	70.81	1.71	19.82	1.96	71.46	1.75	18.56	2.20	70.86	2.55	20.81	2.39	70.68	1.78	20.06
Mordanted																
5	2.05	68.17	–1.21	16.83	1.93	68.55	–0.13	16.72	2.14	67.46	–3.07	16.10	1.79	68.89	–0.36	16.51
10	2.29	67.60	–1.90	18.06	2.23	68.59	1.13	18.32	2.30	68.42	–2.79	18.56	2.29	69.07	–1.98	18.26
20	2.37	67.52	–1.29	17.91	2.27	69.04	–2.43	18.09	2.37	66.85	–1.90	17.41	2.28	68.52	–1.99	17.94
30	2.44	66.13	–4.00	16.74	2.39	69.12	–0.46	18.99	3.70	66.50	–0.57	20.69	3.25	66.10	–1.98	23.41

Table 28.9 Colour fastness to washing (ISO 105-C06)—unmordanted samples

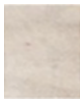
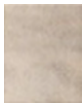
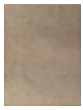
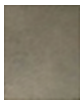
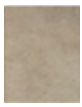
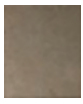
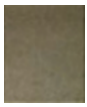
Concentration g/L	Unmordanted			
	Pad dry		Pad batch	
	With resin	Without resin	With resin	Without resin
5				
10				
20				
30				

Table 28.10 Colour fastness to washing (ISO 105-C06)—mordanted samples

Concentration g/L	Mordanted			
	Pad dry		Pad batch	
	With resin	Without resin	With resin	Without resin
5				
10				
20				
30				

Colour strength value grows evenly with the increase of dye concentration for both padding techniques and for all studied tinctorial conditions. Colour strength is always higher when mordant agent is added to the dyebath.

Table 28.11 Colour fastness to washing (ISO 105-C06)

	Unmordanted				Mordanted			
	Pad dry		Pad batch		Pad dry		Pad batch	
	Without resin		Without resin		With resin		With resin	
Concentration g/L	Colour change	Stain	Colour change	Stain	Colour change	Stain	Colour change	Stain
5	2/3	3	2/3	2/3	3/4	4/5	4	3
10	2/3	3	3	3/4	4	4	3	4
20	3	3	3	3/4	4	4	3	3
30	3	3	3	2/3	3/4	4/5	3/4	3/4
5	3	2/3	3	3	3/4	3/4	3	3/4
10	2	2	2	2/3	3	2/3	2/3	3
20	2/3	2	2/3	3	2/3	2/3	3	3/4
30	2/3	3	2/3	2/3	2/3	3	2	3

Padding processes are streamlined when resin is added to the dyebath.

Colour strength is slightly higher in the pad-batch padding process. However, the small difference between these two padding processes does not justify the option for the more time-consuming and discontinuous process.

The highest *K/S* results were obtained with the pad-batch process for mordanted samples with resin.

On the overall, washing fastness values may be classified as low to medium, with the best results being attained when resin is added to dyebath. Pad-batch process presents slightly better washing fastness results when compared to pad-dry method.

Rubbing fastness values are excellent for both tested conditions and padding techniques. Mordanted samples with resin provide the best results.

28.5 Future Prospects

Natural colourants have been used in the colouration of textile materials since ancient times. After the appearance of the first synthetic dye, Mauveine, by Henri Perkins in 1856 and following commercialization, the importance and consumption of natural dyes strongly decline. Over the last two decades with the continuous growing of an environmental consciousness by the consumers regarding the ecological impact of the textile industry, which is the second most pollutant in the world, and particularly, to what concerns to textile colouration, natural dyes have regained importance and, currently, is an increasing interest and trend in natural dyes and natural dyeing (Ganglberger 2009).

Natural dyes, as stated by the name, have a natural origin from plants, animals, minerals or even from fungi and bacteria which may be, in the future, potential sources of tailored colourants. They are renewable and biodegradable and represent a sustainable alternative to the colouration of textile goods.

Table 28.12 Colour fastness to rubbing (ISO 105-X12: 2013)

Concentration g/L	Unmordanted			
	Pad dry			
	With resin		Without resin	
	Dry	Wet	Dry	Wet
5	4/5	4/5	3	3/4
10	4	4/5	3	3/4
20	4/5	5	4	4
30	4/5	5	3	4
	Mordanted			
	Pad dry			
	With resin		Without resin	
	Dry	Wet	Dry	Wet
5	4	4/5	3	4
10	4/5	5	4	4
20	4	5	3/4	3/4
30	4/5	5	3	3/4
	Unmordanted			
	Pad batch			
	With resin		Without resin	
	Dry	Wet	Dry	Wet
5	4/5	4/5	3	3/4
10	4	5	3	3/4
20	4/5	4/5	4	3/4
30	4/5	5	3	4
	Mordanted			
	Pad batch			
	With resin		Without resin	
	Dry	Wet	Dry	Wet
5	4	4/5	3/4	4
10	4/5	5	3	3/4
20	4	4/5	4	4
30	5	5	4	3

Most natural dyes are environmentally safe, possess medicinal properties and exhibit an intrinsic anti-bacterial effect onto dyed textiles.

Nowadays, the scientific community, at worldwide level, is combining efforts to attain ready-to-use technical solutions, minimize the disadvantages of natural dyes and enable their application in an industrial level for specific applications and products.

Textiles coloured with natural dyes are preferred by consumers as suggested in market research study. The growing number of environmental conscious consumers is driving the expansion of this niche market, increasing their economic importance and creating the basis for a new eco-dyes industry. Nonetheless it should be stressed

that due to certain inherent drawbacks and low availability natural dyes represents about 1% of the market as suggested by (Saxena and Raja 2014).

According to Samanta and Agarwal (2009) figures of textiles consumption are enormous and not all of them can be coloured with natural dyes due to lack of availability and technical issues. Thus, the need to use synthetic dyes will endure in a near future. Nonetheless, a portion of coloured textiles can be achieved using eco-safe natural dyes.

Natural dye-bearing materials and purified extracts are already available and in use in certain SME textile units, particularly, in the USA, India, China, Germany and France. It is clearly perceived by now that textile companies are slowly moving towards more sustainable alternatives, and they are ready to meet the market demands as soon as consumers impose this change.

Revision of traditional mordants, mordanting and dyeing recipes is of paramount importance to become more sustainable (Shahid-ul-Islam and Mohammad 2017; Forman and Figueiredo 2016). The ban of some metallic salts used as mordant along with the utilization of enzymes, organic wastes and by-products from the food and agricultural industry as biomordants represent a major leap ahead to overcome poor fastness properties and to streamline the natural dyeing.

The optimization of the natural dyeing process with less energy consumption, lower materials-to-liquor ratio, and innovative non-conventional dyeing technologies, such as the usage of nanopigments for textile colouration (Mongkholrattanasit et al. 2011; Yusuf et al. 2012), use of ultrasonic energy, microwaves and supercritical carbon dioxide are becoming potential solutions, capable to surpass some of the natural dyes limitations and to establish, in a near future, a set of technical and cost-effective alternatives for textile coloration with secondary metabolites.

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Part VII
Future of Secondary Metabolites

Chapter 29
New Challenges and Opportunities from Secondary Metabolites

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Abstract Secondary metabolites (SMs), usually of complex structure and low molecular weight, have remarkable biological activities and, unlike the primary metabolites, are presented in low concentrations and in certain groups of plants. These, in turn, arouse great interest, not only for the biological activities exerted by plants in response to environmental stimuli, but also for the immense pharmacological activity they possess. Many are of commercial importance, not only in the pharmaceutical area, but also in the food, agronomy, perfumery, and other important sectors. In general, secondary metabolites are natural compounds produced in plants with the main objective of protection against abiotic and biotic stresses, besides having important nutritional and pharmacological aspects in human nutrition, they are also sources of aromatic additives, dyes, antioxidants and exert numerous functions. There are many ways to extract these compounds, but green chemistry can generate economic benefits in industrial chemical processes, such as reducing the need for investments in storage and effluent treatment, as well as the payment of compensation for any environmental damage. In addition, the use of bioresidues as promising sources of secondary metabolites is a promising way to promote the circular economy and take advantage of these by-products through bioactive compounds and their application as additives in new food sources. Currently one of the challenges is to produce sources of secondary metabolites from domesticated plants, since these have differences from wild ones, and the domestication process could preserve defense traits, such as changing the significantly, in addition to domestication-related climate changes, which may also attribute other characteristics to the secondary metabolites. Moreover, another interesting biotechnology applied to obtain large-scale SMs naturally produced by fungi is heterologous expression, which consists in the transcription of one or more genes from the cluster data gene of

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the fungi producing the target compound in a secondary host, which in most studies are yeasts or filamentous fungi.

The use of these compounds in turn must be in force within the parameters allowed by legislation for food incorporation and in the industry in general, since they differ according to the sources, uses, toxicological levels, and the regulations regarding their certifications. However, secondary metabolites emerge as a promising source for new opportunities of application, emphasizing their potential to act as strong and natural additives for several industrial purposes.

29.1 Introduction

Secondary metabolites are divided into three main groups: terpenes, phenolic compounds, nitrogen compounds and glycolysis (acetyl-CoA). The major groups are divided into subgroups with specific biosynthesis, function, and importance. Terpenes are made from mevalonic acid (in the cytoplasm) or from pyruvate and 3-phosphoglycerate (in the chloroplast). Phenolic compounds are derived from shikimic acid or mevalonic acid.

Finally, alkaloids are derived from aromatic amino acids (tryptophan, tyrosine), which are derived from shikimic acid, and from aliphatic amino acids (ornithine, lysine). The discovery and studies of most of these compounds are recent. Because they represent a huge set of substances, studies are needed to elucidate the role of these compounds in plants and their function in the plant/animal interaction for a better utilization (Borges et al. 2020).

The diversity of secondary metabolites produced by plant and animal species represents a broad portfolio of generally complex chemical structures that are targets for study and sources for the development of new treatments for ailments and maintenance of physical well-being, covering flavonoids, alkaloids, essential oils, tannins, anthraquinones, steroids, carotenoids, seaweeds, insects, fungi, and others (Moro 2016).

In this sense, this chapter comes with the aim of highlighting the importance of these metabolites, as well as showing the complexity of these existing compounds and their contribution as natural agents, also for the substitution of existing molecules, providing natural alternatives to the use of synthetic additives, known for their adverse secondary effects. This chapter focuses on the importance of obtaining these molecules from biowastes and by-products and in the urgency of using less aggressive and toxic extraction techniques, elucidating the legislations regarding their certification and use, providing a compilation of relevant research that can help the reader to identify the trends of natural products and their importance for the various areas of the industrial sector.

29.2 Replacement of Synthetic Additives by Natural Ones

With the aim of enabling storage for longer periods and maintaining the same intrinsic qualities of foods, food additives are used for various purposes during the preparation of foodstuffs, playing an important role in maintaining the quality and organoleptic characteristics, since the environmental conditions to which the food is subjected, from manufacturing to marketing, such as temperature changes, lipid oxidation, and exposure to microorganisms, can alter the original composition of the product. The use of additives has contributed to ensure product's integrity, both in terms of safety and availability (ASAE 2013).

According to the European Union legislation present in Regulation (EC) n° 1333/2008, food additives are defined as "substances that are not normally consumed as foodstuffs in themselves but are intentionally added to foodstuffs to achieve a certain technological objective." It is important to note that food additives do not cover contaminants or substances added with the aim of maintaining or improving the nutritional properties of a particular foodstuff (ASAE 2013). According to their industrial use, additives are classified into 25 classes, which include a multitude of compounds with specific functionalities that can be used following the specific legislation of each country and a food safety policy (Carocho et al. 2014; Carocho et al. 2018; Martins et al. 2019).

Food additives are food ingredients and must be mentioned in the ingredients list. Additives must be named by the name of their functional class followed by their specific name or European Community (EC) number. This letter E (for Europe) followed by three or four digits, being the same valid in all European Union countries, facilitates the labeling of the substances present in the composition of the foodstuff, governed in accordance with Directive 2000/13/EC (ASAE 2013).

Food additives can be from natural or synthetic origin, meaning that the former can occur naturally in food or be recovered from a natural source, while the synthetics are defined as artificially synthesized substances (Zeece 2020) and represent the class of additives most used in the industry due to its chemical stability, easy application, and low cost. However, in recent years, the use of natural substances in food systems as substitutes for synthetic substances represents one of the main trends and challenges of the food industry, mainly due to the high toxicity and allergenic problems associated with artificially synthesized additives.

Food additives are seen as chemicals added to a food, diminishing the fullness and purity of the product, which presents a contradiction since the added chemical may be of natural origin and may provide benefits to the consumer even though it carries the designation E. This designation means that the additive has been fully assessed for safety and is strictly controlled in the way it can be used. There are around 2500 food additives currently used by the food industry, and about 12–15% are natural products, such as beta-carotene (E 160a), also referred to as pro-vitamin A, which has an E number and as such is not considered a "clean label" when present as an ingredient. It should be noted that many of the foods are also seen as clean labels, however, they contain added E numbers as it is permitted to

use the name instead of the E name, as it is the case of rosemary/rosemary extract, being used instead of the E392 name, and ascorbic acid or vitamin C instead of E300 (Baines 2010). Some of the ingredients are pure chemicals isolated from plants, animals, or microorganisms. However, most are in the form of extracts, oleoresins (ORs), fixed oils, and volatile oils, among other forms (Khan and Abourashed 2010).

There is a big difference between the preparation of natural and synthetic additives for application in foods during their processing. Synthetics are produced as pure substances of constant composition and are applied as such or in well-defined mixtures with other substances. Naturals, on the other hand, are available from raw materials of varying composition. Both the content of active substances and the contents of various other compounds present depend on the plant variety, climatic conditions, degree of ripeness as well as many other factors. The most widely used natural additives are not exactly purely natural but are identical in nature. This means that their structure is the same as that of natural substances but obtained by synthesis and supplied in a relatively pure state, as is the case with synthetic tocopherols, ascorbic and citric acids (Pokorný 2001).

Consumers have become increasingly exigent in terms of their food choices, demanding for products that are as natural as possible, with "clean" labels, i.e. without the presence of synthetic additives and without compromising the quality and food safety. Thus, the food industries are trying to meet these demands by developing more natural, appealing, and low-cost products for the consumer. As possible substitutes, fruits, vegetables, spices, herbs, essential oils, oleoresins, yeasts, fungi, seaweed can be mentioned, among the most diverse extracts that can be obtained from natural products. Although full of challenging aspects, numerous research has been conducted and several natural sources have been tested to obtain new substitutes of natural origin.

29.2.1 Safety of Food Additives

Chemical additives can cause damage to the microbial cell, interfering in its development, adaptation, or multiplication process, which reduces food deterioration processes and thus increases its shelf-life. However, due to teratogenic, carcinogenic, and toxic problems, and because they are also related to insect, pest, and fungus resistance caused by their indiscriminate use, they have lost interest (Ootani 2013; Abbaszadeh et al. 2014).

European Union legislation stipulates that food additives must have advantages and benefits for the consumer and must be safe, as well as meeting criteria such as preserving the nutritional quality of foodstuffs, increasing stability or improving their organoleptic properties. The use of food additives is not free of risks to public health, since a misuse of these substances, either by applying excessive levels or by including an undeclared additive may lead to some hazards, however, when these are used correctly, in accordance with the legislation, they do not represent a risk to consumer's health (ASAE 2013). The control and regulation on food additives is

defined in Regulation (EC) n° 1333/2008, which establishes the general framework, guidelines, principles, and objectives that refer to all specific legislation on the use of additives, specifying maximum limits and the respective use of additives applied in foodstuffs in order to achieve the desired technological target and simultaneously ensure consumer's safety.

Toxicology plays an important role in the use of new ingredients of natural origin, since toxicological information is the central point that determines the risk associated with the substance in question. Consequently, the release of these compounds is carried out by conducting toxicological studies (Jansen et al. 2020).

In Europe, the Scientific Committee on Food (SCF) and/or the European Food Safety Authority (EFSA) evaluate the safety of food additives based on information regarding the manufacturing process, fate in food, and toxicological data. In general, before an additive is submitted as being effective, tests on genotoxicity, reproductive toxicity, pharmacokinetics and metabolism, repeated dose toxicity, subchronic and chronic toxicity, neurotoxicity, immunotoxicity, and carcinogenicity are carried out. After this first step, the new additive goes for human testing respecting all age groups, from children to the elderly, of both sexes and includes biochemical examinations, organ function, enzyme reactions and assays of absorption, metabolism, and excretion. Based on this data, EFSA determines the level below which the intake of the substances can be considered safe—called the Acceptable Daily Intake (ADI) and calculates the possible exposure to a food additive, considering the maximum level of addition in foodstuffs, which will depend on the frequency of intake and the possible side effects of each additive, as well as its chemical characterization and degree of purity. If the ADI is not exceeded, the use of the food additive is considered safe (Regulation (EC) n° 1333/2008, ASAE 2013).

When it comes to food substances, such as additives of natural origin, the vast majority of experiments carried out are in vivo, using animals such as rodents (rats and mice) or non-rodents (dogs). The substance to be tested must be added to the diet of the animals, meeting the nutritional requirements of the species used in the study. Usually, the substance is administered to experimental animals at three levels of doses and the control group: (1) high, with the intention of producing toxicity; (2) low, where no toxicity is observed; (3) intermediate, in order to identify minimal toxic effects; (4) control, which receives no dose of the substance tested (WHO 2009).

According to the Regulation (EC) No 1333/2008, the term "*Quantum satis*" means that no maximum level is specified, but that additives are used in accordance with good manufacturing practice at a level not higher than the one necessary to achieve the intended result.

29.2.2 Food Enrichment

Fortification, enrichment, or simply addition is a process in which one or more nutrients, from natural origin or not, are added to food, within legal parameters, with

the objective of reinforcing its nutritional value and preventing or correcting eventual nutritional deficiencies presented by the population in general or by specific groups of individuals. This process has been used as a low-cost resource in the prevention of nutritional deficiencies in many countries. Several foods have been used for fortification, showing to be efficient and well tolerated. The addition of fortificants must occur in foods that effectively participate in the regional diet routine. Its use should be inserted only after an evaluation of the nutritional status of the target population (Vellozo and Fisberg 2010a, b).

The direct incorporation of bioactive compounds in food products should be very well studied, since, although it is possible to import the idea of the action of these bioactive compounds through pharmaceutical sciences to food science, the question of the interaction of this compound with the food matrix is quite unique for each food (Mohammadian et al. 2020).

It should be considered that biomolecules, before extraction, are dispersed in a complex environment, being able to behave in a synergistic way and, in many cases, stabilized by the mixture of elements present in their natural environment. However, after their extraction and isolation, their physiological activity can be affected, reduced, or even completely inactivated. To avoid this, new technologies can be proposed to reduce or even prevent the loss of functionality of these compounds. Some techniques, such as nanotechnology, can be used in a promising way in order to turn bioactive compounds more stable, solving their restrictions and increasing their industrial application (Giaconia et al. 2020).

29.2.2.1 Essential Oils

Essential oils are formed by a complex mixture of compounds not very soluble in water (phenylpropanoids, terpenes, and terpenoids). In plants, these compounds are synthesized as secondary metabolites to provide protection against external agents such as UV light, herbivores, insects, and pathogens. They are produced by different parts of plants and can be extracted from different plant materials, such as leaves, bark, stems, roots, flowers, and fruits by steam distillation, mechanical extraction processes of the epicarp of citrus fruits for instance, or by dry distillation, followed by separation of the aqueous phase, if any, by physical processes, and can also be physically treated without altering their composition. More than 3000 thousand types of essential oils are known but only 300 are being used in food industries and other formulations (Ribeiro-Santos et al. 2017; Hassoun and Çoban 2017; Sharma et al. 2020).

The incorporations of oils in product formulations have been highlighted by their biological properties, especially antimicrobial, antitumor, antioxidant, anti-inflammatory, insecticidal, antitumor, analgesic, and antidiabetic. However, it is notorious some limitations of the essential oils in industrial scale as for example the great variability of the chemical composition, interactions with the food matrix, toxicity, peculiar sensory characteristics, need of previous standardization for application of the product, susceptibility to oxidation, volatile nature, and ecological

biodiversity. The antimicrobial activity of essential oils is not the same for Gram-positive and Gram-negative bacteria due to the limitation of the external membrane in Gram-negative bacteria to the entry of hydrophobic components into the cells. Phenolic compounds are mainly responsible for the mechanisms of action of essential oils, and the microbiological activity is related to the position of the hydroxyl group. However, the incorporation as preservatives in foods is restricted due to the use of high quantities and alteration in the sensory qualities of products (Pateiro et al. 2021).

Among the technologies developed as an alternative to overcome these barriers it is possible to highlight the encapsulation or production of nanoemulsions of oils contributing to a higher stability, targeting active compounds, extending shelf-life, facilitating the incorporation into foods and with other ingredients masking the perception of strong odors improving availability. In addition, the development of active packaging systems with antioxidant and antimicrobial properties also allows the interaction between food and packaging systems, promoting a higher stability (Stevanović et al. 2018; Ribeiro-Santos et al. 2017; Prakash et al. 2018).

29.2.2.2 Microalgae and Cyanobacterial Biotechnology: Food Application

Microalgae and cyanobacterial biotechnology have been significantly developed and diversified for various commercial applications (Vaz et al. 2016). Microalgae and cyanobacteria stand out for presenting characteristics of interest to the industry, including pharmaceutical, cosmetic, and food industries, because some of these organisms produce biologically active compounds, such as antioxidants and antibiotics (Harun et al. 2010; Barra et al. 2014). The advantages in the use of microalgal biomass, photosynthetic microorganisms can double their biomass ranging from 2 to 5 days on average and can achieve high yields without the application of pesticides, herbicides, or fungicides. In addition, they do not require arable land for cultivation, and the nutritional requirements for the cultivation of these organisms can be found in industrial waste, which can transform what is considered a problem, into raw material to produce formulations with high added value (Vaz et al. 2016).

In this context, *Arthrospira platensis*, for instance, can be exploited by the food industry to produce food supplements that promote health benefits by presenting substances such as phycobiliproteins that may exhibit antioxidant and anti-inflammatory, hypocholesterolemic, as also anticancer activities (Benelhadj et al. 2016; Hernandez et al. 2017). Food supplements produced by these microalgae can be of great interest for the food industry since they can be used in health promotion through the capacity to produce high contents of antioxidant carotenoids (lutein, astaxanthin, zeaxanthin, lycopene, or beta-carotene) (Tinoco et al. 2015).

Microalgae and cyanobacteria are one of the most interesting sources of food ingredients and functional foods, since they can be used to improve the nutritional value of foods, due to their richness in compounds with beneficial attributes (Benelhadj et al. 2016). Its balanced chemical composition (good quality proteins,

fatty acid's profile, vitamins, antioxidants, and mineral salts) and its valuable attributes can be applied in the formulation of new food products. The use of photosynthetic microorganisms as a source of functional foods is a priority area in algae technology allowing the establishment of an effective production system with beneficial environmental and health-related effects. Important research has been carried out regarding the development of different healthy and more attractive food products, prepared from microalgae and cyanobacteria, including pasta, ice cream, cookies, among others (Benelhadj et al. 2016; Rodríguez De Marco et al. 2014).

29.2.2.3 Endophytic Fungi

Endophytic fungi have been highlighting interest in science due to the production of secondary metabolites with biotechnological applications in the food and pharmaceutical industries. The various classes of molecules produced by endophytic fungi may possess hormonal, antibiotic, antitumor (Peixoto et al. 2002), antifungal, cytotoxic (Silva 2010), antiviral, immunosuppressive, and antiparasitic activities (Demain and Sanchez 2009). A diversity of secondary metabolites with antimicrobial activity are produced by endophytic fungi and can be cited as: aliphatic compounds, phenolic compounds (phenols and phenolic acids, isocoumarin derivatives, flavonoids and lignans, quinones), alkaloids (indole derivatives, amines, and amides), peptides, polyketides, steroids, terpenoids (mainly sesquiterpenes, diterpenes, and triterpenes). The ability of the endophyte to produce the same bioactive metabolites as its host plant may reduce the collection of rare, low-growing, and endangered plants, thus preserving biodiversity (Stierle et al. 1993).

When a microbial metabolite is considered a candidate for a new drug, its production can be carried out on a larger scale, using the manipulation of parameters of the fermentative process for the expression of metabolites, aiming at the improvement of its yield (Tejesvi et al. 2007). Increased production of these compounds can also be achieved by subjecting the microorganism strain to breeding programs (Penalva et al. 1998) that can isolate more easily cultivable mutants that generate additional or modified products with higher therapeutic index (Piepersberg et al. 1994).

The variety of secondary metabolites produced by a single endophyte has not yet been calculated, but it is estimated to be high, due to the versatility and easy adaptation of the fungi (de Azevedo et al. 2002). Some endophytic isolates have the ability to produce different substances, such as bioactive volatile organic compounds, which can act with other classes of compounds, being lethal to other microorganisms, such as pathogens and Gram-positive and Gram-negative bacteria inhibiting or killing a variety of harmful disease-causing agents, such as bacteria, fungi, viruses, and protozoa that affect humans and animals. In addition to antibiotics, several high value-added drugs can be produced from endophytic microorganisms, extracted from a small portion of plant tissue, thus maintaining the

production of vital compounds for people affected by numerous diseases (Strobel 2006).

The investigation of microorganisms by pharmaceutical and food industries to obtain different compounds of interest is still modest. Antioxidants have become the topic of interest recently, however, endophytic fungi represent an abundant and reliable source of new antioxidant compounds (Rajamanikyam et al. 2017).

29.3 Extraction Techniques

The attempt to adapt to the current consumer demand has driven the search for natural additive sources and emerging recovery techniques. Recent research has demonstrated the potential of several natural sources for extraction or recovery of additives, even though full of challenging aspects, such as stability and its interaction with food compounds as well as in the organism. Plant sources have represented the main matrix for obtaining natural aromatic compounds (Giuffrida et al. 2020; He et al. 2020; Jahurul et al. 2020; Kim et al. 2020; Mohd Ali et al. 2020), while pigments can be obtained mainly from industrial waste (Lombardelli et al. 2020), algae or marine microalgae (Böcker et al. 2020; Pardilhó et al. 2020), among others (Dini et al. 2020; Silva et al. 2019). Research for extraction of natural antioxidants has been extensive and numerous matrices have been evaluated. As for pigments and aromas, plant sources (De Biaggi et al. 2020), by-products, and industrial waste (Fidelis et al. 2020; Schneider et al. 2020) are the most studied. Algae or microalgae and plant matrices such as flowers have been studied for the recovery of natural additives that can be used as body agents (Bernaerts et al. 2019).

29.3.1 Benefits of Using Green Solvent Techniques

The main concept of Green Chemistry is the use of skills and knowledge to reduce or eliminate the use or generation of hazardous substances during the planning, manufacturing, and application of chemicals in order to minimize threats to the health of operators and the environment (Anastas and Eghbali 2010). Green chemistry can generate economic benefits in industrial chemical processes, such as the reduced need for investments in storage and effluent treatment, as well as the payment of compensation for any environmental damage (Prado 2003).

29.3.1.1 Green Solvents

A green solvent is a solvent that does not pose health risks to the analyst, is safe to handle, biodegradable, and environmentally friendly (Lesellier and West 2015). In summary, the 12 principles of Green Chemistry are based on the minimization or

non-use of toxic solvents in chemical processes and analysis, as well as the non-generation of waste resulting from these processes. To this end, atomic energy and savings take pride of place, as does the use of renewable and harmless raw materials. In addition, the acceleration of chemical reactions by catalysis can help, for example, in energy savings and less waste generation. One of the principles is also concerned with the conscious development of chemicals, so that after their useful life they should decompose and become environmentally harmless degradation products, also avoiding bioaccumulation. Thus, it is observed that these principles concern with the planning of the product, through its synthesis, processing, analysis, and its destination after use. The main objective is to minimize environmental risks and inherent industrial activities (Prado 2003).

Regarding the use of organic solvents in the extractions, there has been a growing concern to replace them by solvents considered "green," i.e., with less or no proven toxicity, non-aggressiveness to the environment, among other factors. The most common are water, supercritical fluids, ionic liquids (ILs), and also deep eutectic solvents (SEPs) (Shabani et al. 2020). The importance of the use of new technologies is perhaps evident when considering that in a production process of a pharmaceutical active ingredient, approximately 50% of the mass in the process corresponds to solvents. A large part of these solvents is toxic, flammable, and/or corrosive, collaborating to intensify the pollution of water, air, and soil, in addition to the risks of exposure of workers (Anastas and Eghbali 2010).

The most innovative ones, such as the LIs, emerge as a clean alternative to almost all solvents and can be used in their pure form or together with another solvent. Basically, ionic liquids are composed of an ammonium and phosphonium cation that has the ability to form ionic pairs or complexes with metal ions in an aqueous matrix, achieving high efficiency and unique physicochemical properties, such as negligible vapor pressure, high thermal stability, and non-flammability (Guleria et al. 2020).

As for the stability of natural food additives, whether in the form of isolated compounds or extracts, often during or immediately after extraction, many compounds start to degrade. This degradation can be caused by exposure to high temperatures, enzymes, chemicals, or even the passage of time (Seibert et al. 2019). As an alternative, encapsulation in its various forms has proven to be a solution, albeit with limited results and difficult to implement in a wide variety of foodstuffs. Micro- and nano-encapsulation, which are techniques employed, have as principle the creation of an external membrane to the compounds of interest to protect them. The difference between the techniques is based on the size of the formed particle (1–1000 nm for nanoencapsulation and 1–1000 μm for microencapsulation). These processes are effective because after encapsulation, the final products can be a powder, a paste, or a liquid. This facilitates their use by the food industry as they are stable, easy to weigh, transport, and store.

The SEP solvents have the ability to dissolve organic compounds, have also low cost, and present low toxicity. These solvents are based on the mixture of one or more pairs comprising a hydrogen bond acceptor and a hydrogen bond donor which, in appropriate molar ratios, generate strong intermolecular interactions. This mixture provides favorable physical properties that resemble those of ionic liquids, such as

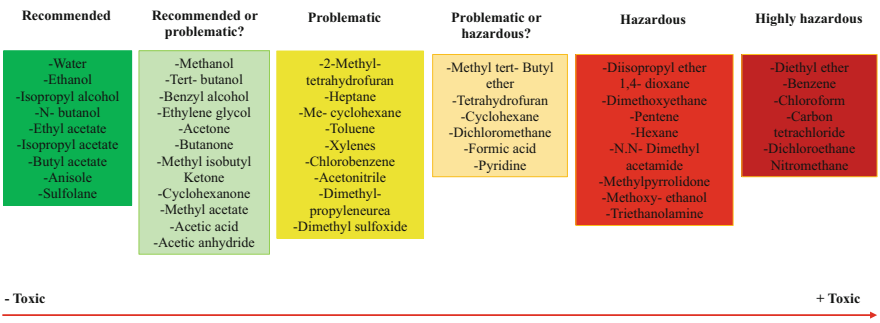

Fig. 29.1 Solvent's classification

low melting point, low volatility, high viscosity, high surface tension, and high thermal stability (Jiang et al. 2019; Shabani et al. 2020).

Prat and collaborators, in an article published in 2014, made an interesting comparison regarding the toxicity of solvents by dividing them into categories (Fig. 29.1).

29.3.1.2 Deep Eutectic Solvents (DES)

Deep eutectic solvents (DES) have emerged in the context of green chemistry because of their biodegradability, low or no toxicity, easy synthesis, and low cost (Dai et al. 2013). DES have been applied as an alternative to various other solvents and are identified as being a mixture between a salt or hydrogen acceptor (HBA) and a hydrogen donor (HBD). DES share many properties of LIs with the advantage of being low cost, chemically inert, and easy to prepare. Moreover, depending on the precursors (HBA and HBD) employed in their synthesis, DES can be biodegradable and non-toxic. Due to the strong hydrogen bonds that form DES, there is a large decrease in the melting temperature of the complex formed, making them liquid at relatively low temperatures (Liu et al. 2008).

The interest of DES in the extraction of phenolic compounds from plants is due to its biocompatibility and also because of the possibility of a greater stability of phenolic compounds in these solvents. This effect results from molecular interactions between the solvent (DES) and the phenolic compound, preserving in this way the functional characteristics of the biomolecule, such as antioxidant capacity.

One of the most used hydrogen receptor components for DES formation is choline chloride (ChCl), a non-toxic quaternary ammonium salt (Yadav et al. 2014). This salt is considered an essential component for the diet and normal functioning of all cells, assisting in various metabolic mechanisms (Fu et al. 2017). Choline cation has recently been approved with no maximum limit, by the Directive Council 70/524/EEC, for use as a nutritional additive, and can be incorporated into feed and food (Hammond et al. 2016; Yadav et al. 2014).

Ruesgas-Ramon et al. (2017) cite that the most common deep eutectic solvents (DES) are those formed by mixing choline chloride as hydrogen acceptor (HBA) and urea, ethylene glycol or glycerol as hydrogen donors (HBD). Maugeri and Domínguez (2012) show that recent studies report that the mixture of ChCl with glycerol tends to decrease the viscosity and density, further evidencing that a simple binary mixture of compounds can form a DES.

There are two most commonly used methods for the synthesis of DES: by heating and by evaporation. In the heating method, the constituents are heated to about 50 °C under gentle agitation. In the evaporation method, the components of the mixture are dissolved in water, heated up to 60 °C, under gentle agitation and after the formation of DES the water is removed by vacuum evaporation. However, since the components may be slightly unstable, the heating method is generally the most used (Dai et al. 2013).

29.3.2 Impacts of Green Chemistry

29.3.2.1 Pharmaceutical Analysis

The method of choice for the determination of active pharmaceutical ingredients as well as the investigation of impurities and degradation products is high performance liquid chromatography (HPLC). Most of these methods use as organic solvents acetonitrile and/or methanol (Marco and Salgado 2017). Toxic organic solvents such as acetonitrile and methanol, in addition to harming the health of the operator who is exposed daily to these solvents, also require proper waste management for the disposal of this contaminant. This has a cost that will certainly be included in the final product (Pedroso et al. 2016).

Buffer solutions, besides requiring a certain amount of preparation time, have a low shelf-life, which requires re-preparation and therefore a longer dispensing time. Their use also requires an extensive cleaning process of the column and the entire chromatographic system (Kogawa and Salgado 2012).

The growth and importance of using green chemistry is inevitable, as it is not only limited to the use of a less toxic solvent, but also to the whole process that will minimize reagents, steps, and costs.

29.3.3 Analysis of Secondary Metabolites Produced in Plant Cultures

There are several methods for the analysis and identification of known secondary metabolites produced in plant cultures. The analysis of complex mixtures is usually performed by thin layer chromatography (TLC), by comparison with the retention factor (Rf) values of known substances in different eluent systems and by their

reactivity against different chromogenic products (Van Beek and Van Gessel 1988). CCD still remains one of the preferred methods for qualitative analysis of known compounds because it does not require sophisticated equipment or laborious sample preparation.

For quantitative analysis, high performance liquid chromatography (HPLC) systems coupled with a UV-VIS detector are the most widely used technique (Theodorides et al. 1998; Giroud et al. 1991). Capillary gas chromatography (GC) is also widely used (Dagnino et al. 1991) and an important characteristic of GC is its great power of detection and separation of compounds. The use of GC coupled with mass spectrometry (GC/MS) allows the identification of compounds present in minute quantities, even in complex mixtures (Zocoller et al. 2005).

Supercritical fluid chromatography (SFC) is another method employed, which uses packed columns similar to those of HPLC or capillary columns similar to those used in GC/MS (Choi et al. 2002). CFS has a great potential applicability due to its high resolution and high stability of the compounds under the conditions of use.

Capillary electrophoresis (CE) is a technique that has been used with relative success because it presents great sensitivity associated with high resolution. CE is applicable in the determination of a wide variety of samples, including water-soluble vitamins, amino acids, inorganic ions, organic acids, drugs, catecholamines, chiral substances, proteins, peptides, and many others. One feature that differs CE from other techniques is its unique ability to separate electrically charged macromolecules of interest in both biotechnology industries and biological research. Another important aspect is the possibility of coupling CE to mass spectrometry using soft ionization techniques such as electrospray, thermospray, and atmospheric pressure ionization (Guttman et al. 2004).

The use of plant cell culture to produce chemicals and drugs has greatly contributed to advances in several areas of plant physiology and biochemistry. The use of genetic tools and the current extensive knowledge about the regulation of secondary metabolism pathways could provide the basis to produce these metabolites at commercially acceptable levels.

29.4 Opportunities

29.4.1 Synergistic Effects

Secondary metabolites from plants and fungi are widely used for their beneficial effects on health, such as food additives to preserve or improve food properties (López-Malo et al. 2006; Keller et al. 2005). The mixture of synthetic or natural compounds, or both, can result in three different mechanisms: (1) additive, in which the result is the effect of the sum of each individual effect; (2) antagonistic, defined by the reduction in efficacy when compared to the sum of individual effects; and (3) synergistic, being the increase or enhancement of the effect (López-Malo et al. 2006). Among the synergistic mechanisms can be: (1) increased efficacy;

(2) reduction of undesirable side effects; and (3) an increase in the stability and/or bioavailability of the components, in which it is difficult to predict the pharmacological mechanisms of action and their effects when combined (Inui et al. 2007).

Cheesman et al. (2017) discuss the combination of plant extracts and synthetic antibiotics, justifying the decrease in effectiveness due to the misuse of antibiotics. The authors mention that plant extracts are not as expensive as synthetic antibiotics, and that mixtures of compounds can result in new potential antibiotics. *Staphylococcus aureus*, for example, is one of the most researched bacteria regarding its resistance to antibiotics, with MRSA (methicillin-resistant *Staphylococcus aureus*) being the strain of greatest interest (Vestergaard et al. 2019). Zuo et al. (2011) investigated the synergistic antibacterial and antibiotic effect between tetrandrine, a bisbenzylisoquinoline alkaloid isolated from *Stephania tetrandra*, and cefazolin, generating a MIC reduction against MRSA of up to 94%. Likewise, Zuo et al. (2012) evaluated the synergistic effect between berberine, an isoquinoline alkaloid from *Berberidaceae* spp., and azithromycin, providing a MIC reduction of up to 96.9% for three of ten MRSA strains, with a minimum of 75% reduction for the combination between the two compounds.

Chandarana et al. (2005) compared the antibacterial effects of ginger, mango ginger, and turmeric extracts, as well as their combinations, in which the mixture of ginger and turmeric showed a greater zone of inhibition for *S. aureus* (14.5 mm) when compared to the individual extracts (12.33 and 0 mm for ginger and turmeric, respectively). On the other hand, the authors reported no inhibition for *S. aureus* in the mixture between turmeric and mango ginger, in which the individual extract of mango ginger showed an inhibition zone of 11 mm. These results demonstrate the difficulty of predicting whether the effects will be synergistic of antagonistic, since for the same extract with no inhibition for *S. aureus*, two different effects occurred in their mixtures. Alves et al. (2013) combined wild mushroom extracts with commercial antibiotics in order to evaluate the antimicrobial effect of the mixture, in which they showed positive synergistic effects against MRSA (MIC reduction from 8 to 0.8 μg/mL for penicillin and a mixture between penicillin and *M. rosea*, respectively), suggesting a reduction in therapeutic doses of synthetic antibiotics.

Natural antioxidants in food products are also already used, in which rosemary extract (E392) has already been approved by the European Food Safety Authority, with carnosic acid and carnosol responsible for most of the antioxidant effect (EFSA 2008). As well as antimicrobials, the mixture between compounds can be an alternative to increase the effectiveness of antioxidants. Among the antioxidant compounds, the synergistic effect may occur due to: (1) formation of new compounds with greater antioxidant activity; (2) solubility and phase distributions of antioxidants; (3) formation of stable intermolecular complexes between antioxidants; (4) regeneration of stronger antioxidant through weaker antioxidant; and (5) unpredictable interactions between compounds (Olszowy-Tomczyk 2020).

Misan et al. (2011) evaluated the antioxidant effect of plant extracts such as parsley, buckthorn, mint, caraway, and their mixture, when applied to cookies in order to delay lipid oxidation. The mixture showed high antioxidant activity in most tests in addition to oxidation stability, justified by the synergistic effect between the

compounds present in the isolated extracts. Ribeiro et al. (2015) investigated two mushroom extracts (*Suillus luteus* and *Coprinopsis atramentaria*) and found a synergistic effect when used in equal proportions (1:1), showing better antioxidant activity results in the DPPH radical scavenging activity and reducing power assays.

29.4.2 Use of By-Products and Biowaste

Food loss and waste occurs at all stages of food processing, causing environmental impacts, waste of natural resources and problems with food safety, so it is essential to evaluate possible ways of food waste and how to avoid or reduce them to a minimum possible (Fusions. 2016; Xue et al. 2017). According to Fusions (2016), in 2012 about 88 million tons of food were wasted in Europe (approximately 173 kg/person), in which processing and production accounted for 30% of the total amount. Among the alternatives to reduce waste, the use of by-products and biowaste becomes a solution for extracting bioactive compounds from parts of the plant that would otherwise be discarded, such as bark, seeds, peel, bagasse, and leaves, which is generally incorrectly disposed, causing environmental pollution and public health problems (Helkar et al. 2016; Lyu et al. 2020).

In addition to these discarded parts being carbohydrate sources, they also contribute largely due to their bioactive compounds, being potential ingredients for the food industry (Lyu et al. 2020). Table 29.1 presents by-products and biowaste with potential for food and pharmaceutical applications.

Barreira et al. (2019) investigated the bioactive compounds present in apple pomace, in which the major phenolic compounds found were flavonoids, hydroxycinnamic acid, and dihydrochalcones, known for their antioxidant, anti-inflammatory, and antimicrobial activities, being a possible potential by-product to be used in pharmaceutical applications (dermal formulations). Leal et al. (2020) studied grape stem as a by-product of the wine industry, showing good antioxidant (0.64 mmol Trolox/g in DPPH assay), antimicrobial (effectiveness against some Gram-positive bacteria), and anti-inflammatory activity (35.35% inhibition activity of nitric oxide), demonstrating high potential for food and pharmaceutical purposes.

Banerjee et al. (2020) discussed the importance of evaluating mushroom by-products, being, like plant by-products, source of bioactive compounds, in which the authors evaluated enoki mushroom stem as a good source of protein (13.5%), as well as strong antioxidant capacity due to its high free radical scavenging activity (84.2%). Kang et al. (2012) also evaluated another mushroom by-product, namely oak mushroom stipe, showing high hydroxyl radical scavenging (79.75%) when used at a concentration of 5.0 mg/mL.

It is also fundamental to assess the toxicity of these by-products/biowastes as they may not be fit for human consumption and are generally discarded. Chichiricco et al. (2019) evaluated by-products from saffron, in which the aqueous extracts were able to modulate reactive oxygen species levels, without producing any genotoxic or cytotoxic effects. Costa et al. (2019) investigated the potential of grape pomace

Table 29.1 By-products and biowaste as a source of value-added compounds

Food by-product or biowaste	Secondary metabolite	Health benefits	Possible applications	Reference
Okara	Phenolic compounds	Decrease in plasma cholesterol, prevention of cancer, diabetes, and obesity (soy-based foods)	Enriched vegetable paste	Guimarães et al. (2018)
Apple pomace	Polyphenols, quercetin glycosides	Prevents constipation and hypertension	Bakery products	Lyu et al. (2020)
Cocoa husk	Polyphenols	Dietary fiber source, antioxidant activity	Snack products	Jozinovic et al. (2019)
Rice bran	Phenolic acids, tocopherols	Hypoglycemic and hypolipidemic effects, immunomodulatory effects, and oxidative stress reduction	Gluten-free products	Spaggiari et al. (2021)
Cocoa bean shell	Vitamins, polyphenols, flavanols	Anticarcinogenic, antidiabetic, and anti-inflammatory effects	Cocoa flavoring, functional beverages	Rojo-Poveda et al. (2020)
Grape stem	Phenolic compounds	Antioxidant, antimicrobial, anti-inflammatory, and anti-aging activity	Cosmetic, pharmaceutical, and food products	Leal et al. (2020)
Paeonia veitchii seeds	Oligostilbene, monoterpene glycosides	Antibacterial activity and anticancer effects	Functional foods	Zhang et al. (2018)
Oak mushroom stipe	–	Antioxidative and antimutagenic effects	Health-promoting food products and animal feeds	Kang et al. (2012)
Pineapple peel and pomace	Phenolic compounds	Antioxidant activity, prevents constipation and decreases intestinal absorption of cholesterol	Low-fat beef burger	Selani et al. (2016)
Cupuassu seeds	Polyphenols, flavonoids	Reduces cardiovascular diseases chances and degenerative diseases	Food preservative	Costa et al. (2020)
Tomato pomace	Phenolic compounds, lycopene	Antioxidant activity	Functional ingredient	Silva et al. (2019)
Apple pomace	Phenolic compounds	Antioxidant, anti-inflammatory, and antimicrobial properties	Pharmaceutical (dermal formulations)	Barreira et al. (2019)
Enoki mushroom stem	Phenolic compounds	Antioxidant activity, decreases cholesterol and blood pressure levels	Goat meat nuggets	Banerjee et al. (2020)

extract, showing strong antioxidant and antimicrobial activity against certain strains (*S. aureus*, *E. coli*, and *P. aeruginosa*), and shown to be safe against Caco-2 intestinal cells. Finally, mushroom toxicity is also studied, in which Kang et al. (2012) evaluated the cytotoxicity of oak mushroom stipe, which showed no cytotoxic effect (>90% cell viability) at the maximum concentration tested for RAW264.7 cell line.

29.5 Main Challenges for Secondary Metabolites

29.5.1 Domestication of Wild Species

The domestication of wild plants is considered one of the greatest achievements of the human being, which is defined by the exposure of wild species in selected environments, associated with human cultivations, controlling reproduction and generating an increase in the population size of the domesticated species (Purugganan 2019).

Domestication is also associated with changes in plant phenotypes, results of the adaptive evolution of plants in new environments, in which this change can be called intentional selection (desirable characteristics and artificially placed in the plant) or unconscious selection, because of the harvesting methods (Ensslin and Godefroid 2019). Among the changes that a domesticated plant can have, there are: less genetic diversity, changes in the plant (genetic, genomic, morphological, physiological, and anatomical changes), differences in disease resistance, competition for water, light, heat, and nutrients, in addition to having lower toxicity (Begna 2020). Adaptability to cultivation is one of the parameters that define the difficulty of domesticating wild species, in which their genetic change depends on the wild plant to be domesticated. While some crops adapt to conditions easily, such as good humidity, high soil fertility, and a positive reaction to the sun, others need some external stimulus (Kupzow 1980).

The simultaneous seed germination is defined as a fundamental characteristic to evaluate the success of the field culture, which can be divided into three types for wild plants: (1) seeds that germinate quickly and simultaneously under favorable conditions; (2) seeds with prolonged germination even under favorable conditions; and (3) seeds that do not germinate without some external stimulus (temperature, humidity, or dryness). Of the three, only the first type can successfully germinate under favorable conditions, and the second and third types require genetic reconstruction or seed treatments (Kupzow 1980). Gene isolation also becomes a problem as plant domestication reduces genetic diversity, in addition to the difficulty in this process involving recent demographic events, requiring, for example, reverse genetics or population genetics to correlate genes to a specified desired phenotype (Ross-Ibarra et al. 2007).

As for secondary metabolites, several plant characteristics can be noted between wild and domesticated species, such as color, for example, which is regulated from

secondary metabolites (Ku et al. 2020). Otieno et al. (2007) investigated the effect of domestication on the bioactivity of 27 medicinal herbs, in which there were no significant differences regarding the increase or decrease of the antimicrobial activity of the plants. On the other hand, the authors found that there was a significant increase in the vegetative biomass of some domesticated species. In contrast, Petropoulos et al. (2020) found a decrease in the antimicrobial activity of domesticated species of *Centaurea raphanina* subsp. *mixta* when compared to wild plants. Chitindingu et al. (2012) investigated that among the ten evaluated cereal species from Zimbabwe (six wild and four domesticated species), the one with the highest yield of phenolic compounds was a wild cereal, namely *Eleusine indica*. Finally, Ceccanti et al. (2021) evaluated wild and domesticated species of *Sanguisorba minor* Scop, in which, in general, wild samples showed greater antimicrobial effect, such as greater cytotoxic activity against HeLa cells.

Therefore, in general, the domestication of plants results in the reduction of secondary metabolites as they are related to the resistance of plants to biotic stresses. However, the domestication process can also preserve defense traits, such as changing the defense method significantly, in addition to climate changes related to domestication, which can also attribute other secondary metabolite characteristics (Ku et al. 2020; Gaillard et al. 2018).

29.5.2 Stability

Although secondary metabolites are described as being stable defensive agents produced by the organism, they are also susceptible to several factors, such as high temperatures, exposure to oxygen, among others, that can change their chemical structure, decreasing their stability, an important issue if it is intended to use these compounds in foods for instance (Zorenc et al. 2017). Carotenoids, for example, have their bioaccessibility and bioavailability affected by factors such as the matrix that will be incorporated and the microstructure of the food (Lemmens et al. 2014).

Albuquerque et al. (2021a, b) discussed the difficulties regarding the use of natural colorants in food matrices, since many variables can cause instability during their extraction of application, such as the presence of light, oxygen, heat, pH changes, and others. Ruiz-Rodriguez et al. (2008) report on caution regarding the use of carotenoids, secondary metabolites known for giving color to some foods, since they are compounds with many unsaturated structures and consequently sensitive to heat and oxygen.

As for extraction of bioactive compounds technologies, Wen et al. (2020) discuss the use of efficient methodologies, with minimal degradation and consequently higher yield. Ahmed and Ali (2013) evaluated different processing methods of white cauliflower (steam-blanched, steam-boiled, stir-fried, and microwaved cauliflower), obtaining an antioxidant activity reduction of approximately 10% in all processes when compared to the fresh product.

Wen et al. (2020) mention the effect of temperature, in which some classes of bioactive compounds are heat sensitive, but higher extraction temperatures result in a faster mass transfer rate, making it necessary to evaluate the best conditions to obtain a higher yield without the degradation of bioactive compounds. Getachew and Chun (2016) evaluated different temperatures (180 and 220 °C) for the extraction of bioactive compounds using hydrothermal process, reporting that at lower temperatures the degradation of phenolic compounds and flavonoids is lower. Casagrande et al. (2018) investigated the influence of temperature (40, 60, and 80 °C) for the extraction of bioactive compounds from *Baccharis dracunculifolia* and found that, in this temperature range, the highest temperature showed an increase in the extraction of phenolic compounds by up to 50%. Therefore, it is possible to verify that, at lower temperatures (less than 100 °C), the compounds degrade to a lesser extent while reaching good temperatures for the extraction of bioactive compounds, while at extreme temperatures (close to 200 °C) despite having good extraction temperatures, they destroy the bioactive compounds present in the food matrix.

29.6 Legislation for Legal Use of Secondary Metabolites in Different Industries

The effective use of secondary metabolites (SMs) as active principles in several fields, such as the food, cosmetic, and pharmaceutical industries, has been an exhaustive study in the last decades. However, the practical use of these compounds for a certain purpose depends on prior authorization of the competent agencies in each industrial sector and region where the new SMs-based product is intended to be marketed. The lack of homogenization of legislation between different global regions, unclear regulations, and the long time of the approval process are limiting factors for the commercialization of new products based on SMs (Gubser et al. 2021). Below, we will present by category some requirements and steps for the approval of new SMs-based products by the main regulatory agencies in the world, namely from the USA (US) and in the European Union (EU).

29.6.1 Food Ingredients, Food Supplements, and Food Additives

The legislation and approval process for SMs for food industry products may differ depending on their intended use. For example, in the USA food additives and food ingredients are in the same category of products and require the same rigorous processes for their approval. On the other hand, ingredients for food supplements are evaluated more superficially. In Europe, food additives are distinguished from food ingredients and are strictly legislated by the EFSA, while food ingredients and

food supplements may have some similarities in their approval processes. Next, more details will be presented on the approval process for these compounds in these three different sectors of the food industry.

EFSA (European Food Safety Authority) and FDA (Food and Drug Administration) are agencies responsible for legislating and monitoring food ingredients, food additives, and food supplements in the EU and the USA, respectively. The conflicts between the standardization of these two agencies start from the definition of food additives and food ingredients. For example, in the USA food additives are considered as which substances are used during processing, treatment, packaging, storage, and distribution. Food additives require a pre-marketing authorization provided by the FDA; however, it is not necessary if the ingredient/substance is known as safe (GRAS—Generally Recognized as Safe). Furthermore, food additives, according to FDA, can be divided into two groups: direct food additive—added to food for a specific purpose, and indirect food additive—which can be found in the food in trace amounts due to packing, storage, or other processes (Rulis and Levitt 2009; US FDA 2018). According to EFSA, food additives are defined as substances added intentionally to food to coffer a specific technological function, such as color, texture, sweeten, among others (Carocho et al. 2014; Vilas-Boas et al. 2021).

In the European Union, the approval process for new food ingredients based on plant/fungi SMs may differ according to their origin. Products derived from a traditional natural source (used before May 15, 1997) may have pre-market approval based on their history of safe use. However, according to EU regulation 2015/2283, derivatives of matrices not used before that date are defined as novel foods isolated or produced from plant cells and tissue cultures. This category also includes food ingredients obtained by emerging technologies used after this data, which are necessary to the evaluation performed by the EFSA to determine its safety and define if it is or is not its pre-market authorization (Vilas-Boas et al. 2021). However, SMs obtained from a natural source that have a purpose used as food additives should attend the Regulation (EC) N° 1331/2008. In this case, EFSA's assessment of new food additives is divided into four parts: the first is to evaluate the "chemistry and specification," which consider the origin of the substance (for example, derived from a botanical source, if it is a nanoparticle, among others), as it is a single compound or complex mixture, its chemical classification, its process of obtaining, methods of analysis, and its stability. The second part consists in the "Existing Authorization and Evaluation"; then the "Proposed uses and exposure assessment," where the dose level was determined considering the groups and population of all EU member states, and, in the last step, the "Toxicological Studies," which includes three tiers: 1—evaluation of bioavailability, in vitro studies for determining gastrointestinal toxicity, subchronic toxicity (animal model assay), genotoxicity; 2—determination of bioaccumulation, genotoxicity, chronic toxicity, carcinogenicity, reproductive and developmental toxicity; and 3—Toxicokinetic assays performed in animals, with repeated administration until approximately five terminal half-lives, human clinical trials, toxicity tests in animal, studies for endocrine, developmental neurotoxicity, of studies on the effects of the proposed additive in vitro and in vivo assays, the latter includes metabolism, acute subchronic and chronic toxicity in an

animal model. Human trials are performed only after acquiring reliable data from animal model studies, in tier 3. Besides this data, other safety and/or specific studies may be requested during the evaluation of the food additive (Carocho et al. 2014; Vilas-Boas et al. 2021). In the case of the American approach, new food additives must also be evaluated against these topics, and a team of scientists from the Office of Food Additive Safety (OFAS) must carry out these evaluations (Rulis and Levitt 2009).

Food supplements/dietary ingredients, in the EU legislation (directive 2002/46/EC), are defined as an addition to a normal diet, with a high concentration of nutrients (or other substances) with nutritional or physiological effects (Ratajczak et al. 2020). Also, botanical agents applied to food supplements may attend to the regulation 258/97/EC, in wich food suplements can include substances derived from botanical matrices, including botanical extracts. To ensure a better quality of products derived from botanical sources, in 2009, EFSA released a guide entitled "Safety assessment of botanicals and botanical preparations intended for use as ingredients in food supplements," which can also be applied in other sectors. According to this guidance, botanicals and botanicals preparation must be evaluated regarding their safety following a Hazard Analysis and Critical Control Point (HACCP) determined by Codex Alimentarius, considering all processes since the primary production (Vilas-Boas et al. 2021). Toxicological studies required for botanical ingredients are not defined and can be changed depending on the botanical source (for example, if it has a long-term history of food use), its intended uses, and levels of the use in the food supplements. In addition to being safe, the botanical ingredient must be tested for its shelf-life. And information about its degradation must be provided (Vilas-Boas et al. 2021). In the USA, this class of food products are defined as a product/ingredient intake by mouth that has in its composition vitamins, fatty acids, and herbs or vegetal (plant material, macroscopic fungi, or combination of these materials), and products not sold in the USA before 1994 are classified as "new dietary ingredient." Contrarly to pharmaceuticals and food additives, dietary ingredients do not need FDA marketing approval, however, their production process must meet good manufacturing practice criteria, and the manufacturers and distributors of dietary supplements are responsible for the safety of their products before introducing them to in the market, and the FDA is responsible for inspecting the production process when necessary, has the potential to declare recalls products if it shows some hazardous (Ratajczak et al. 2020). In the specific case of dietary supplements produced from botanical sources, these can contain the whole plant, part of them, powdered vegetal material, or extract of vegetal. This type of supplement is widely used in the USA, and there is more than 2000 supplements based on botanical source marketed there (Shipkowski et al. 2018).

Despite being widely marketed, especially in the USA, data on the toxicity of some of these supplements are scarce, and studies that assess the toxicity of each compound individually may have irrelevant results since in many cases food supplements are made up of a complex mixture of matrices or part of them, another factor that hinders the determination of the toxicity of dietary ingredients based on SM is the lack of standardization of the raw material (higher plant is subject to different environmental and biological factors that affect its chemical profile) which

makes it impossible to extrapolate the data globally. Furthermore, the production of dietary supplements is more susceptible to microbiological contamination, which puts the health of the consumer at risk (Ratajczak et al. 2020; Shipkowski et al. 2018). However, the major challenge to the development of food supplements based on SMs is to ensure that the final product is free of contaminants, safe for consumption and that there is standardization throughout the production chain (Shipkowski et al. 2018).

29.6.2 Pharmaceuticals

According to FDA, a drug is defined as a substance other than a food, intended to be used in the diagnosis, cure, mitigation, treatment, or prevention of diseases, acting over the structure or over the body functions. Natural pharmaceuticals can be recognized as: natural substances or organisms unregulated; unmodified natural compounds or materials FDA-regulated; semisynthetic compounds obtained from a natural substance that has undergone chemical modifications; and a purely synthetic medicinal compound inspired by a natural compound (Patridge et al. 2016). However, herbal products are not included in the FDA drug regularization and are considered as dietary supplements (Van Norman 2016).

The study performed by Patridge et al. (2016), regarding the approval of natural products by the FDA between the years 1931 and 2014, describes a continuous increase in the approval of derived natural compounds until 1930, with its maximum in 1990, and from then onward there was a decrease in the number of natural compounds without modification, and the number of compounds derived from natural sources began to increase. This advance reflects a response to the challenges of using unmodified natural compounds, but, their use have several barriers that need to be overcome, such as establishing a sustainable production of these compounds, purification of the target compounds and stabilization techniques. Therefore, to overcome these barriers, several studies, investments are necessary and require too much time, which has led pharmaceutical industries to investigate and invest in semisynthetic products derived from plants and other natural sources (Patridge et al. 2016). Currently, about 25% of the drugs approved by the FDA are produced from plants, 16% from bacteria, and 12% from fungi (Patridge et al. 2016). Morphine and phenoxymethylpenicillin were the first pharmaceutical compounds approved for use obtained from a plant (1827) and fungus (1953), respectively (Patridge et al. 2016), and until 2019, 185 molecules obtained from the plant were allowed to their use as therapeutics for the treatment of cancer (Süntar et al. 2021).

Regarding the approval processes, the evaluation of new pharmaceuticals can take many years. For instance, it is estimated that the FDA evaluation time for a new drug can vary between 10 and 15 years that corresponds to the time between the pre clinical tests, passing through complex clinical tests, and posttrial until its approval or not. Besides that, high investment is required for its development until its

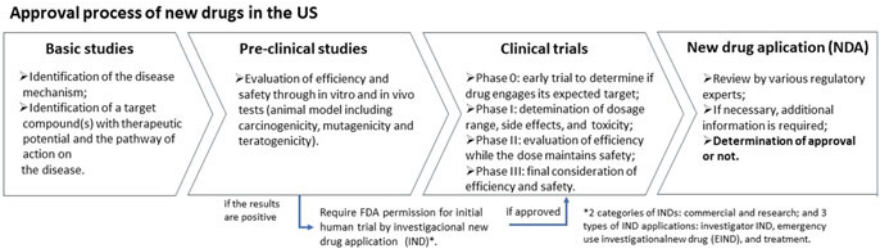

Fig. 29.2 Summary of steps in the FDA approval process for new drugs

commercialization, which can exceed more than 1 billion dollars (Van Norman 2016). The long time to approval and the high cost of a new drug are due to the different studies, processes, and protocols that must be carried out step by step. Figure 29.2 summarizes some of these steps required for the approval of a drug by the FDA. The approval process requires basic studies from the characterization of the substance with evaluated medicinal properties, such as studies in animal models to determine mechanisms of action and toxicity, from then on, studies in humans are needed, but these must be authorized by the regulatory bodies, in the FDA, for example, authorization must be requested through the IDN. Clinical studies are divided into phases, in phase 0 the study is carried out in a small group of healthy patients (10–15 individuals) who receive a small dose of the substance, and the main objective in this phase is to determine whether the substance has harmful effects. Phase I (20–50 patients) aims to determine the form of administration, minimum and maximum dosage, treatment time, side effects. Phase II is conducted in a larger group of people (>100) where it seeks to determine the effectiveness of the treatment and possible side effects. Phase III is a large-scale study (>1000 subjects), which requires a long evaluation time (1–4 years) and aims to evaluate and monitor the effectiveness of treatment and adverse reactions. It is estimated that only 25–30% of proposed new drugs make it to phase III. If the drug has satisfactory results in all phases of the clinical trials, it will then be submitted as a new drug application (NDA). All documents for the production and evaluation process of the new pharmaceutical will be evaluated by a team of specialized reviewers, and new information may be required, as well as a new clinical trial (phase IV). At the end of the process, the new drug receives marketing authorization or not (Van Norman 2016).

In the EU, a specific committee was created in 2004 to evaluate herbal medicines, the "Committee on Herbal Medicinal Products (HMPC)", which is part of the European Medicines Agency (EMA), with the aim of harmonizing the European market. It is responsible for compiling and evaluating scientific data on proposed herbal substances. All information including intended use, safety, undesirable effects, and interaction with other medicines, about the herbal product, can be found in the European Union Herbal Monographs (Knoess and Wiesner 2019). The HMPC distinguishes herbal products into two sections: (1) well-established use, which receives marketing authorization if the product presents sufficient data to

prove its efficiency and safety); and (2) traditional use, which requires simplified registration and is accepted based on sufficient and plausible efficacy. For the registration of a new herbal product, the guidance of HMPC requires data on the quality (preparation of the product, good agricultural and collection practice for starting the materials of herbal origin, specifications of the test procedures and acceptance criteria for the herbal substance evaluated), no clinical studies to evaluate the genotoxicity of the herbal substance; clinical trials to evaluate the safety and efficacy of the herbal product, and submit the documents regarding the safety of herbal substances, which include allergenic potency, contamination of the herbal medicinal product with toxic substances, environmental risk assessment of the herbal medicinal product. Contrarly to the US approval process, the HMPC considers literature data to establish a consensus on the safety of the herbal substance, and at least one clinical study of quality is necessary to prove its efficacy, however, the time to marketing approval can be lest 10 years. According to Directive (2004/24/CE), for the herbal product to be considered of "traditional use," it has to be traditionally used for a minimal of 30 years, being that for 15 years this product is traditionally used in Europe (Knoess and Wiesner 2019).

29.6.3 Cosmetic Ingredients

Cosmeceuticals are defined as cosmetics that have biological properties, like pharmaceutical products, and are formulated with bioactive ingredients, and their beneficial health effects have been demonstrated in clinical trial studies. Although the term cosmeceuticals is spread worldwide in the scientific society, at the regulatory level, both the US and EU committees do not recognize them as cosmetic products and must be legislated as pharmaceuticals (Cornell et al. 2019).

Regarding cosmetic ingredients, in the USA, the evaluation of the safety of these products is performed by a committee called Cosmetic Ingredient Review (CIR), however, it does not have regulatory authority and works to publish ingredient safety data from its review process, and this information can be considered by FDA in producing their guidelines (Cornell et al. 2019). In the EU, pre-marketing authorization is not necessary for cosmetics, however, before its commercialization, details about the product, including its safety data, production steps, and quality parameters, have been submitted to the European Commission via a centralized system called as Cosmetic Products Notification Portal (CPNP). The EU system also has a post-market surveillance system and companies are required to establish the safety of their products through testing. Another different point of the US and EU regulations is the evaluation of the safety in animal testing, it has been banned in European countries, and allowed in the USA (Cornell et al. 2019).

In European Union, essential oils, also known as "odorous products," sold in quantities equal or higher than 1 ton/year and destined for cosmetic products, household care, cleaning products, and other applications, must be represented by the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH)

(Ellis 2010). An essential oil obtained from natural sources is susceptive to variation in its chemical composition due to various factors that can affect the condition of development of the matrices source and different types of processes that the raw material can be submitted. Therefore, the REACH cannot accept data from literature of physical-chemical and toxicologic determination for botanical complex matrices. Furthermore, essential oils from a botanical source are considered as natural complex substances with substances of unknown or variable composition (UVCBs). For commercialization, all substances present in a content >10% of the essential oil must be identified, as well as all relevant constituents present at > 0.1% have to be described. According to identification, essential oils can be classified as type 1—are well-defined NCSs with >90% of the composition known; and type 2—that as labeled incompletely (<90% of composition determined) (Ellis 2010). Regarding toxicological determination, are necessary studies about its allergic potential, acute toxicity, mutagenicity/genotoxicity studies (Ellis 2010). The FDA does not require approval for the use of fragrances in cosmetic products. Therefore, the cosmetic producers become the main responsables for the safety and quality of those products produced with secondary metabolites belonging to the terpenoid class. To control, standardize, and guarantee the safety and quality of essential oils, the International Organization for Standardization (ISO), through the technical committee ISO/TC 54, has established composition parameters and standardized analytical methods for evaluation of the quality, including step control in labeling, transport and marking, of essential oils (Sharmeen et al. 2021).

29.7 Feasibility of SM Production in Plants and Fungi at an Industrial Level

29.7.1 SM Obtained from Plants

The laboratory synthesis of SMs can become complex and expensive, so obtaining these compounds from the plant/fungus matrix may seem more viable, however, the low concentration of the bioactive compound in the matrices can lead to the processing of a large amount of plant material. In addition, due to factors that can influence the growth of the matrix (e.g., geographic localization, soil conditions, climate, season, aging of the culture, stress suffered by chemical, physical, and biological attacks, among others), there is variability in the concentration and quality of the target compound, which is a problem for the standardization of the industrial process, reducing productivity and increasing the cost of obtaining the product (Farjaminezhad and Garoosi 2021; Wawrosch and Zotchev 2021). Furthermore, the exploitation of the flora to obtain SMs for the application in pharmaceutical products has led some medicinal plants (~15,000) to extinction (Süntar et al. 2021). To cover these limiting factors to effective use of SMs obtained from traditional/natural plant/fungi culture, studies have proposed diverse ways to increase the

synthesis of these compounds by biotechnology technics, including elicitation, and plant culture metabolic and engineering (Dias et al. 2016; Keshavan et al. 2022; Süntar et al. 2021).

29.7.2 Elicitation

Several factors can cause stress conditions and biological attacks in plants, which may lead to a higher production of SMs. From this principle, purposeful stimulation of the defense mechanisms through controlled conditions is determined as elicitation. This technic consists of the introduction of one or more conditions/factors able to start the defense mechanism of the plant cells. These conditions/factors are denominated as elicitors and they can be divided into: abiotic, that includes the physical and chemical environment conditions (e.g., radiation, temperature, humidity, water restriction, salinity, carbon dioxide concentration, heavy metals, mineral salts, among other), and in also hormone stimulators, such as salicylic, jasmonic and arachidonic acids, that are used as abiotic elicitors, and biotic elicitors, that are the substances of biological origin, for example, polysaccharides, fungi, bacteria, yeast extract, among others (Albuquerque et al. 2021a, b; Oluwakemi et al. 2018).

Some studies (Table 29.2) have shown that the elicitation in plants has a positive effect on the production of SM. The production of essential oils in *Ocimum gratissimum* L. increased about 39.8% after biostimulation with a salicylic acid solution, with an increase in the Eugenol concentration (Alvarenga et al. 2022). Besides the elicitation in high plants, this also can be applied in the isolated parts of the plant after harvest, for example, the elicitation with UV light during 24–48 h on mature grapes resulted in the increased amount of stilbenes (Adrian et al. 2000).

29.7.2.1 In Vitro Culture for SMs Production

The production of SM from plant tissue culture has been studied since the beginning of the twentieth century. In 1950 plant tissue culture was used for the first time by the Charles Pfizer Company for the synthesis of phytochemical compounds from in vitro grown watermelons. Years later, namely in 1987, an important study with 30 cell different culture systems showed that the in vitro plant culture for production of SM could be more efficient than normal cultivation (Dias et al. 2016). The main advantages of the in vitro culture in front of normal cultivation are reliability, predictability, scalability since that uncontrollable environmental factors and geographic conditions do not influence the plant development, stability of plant genetic, sustainable, and quicker production, and easier extraction and purification (Dias et al. 2016; Farjaminezhad and Garoosi 2021; Wawrosch and Zotchev 2021).

This biotechnology consists in the cultivation of plant tissue in aseptic conditions, under controlled conditions, such as optimized medium composition, temperature, and humidity controlled, which allow the production of biomass standardized in

Table 29.2 Biotechnological strategies to increase the production of SMs in botanical matrices

Elicitation				
Plant species	Target compound	Elicitor	Outcome	References
Ocimum gratissimum L.	Essential oil	SA (1.0 mM)	39.8% increase	Alvarenga et al. (2022)
Vitis vinifera L. grapes	Stilbenes	UV light	18-fold	Adrian et al. (2000)
V. vinifera L. cv. Tinto cão grapes	Anthocyanins Catechin Flavonols	Chitosan	47–82.45% increase 133% increase 59.0–105.8% increase	Singh et al. (2020)
V. vinífera L. cv. Tempranillo Blanco grapes	Catechin Quercetin Isorhamnetin	Ascophyllum nodosum	1.48–1.93-fold 1.54–2.12-fold	Gutiérrez-Gamboa et al. (2020)
Brassica oleracea	Flavonoids	MeJA (10 μM) SA (100 μM)	31% increase 33% increase	Pérez-Balibrea et al. (2011)
Salvia verticillata	Phenolic acids	MeJA (50 μM) AgNO3 (15 μM)	1.21-fold increase 1.52-fold increase	Pesaraklu et al. (2021)
S. officinalis	Phenolic acids	MeJA (50 μM) AgNO3 (15 μM)	1.64-fold increase 1.60-fold increase	Pesaraklu et al. (2021)
Melissa officinalis L.	Rosmarinic acid	Heat stress (38 °C/5 h)	59% increase	Pistelli et al. (2019)

In vitro culture					
Plant tissue	Target compound	Type of production	Medium/Elicitor	Outcomes	References
Trigonella foenum-graecum	Diosgenin (sapogenin)	Callus culture	MS medium/ coconut milk (150 mL/L)	27% increase	Oncina et al. (2000)
Hylocereus costaricensis	Betalain	Callus culture	Red light	3.8–4.8-fold increase	Winson et al. (2021)
Fagonia indica L.	Kaempferol Apigenin Myricetin Isorhamnetin	Callus culture	UV-C radiation	Increases compounds production	Abbasi et al. (2021)
Nothapodytes nimmoniana (J. Graham) Mabb	Camptothecin	Cell suspension culture	MS medium/ Chitan	11.48-fold increase	Keshavan et al. (2022)
Azadirachta indica	Azadirachtin Squalene	Cell suspension culture	Yeast extract	219.78 mg/L yield 4.53 mg/L yield	Farjaminezhad and Garoosi (2021)
V. vinifera L.cv Gamay Fréaux	Resveratrol	Cell suspension culture	GC-2 medium, JA, GLU	2400 mg/L yield	Vuong et al. (2014)
Vitis vinifera cv. Monastrell	*trans*-resveratrol	Cell suspension culture	Gamborg B5 medium/ diadenosine triphosphate	Increase the accumulation of trans-resveratrol	Pietrowska-Borek et al. (2014)
Salvia miltiorrhiza	Salvianolic acid Caffeic acid	Cell suspension culture	MS media/JA, SA, MeSA	Increases the accumulation of the compound	Dong et al. (2010)
Hypericum perforatum	Flavonoids	Cell suspension culture	MS medium/ MeJA (100 μmol/L)	2.7-fold increase (280 mg/L)	Wang et al. (2015)

(continued)

Table 29.2 (continued)

In vitro culture					
Plant tissue	Target compound	Type of production	Medium/Elicitor	Outcomes	References
V. vinifera subsp. *sylvestris*	Resveratrol	Hairy Root culture	MS medium	31-fold increase	Hosseini et al. (2017)
Taxus × *media* var. Hicksii	Paclitaxel 10-deacethylbaccatin III	Hairy Root culture	DCR-M medium/ L-phenylalanine and MeJa	568.2 μg/L yield 422.7 μg/L yield	Syklowska-Baranek et al. (2009)
Beta vulgaris	Betalains	Hairy Root culture	MS medium	330.5 mg/L yield	Pavlov et al. (2007)
Echinacea purpurea	Caffeic acid Cichoric acid	Hairy Root culture	MS medium/ Ultrasound (40 kHz, 240 W)	Increase of production after ultrasonic treatment	Liu et al. (2012)
Fagopyrum tataricum Gaertn.	Rutin	Hairy Root culture	MS medium/UV-B radiation	9.35-fold increase	Huang et al. (2016)

JA jasmonic acid, *MeJa* methyl jasmonate, *SA* salicylic acid, *GC* GLU – β-glucan

terms of amount and chemistry composition. To start the bioproduction of SM by in vitro culture, studies must be performed to select a potential source of the target compound that allows the obtaining of high yield in callus culture, followed by the determination of the explanted part (leaves, seeds, root, stem, among others) (Süntar et al. 2021). In principle, any plant can be introduced into a type of in vitro culture, as long as the plant tissue has all the necessary genetic material to maintain the metabolic process just like its parent plant (Dias et al. 2016). Another important step is the selection of medium. The concentration of nutrients (salt, types, and levels of carbohydrates, nitrate levels, phosphate, growth regulators) in the composition of the medium is crucial to biomass accumulation and synthesis of SM (Murthy et al. 2014). To enhance the production of SM and reduce production time, elicitation with abiotic and biotic elicitors is also used in in vitro culture. For the success of an in vitro SM production, a rigorous optimization process must be previously carried out to determine the elicitor(s), its/their concentrations, biomass growth time, and contact time with the stimulating agents (Gubser et al. 2021; Wawrosch and Zotchev 2021).

The production of SMs by in vitro culture can be done in several ways, and the most used methods at the scientific and industrial level are Callus culture, suspension cell, and hairy root cultures. Callus culture is characterized by the formation of callus with identity thought of small pieces of the matrix (explants), this technique has been used in the recovery of endangered species. Although callus cultures are essential for the transcription of the explant cell genome, this technique is not suitable for the production of SM on a large scale due to the long time required for the development of calluses and the risk of contamination and genetic changes that can occur during the process (Kreis 2019). Suspension cells culture is starting from a formed callus introduced into a liquid medium, from then on, callus proliferation is controlled by adequate conditions of aeration, agitation, light, temperature, nutrients, among

others. This process can be carried out in bioreactors in a continuous process, which allows for a greater processing volume, which makes this method one of the best alternatives to increase the production of SM and make its quantity viable to meet the demand of industries (Farjaminezhad and Garoosi 2021; Wawrosch and Zotchev 2021). Some studies have used callus culture to achieve high yields of SMs. For example, the production of resveratrol by cell culture is largely studied, Vuong et al. (2014) increased the production of this stilbene from *V. vinifera* cells after investigating the effect of seven elicitors, including jasmonic acid (JA), salicylic acid (SA), 3-methyl-salicylic acid (MeSA), betaine (BET), β-glucan (GLU), methyl-β-cyclodextrin (MeCD), and chitosan (CHI). According to the authors, the combination of GLU with JA resulted in the best treatment, which allowed recovery of 2400 mg/L. In addition, compounds of different classes have been produced by these techniques, with triterpenes (Farjaminezhad and Garoosi 2021), alkaloids (Keshavan et al. 2022), phenols, flavonoids, among others.

In hairy root cultures, the synthesis of the SMs occurs in roots after the desired contamination with a natural soil microorganism, the *Agrobacterium rhizogenes*, which increases the hormone biosynthesis, leading to the rapid development of the plant culture in simple media and high secondary metabolites accumulation, and the main advantage of this method is the genetic stability (Wawrosch and Zotchev 2021). This technique is used mainly to study gene expression and biological characteristics. For the production of SMs from plants, some authors refere the biochemical stability, cytodifferentiation, and growth on hormone-free medium as advantages to the cell suspension culture (Srivastava and Srivastava 2007). Diverse compounds can be produced by hairy root culture. Interestingly, although the hairy root culture does not require hormone stimulation, however in the study carried out by Syklowska-Baranek et al. (2009), the production of the anticancer compounds of the taxane class, namely paclitaxel and 10-deacethylbaccatin III, was significantly increased by elicitation with the combination of the l-phenylalanine pathway with the hormone MeJA. In addition, physical elicitation such as ultrasound waves and radiation has shown an efficient combination with this biotechnology to improve the accumulation of SMs (Huang et al. 2016; Liu et al. 2012).

29.7.3 SM Obtained from Fungi

Natural products obtained from fungi represent about 61% of the more than 29,000 substances related in the Natural Products Atlas, and a large part of them are essential to the development of drugs and food ingredients. Despite the fact that fungi, including mushrooms, develop in nature under different conditions, however, many of them are uncultivable in the laboratory. The use of fungi obtained from their natural habitats can bring as a disadvantage the variation in the quality of the target product, low yield, and uncontrollable genetic interference (Meng et al. 2022; Zhong and Xiao 2009).

To overcome these problems, fermentation processes, namely submerged fermentation and solid-state fermentation, are known as a potential way for the large-scale production of bioactive compounds from fungi, however, for the success of these techniques, the optimization of process parameters, such as pH, temperature, medium composition, substrate, aeration, mixing, among others, is essential to achieve optimal biomass generation and better production yields (Xu et al. 2008; Zhong and Xiao 2009). Several SMs have been obtained in high concentrations from fermentation, for example, the meticulous optimization of the medium composition in terms of glucose, peptone, corn flour, and soybean powder concentrations was crucial for the maximum production of ganoderic acid (up to 496 mg/L) from *Ganoderma lucidum* (Xu et al. 2008). Also, elicitation of fungi during the fermentation can increase the synthesis of SMs, such as the addition of phenobarbital at the submerged culture of *G. lucidum* as an inducer of ganoderic acid was capable of increasing its concentration from 28.3 to 41.4 mg/g cell dry weight (Liang et al. 2010). Another positive point of fermentation processes is the possibility of using inexpensive substrates, such as bioproducts and bioresidues from other industries (Zhong and Xiao 2009), for example, erinacine C, an SM of the *Hericium erinaceus*, has been produced in considerable concentrations (569.1 and 591.7 mg/L) from submerged fermentation using as substrate Brewer's spent grain, and wheat bran, respectively (Wolters et al. 2016). Solid-state fermentation has been applied to high production (3 mg/g biomass dry weight) of coconut-like aroma (6-pentyl-α-pyrone) from *Trichoderma* sp. using sugarcane bagasse as substrate. The continuous production of camptothecin, an anticancer agent, was achieved an efficient yield (128 mg/L) in solid-state fermentation of the endophytic fungus *Fusarium oxysporum* with soybean meal and whey substrates (Bhalkar et al. 2016).

Another interesting biotechnological approach applied for the large-scale obtaining of SMs produced naturally by fungi, is the heterologous expression, which consists on the transcription of one or more genes from gene cluster data of the producer fungi of the target compound in to a secondary host, which in most studies are yeasts or filamentous fungi (Alberti et al. 2017; Meng et al. 2022). Fujii et al. (2011) introduced the gene cluster of the biosynthesis of aphidicolin (diterpene) into filamentous fungi, namely *Aspergillus oryzae* using different vectors (pTAex3, pPTRI, pUSA, and pAdeA), as a result, the authors reached the total biosynthesis of aphidicolin, however the process optimization is still necessary. *A. oryzae* heterologously expressed with seven genes of the high fungi *Clitopilus passeckerianus* was capable of producing the antibiotic pleuromutilin with high yield compared to the fungal host (2106%) (Bailey et al. 2016). More details of this biotechnology can be obtained in recent specific reviews that are focused on gene clusters identification and the production of SMs from fungi by heterologous expression (Alberti et al. 2017; Meng et al. 2022).

29.8 Conclusion

Secondary metabolites are of extreme importance and can find a wide range of industrial applications. There are different sources of secondary metabolites that comprise plants, cyanobacteria, fungi, seaweed, and others. Their industrial application comes from the wide range of chemical structures, being able to act, for example, as natural colorants and/or preservatives in the replacement of artificial ones, since consumers are demanding for more natural foodstuff with health benefits.

Despite the difficulties in finding many research indications about secondary metabolites and their action in the human organism, in the last years, the scientific community has been dedicating their studies to the identification of several secondary metabolites and their possible biological action. In this chapter it was possible to demonstrate the importance of secondary metabolites, highlighting their antioxidant, anti-inflammatory, anticancer, and inhibitory action against cardiovascular diseases and other health issues. Thus, it is evident the relevance of studying natural matrices as sources of bioactive metabolites and their possible applications in therapeutic treatments and food formulations. The use of plant cell culture to produce chemicals and drugs has greatly contributed to advances in several areas of plant physiology and biochemistry. The use of genetic tools and the current knowledge about the regulation of secondary metabolism pathways may provide the basis to produce these metabolites at commercially acceptable levels. The capacity in isolating genes that encode key enzymes of secondary metabolism leads to believe that the industrial sector will be greatly enhanced in the future through biotechnology and utilization of different biowaste, prospecting the use of promising sources and use of new metabolites, promoting the concept of circular economy.

In this sense, the importance of precise research to improve the forms of production and availability of different secondary metabolites, ensuring a sufficient production for the industrial application is a hot investigation topic.

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