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Ajay Kumar *Editors*

Phytochemical Genomics

Plant Metabolomics and Medicinal Plant Genomics

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ISBN 978-981-19-5778-9 ISBN 978-981-19-5779-6 (eBook)
https://doi.org/10.1007/978-981-19-5779-6

This Springer imprint is published by the registered company Springer Nature Singapore Pte Ltd.
The registered company address is: 152 Beach Road, #21-01/04 Gateway East, Singapore 189721, Singapore

Dedicated to My
Parents, Family Members
and Teachers
Who Taught Life Lesson

Preface

Plants are considered as the natural chemical factories because they produce a plethora of phytochemicals. Phytochemicals are important for the growth, development and defence of plants. Besides this, many phytochemicals particularly specialized metabolites are known to possess multiple medicinal and nutraceutical properties. Many of these phytochemicals have industrial uses. The biosynthesis of many phytochemicals is highly complex and involves many genes and other micro-regulators, including noncoding RNAs. Due to multiple benefits of these phytochemicals, recently, there is an increase in the interest towards identifying the phytochemicals and their underlying genes. The advancements in the next-generation technologies have resulted in the sequencing of a large number of medicinal plants that have contributed towards the understanding of genes that are responsible for producing a diverse phytochemical in plants. The genes-to-metabolites connections have been explicated, specifically by employing the combined studies of transcriptomes and metabolomes. These approaches are used to understand the functional genomics and the production of phytochemicals in major crops, and also in medicinally valued plants.

Increasing realization of the importance of plant-based bioactive compounds and the quest for finding their genes and other regulatory elements have led to the evolution of "Phytochemical genomics" as an emerging interdisciplinary area of research, which employs genomics and associated '-omics' technologies, such as transcriptomics, proteomics and metabolomics for the investigation of genomic basis of phytochemicals' production and their functions. This field further elucidates the phytochemical biosynthetic pathways and their regulatory aspects. Recent findings have shown a great promise in identifying novel genes that are responsible for the biosynthesis of bioactive compounds in medicinal plants. Further, advancements in computational bioinformatics have accelerated the process of discovery of new bioactive compounds, acquisition of genomic, transcriptomic and metabolomics data and its analysis. Increasing integration of -omics data makes it possible now to understand gene-metabolite networks in medicinal plants, which further enable us to better understand the synthesis, evolution and storage of specialized metabolites

within the plants. The convergence of the results of genomics and metabolomics with the recent revolutionary technologies, such as gene editing can aid and fasten the improvement programs aimed at the medicinal plants for the metabolites of commercial and economic importance.

Phytochemical genomics have led to several progressive outcomes. For instance, co-expression investigation of *Catharanthus roseus* transcriptome data recognized candidate genes that are responsible for the biosynthesis of monoterpenoid indole alkaloid. Similarly, scientists have identified two cytochrome P450 genes in *Glycyrrhiza uralensis* (licorice) for the biosynthesis of a sweetener saponin, glycyrrhizin. This has allowed the possibility of producing metabolites in other organisms, for example, microbial production of glycyrrhetinic acid. The genes involved in the biosynthesis of morphine in *Papaver somniferum* have been recognized by omics approaches. A polyketide synthase enzyme responsible for synthesizing olivetolic acid has been identified in *Cannabis sativa.* The candidate genes involved in the biosynthetic pathways of alkaloids and anthraquinones have been identified using differential transcriptomics and metabolic profiling for engineered cultured cells.

It is noteworthy to mention that the biosynthetic genes in plants are located in the genome, contrasting to microbial genes, where they are clustered together on the genome. Moreover, the expression of these genes is highly regulated. Nevertheless, collective evidences indicate that the genes of few biosynthetic pathways form gene clusters in plant genomes. This enables the detection of functional genes more easily and also offers more comprehensive understandings on evolution and function of specialized metabolites. More research is happening in this advanced scientific area to decipher the role of genes and their products to modulate the biosynthesis of plant secondary metabolites. Thus, surprising discoveries and uses can be achieved in this new field in coming years.

This book titled *Phytochemical Genomics: Plant Metabolomics and Medicinal Plant Genomics* is the first book devoted to the phytochemical genomics of medicinal plants. This book intend to present the latest findings in this area. This book is divided into four sections. The first section introduces the readers to phytochemicals, their diverse sources, roles, applications, bioprospecting, and technological advancements that aid their analysis. The second section introduces the concept of phytochemical genomics and its emergence. It further provided in-depth information on the integration of various -omics technologies for the analysis of the genes that regulate the important phytochemicals. It also provides the metabolomic diversity, databases relevant to medicinal plants and strategies to enhance the production of the metabolites using the information obtained from phytochemical studies. The third section deals with linking phytochemical genomics with recent revolutionary gene editing technologies including CRISPR/Cas for the improvement of the medicinal plant crops. The fourth section discusses the applications of phytochemical genomics such as medicinal plant stress biology, DNA barcoding and computational integration. This book will serve as a reference manual of phytochemical genomics for future. The book gives updated information on the emergence of phytochemicals

as a new interdisciplinary area of research. The book will be helpful to young researchers, graduate students and scientists.

We are highly grateful to all our contributors for readily accepting our invitation and sharing their knowledge and research outcomes to compose the chapters and enduring editorial suggestions to finally produce this venture. We greatly appreciate their commitment despite COVID 19 pandemic. We also thank the team Springer International, especially Dr. Emmy Lee (Associate Editor) and Ms. Shruthi Radhakrishnan (Production Editor) for their generous cooperation at every stage of the publication.

Bengaluru, Karnataka, India — Mallappa Kumara Swamy
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About This Book

It provides up-to-date information on phytochemical genomics, an emerging field on medicinal plants. It systematically provides information on the diverse phytochemicals that medicinal plants yield, their biosynthesis, genetic regulation and their multiple roles in diverse sectors such as medicine, colour, flavour, food and nutraceuticals. The latest information on the bioprospecting of medicinal plants for potential drugs is also highlighted. The book particularly introduces the readers to a new emerging discipline called phytochemical genomics. The first section introduces the readers to phytochemicals, their diverse sources, roles, applications, bioprospecting and technological advancements that aid their analysis. Second section introduces the concept of phytochemical genomics and its emergence. It further provides in-depth information on the integration of various -omics technologies for the analysis of the genes that regulate the important phytochemicals. It also provides the metabolic diversity, databases relevant to medicinal plants and strategies to enhance the production of the metabolites using the information obtained from phytochemical studies. Third section deals with linking phytochemical genomics with recent revolutionary gene editing technologies, including CRISPR/Cas for the improvement of the medicinal plant crops. The fourth section discusses the applications of phytochemical genomics such as medicinal stress biology, DNA barcoding and computational integration. This book will be the reference manual on phytochemical genomics for future research on medicinal plants.

Overall, the information provided in this book will undoubtedly encourage researchers, academics and pharmaceutical industries towards the usage of -omics technologies for the exploration of important bioactive compounds, their applications and identification of the genes that govern the biosynthesis of important metabolites. The book will facilitate the work of new researchers interested in starting their careers in the field of phytochemicals and related aspects. It will also be useful for graduate students of biochemistry, genomics, medicinal chemistry, medicinal botany, biotechnology and bio-engineering. It will also be crucial for scientists interested in the exploration of emerging plant-chemical genomics.

Contents

About the Editors

Mallappa Kumara Swamy is working as a Professor, Department of Biotechnology at East West College of Science (Affiliated to Bangalore University), Bengaluru, India. He has completed his Postdoctoral Research at the Department of Crop Science, Faculty of Agriculture, Universiti Putra Malaysia (UPM), Serdang, Malaysia from 2014 to 2018. Before that, he had worked as an Associate Professor and Head, Department of Biotechnology at Padmashree Institute of Management and Sciences, Bangalore University, Bengaluru, India. He obtained his Ph.D. (Biotechnology) from Acharya Nagarjuna University, Guntur, India in 2013. He is having more than 16 years of teaching and research experience in the fields of plant biotechnology, microbiology, secondary metabolites production, phytochemistry, biomedicine and bioactive studies. To his credit, he has published more than 120 research publications in peer-reviewed journals and 24 book chapters in reputed book publishers. So far, he has edited 13 books with Springer Nature Singapore Pte. Ltd., CRC Press (Taylor & Francis Group), USA and Studium Press, India. He is also serving as the Editorial Board Member and reviewer for few high impact international journals. Presently, he is working in the area of natural products research, plant cell and tissue culture technology for bioactive compounds production and evaluation of their bioactivities. His research is focused on nanobiotechnology for medical applications and plant omics studies.

Ajay Kumar is working as Assistant Professor of Plant Science at Central University of Kerala, India. He has obtained his Ph.D. from School of Life Sciences, Jawaharlal Nehru University, New Delhi under the guidance of Dr. Nirala Ramchairy who is a renowned crop scientist. Dr. Ajay has more than 6 years of teaching and research experience and has published several research papers, review articles and book chapters in reputed journals and books, respectively. He is also currently holding the position of Joint Director, Centre for Sustainability, Corporate Relations and Social Responsibility, Central University of Kerala, Kerala, India. His areas of research include phytochemical genomics, medicinal plants, mangrove genomics, food security and abiotic stresses. He has been a member of Academic Council, Central University of Kerala in the past and currently he is the member of the University Court. He has successfully guided more than 30 post-graduate students for their dissertations.

Part I
Introduction to Phytochemicals

Chapter 1
Phytochemicals: Diversity, Sources and Their Roles

Tareq A. Wani, Irshad Ahmad Bhat, Khushboo Guleria, Mudasir Fayaz, Thattantavide Anju, Kalath Haritha, Ajay Kumar, and Zahoor A. Kaloo

1 Introduction

The bioactive compounds present in plants are known as phytochemicals with high therapeutic and nutritive values (Hasler and Blumberg 1999). They provide protection for the plants from pathogenic infections and harm during the enhancement of the scent, colour and flavour of the plant. These plant chemicals are compounds that protect plants from environmental challenges such as pollution, abiotic stress and pathogen attack (Gibson et al. 1998; Mathai 2000). In addition, they also provide plants with several attributes like protection, growth, reproduction, signalling and allelochemicals against herbivory (Harborne and Baxter 1993; Saxena et al. 2013). Several studies proved that they play an important role in the well-being of human health during proper dietary intake (Samrot et al. 2009; Dai and Mumper 2010). Fruits, vegetables, whole grains, seeds and nuts are the general source of phytochemicals, and they are found in different parts of the plant leaves, stem, root, fruit, flower and seed (Costa et al. 1999). Higher concentrations of some phytochemicals are found in the outer layers of the plant parts, especially pigment molecules such as anthocyanins and flavonoids. Depending on the climatic conditions, type of plants and growing condition, the levels of phytochemicals may differ from one plant to another (Rao 2003). They have biological activities such as antioxidant activity,

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M. K. Swamy, A. Kumar (eds.), *Phytochemical Genomics*,
https://doi.org/10.1007/978-981-19-5779-6_1

antimicrobial effect, anticancer property and immune system stimulation, modulation of detoxification enzymes and hormone metabolism and decrease of platelet aggregation (Hamburger and Hostettmann 1991). Understanding their multipurpose applications, they have focused on the study. Surprisingly, the presence of phytochemical compounds in the medicinal plant provides rich bioresource components in different systems that include medicines, pharmaceutical intermediates, food supplements, nutraceuticals, and chemical entities for semi-synthetic and synthetic drugs (Ncube et al. 2008). Phytochemical compounds that possess nutraceutical features, present in food, are very much important because of their significance expediently related to human health. Phytochemicals present in the plants proved their potential to reduce the risk of non-communicable diseases such as diabetes, cancer, cardiovascular disease and respiratory disorders, as well as diseases such as microbial infections, ulcers, inflammation and hypertension (Liu 2013). Much research work has been done on phytochemicals using in vitro and in vivo models, and this has resulted in understanding their structural and functional potential accountable for disease risk reduction (Dillard and German 2000). Based on the role in plant metabolism, generally, the phytochemicals have been categorised as primary and secondary metabolites. Amino acids, carbohydrates, nucleic acids, proteins and chlorophylls are grouped under primary metabolites. Secondary metabolites constitute plant chemicals such as phenolics, alkaloids, flavonoids, terpenes, steroids, lignans, curcumin, saponins and glucosides (Hahn 1998; Ramawat et al. 2009). Among them, phenolics are common and structurally diverse plant secondary metabolites. The chapter briefly discusses the diversity of phytochemicals present in the plants with their structure and functional aspects in the biological system. It also provides examples of a few plants with specific secondary metabolites and their activities.

2 Defining Bioactive Phytochemicals

The term phytochemical comprises two parts: phyto means plant and chemical refers to the various types of natural compounds present in plants. The chemical compounds with the capability to interact with one or more components of living tissue to induce particular effects are considered bioactive compounds (Guaadaoui et al. 2014). Phytochemicals are grouped into six main types such as lipids, carbohydrates, phenolics, alkaloids, terpenoids and other nitrogen-containing compounds based on their structure and characteristics (Harborne and Baxter 1993; Campos-Vega and Oomah 2013). These phytochemicals are further subcategorised by their biosynthetic origin. Lately, the term phytochemical has been put for various uses for extricating plant chemicals that do not satisfy the classical definition of ‘essential nutrients’. According to Liu et al. (2013), phytochemicals can be defined as non-nutrient bioactive compounds that are found in vegetables, fruits, grains and other food parts. Among plants, around 5000 phytoconstituents have been reported with variable configuration and amount. However, when focusing on the biological

role in humans and the chemical structure of the compound, the major parts of phytochemicals are yet to be discovered (Liu 2013).

Phytochemical compounds have been recognised to possess a broad spectrum of antioxidant potentials and are huge health-care agents to consumers. Regular use of vegetables, fruits and whole grains prevents humans from different diseases associated with oxidative damage. Natural antioxidants are classified into two types: in vivo and in vitro antioxidants. Decomposers of free radicals act as enzyme inhibitors, synergists, singlet oxygen quenchers, electron donors, hydrogen donors, metal-chelating agents and peroxide decomposers. Phytochemical compounds with major health benefits include flavonoids, polyphenols, isoflavonoids, terpenoids, phytoestrogens, carotenoids, phytosterols, fibres, glucosinolates, anthocyanidins and limonoids. Plants synthesise a wide range of phytochemicals (secondary metabolites) that have toxicological effects and have important pharmacological activity in animals and humans (Bernhoft et al. 2010). These include terpenoids, alkaloids, phenolics, flavonoids and glycosides. Table 1.1 represents antioxidant-rich phytochemicals.

3 Main Phytochemicals

3.1 Alkaloids

Alkaloids among the secondary metabolites have the most structural diversity having simple one to complex ones like some neurotoxins. These are usually heterocyclic nitrogen-containing compounds and have played a significant role in plants as a chemical protectant against herbivores, viruses and other microorganisms. Alkaloids have huge pharmacological potential, particularly in medicine and stimulants, and as such have prompted interest in particular alkaloids contained by plants (Bandaranayake 2002). A huge number (12,000) of alkaloids have been identified so far in plants, and a large fraction of these phytochemicals have been found to have tremendous positive effects on human health (Takos and Rook 2013). The presence of these nitrogen atoms causes alkalinity in the compounds, and they generally appear as a ring (cyclic) system (e.g. nitrogen-containing indole ring system in indole alkaloids). Since alkaloids are structurally the most diverse group of secondary metabolites, therefore uniform classification is somewhat unclear. Alkaloids are divided into three major classes such as true alkaloids, protoalkaloids and pseudoalkaloids relying upon precursor and final structure (Singh 2017). True alkaloids have amino acid derivatives and encompass heterocyclic rings with nitrogen-like atropine and nicotine. Protoalkaloids lack nitrogen in their heterocyclic ring and have the same amino acid derivation, for instance, phenylethylamine-derived alkaloids. Caffeine and solanidine are pseudoalkaloids with nitrogen in the heterocyclic ring that are synthesised from amino acids (Singh 2017) (Fig. 1.1). Alkaloids have been significantly utilised in medicines, drugs and clinical environments as shown in Table 1.2 (Royal Society of Chemistry 1971; Cordell 1981;

Table 1.1 Phytochemicals along with their source(s) and biological activities

Phytochemicals	Source	Activity	References
Carotenoids	*Daucus carota*, *Solanum lycopersicums*, *Petroselinum crispum*, *Citrus sinensis*, *Trigonella foenum*, *Spinacia oleracea*, *Brassica oleracea* and *Raphanus sativus*	Antioxidants and anticancerous	Ribaya-Mercado and Blumberg (2004), Agarwal and Rao (2000)
Fibres	Oats, green leafy vegetables and fruits	Cardiovascular diseases and cholesterol reduction	Dillard and German (2000)
Saponins	Green fruits, tomato, oats and leaves	Antibacterial, anti-inflammatory and prevention of ulcers	Mert-Turk (2006)
Glucosinolates	Cruciferous vegetables	Anticancerous	Vig et al. (2009), Hayes et al. (2008), Conaway et al. (2001)
Limonoids	Citrus fruits	Inhibition of phase I enzymes and induction of phase II detoxification enzymes in the liver. Detoxification of enzyme. Protect lung tissues	Willcox et al. (2004)
Phytoestrogen	Berries, red wine, legumes, whole grains, cereals, red grapes and peanuts	Bone loss protection, cardiovascular diseases and anticancerous	Dip et al. (2009), Cos et al. (2003), Morabito et al. (2002), Mense et al. (2008)
Polyphenols	Beverages, oilseeds, vegetables, cereals, chocolates, fruits and legumes	Antioxidant, anti-inflammation, allergies, platelet aggregation and hepatoprotective	Singh et al. (2009), Ko et al. (2010), Prakash et al. (2007), Prakash and Gupta (2011)
Terpenoids (isoprenoids)	Liverworts, lichens, mushrooms mosses and algae	Antispasmodic, antiparasitic, anti-inflammatory, anti-hyperglycaemic, chemotherapeutic, antiviral and antimicrobial	Lee et al. (2003), Paduch et al. (2007), Hammer et al. (2003)
Polysaccharides	Vegetables and fruits	Antiparasitic, antimicrobial, antiallergic, antiviral, anti-inflammatory, enhancing defence mechanism and lowering serum	Schmidgall et al. (2000), Lopez Jr (2007)
Phytosterols	Seeds, nuts, fruits and vegetables	Suppression of initiation through arresting the G1 phase of cell cycle	Dillard and German (2000), John et al. (2007), Von Bergmann et al. (2005)

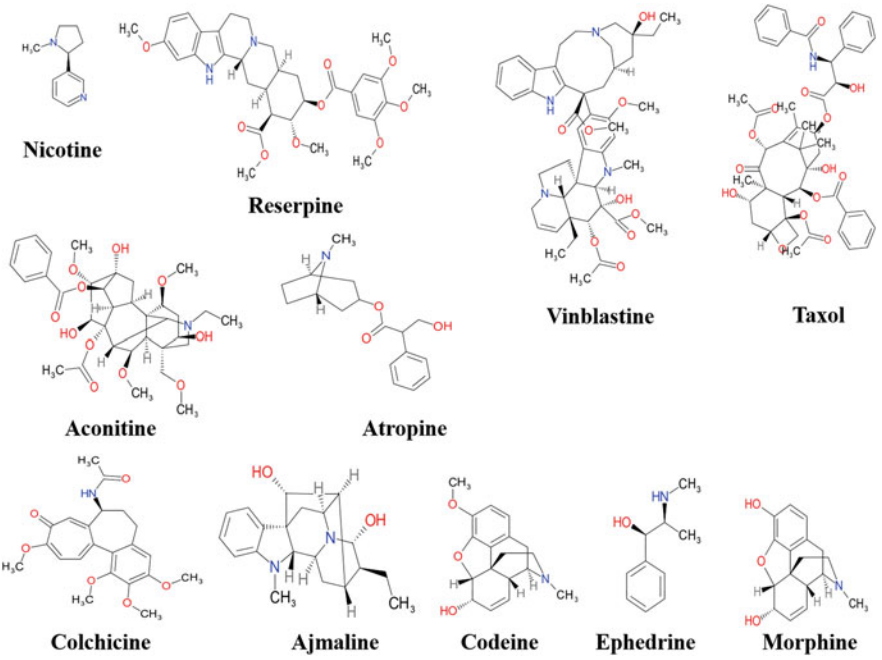

Fig. 1.1 Representative structures of some commonly known alkaloids. (Structures created using ChemDraw addon to MS Word)

Schmeller and Wink 1998; Buckingham et al. 2010). The reaction of alkaloids with acids results in the formation of salts. In general, alkaloids have the suffix -ine. Pure forms of alkaloids are odourless and colourless crystalline solids but rarely appear as yellowish liquids and taste bitter too.

3.2 Terpenes

Terpenes are limited to one of the wide-ranging groups of secondary metabolites and possess isoprene units of five carbons arranged in multiple ways to each other. Terpenes have a simple composition of hydrocarbons. Terpenes undergo different modifications to form terpenoids, oxidised methyl groups and various functional groups added or subtracted at a different position. Terpenoids are grouped into mono, di and tri; sesquiterpenes are based on the number of isoprene units present in them (Kandi et al. 2015) (Fig. 1.2). Terpenoids are effective in various human disease treatments worldwide as they have various biological and pharmacological properties. At present, more than 55,000 terpenes are noted, and this huge number indicates the structural diversification among them (Chávez-González et al. 2016). Furthermore, terpenes also are used in the preparation of perfumes, cosmetics and

Table 1.2 List of alkaloids that are used in medicines, drugs and clinical environments

Alkaloid	Source plant	Activity	References
Aconitine	*Aconitum chasmanthum*	Rheumatism, neuralgia and sciatica	Pullela et al. (2008)
Ajmaline	*Rauwolfia serpentina*	Antiarrhythmic agent	Endreß et al. (1993)
Atropine	*Atropa belladonna*	Antispasmodic, anti-Parkinson and cycloplegic drug	Dimitrov et al. (2005)
Berberine	*Berberis vulgaris*	Eye irritations, AIDS and hepatitis	Imenshahidi and Hosseinzadeh (2016)
Boldine	*Peumus boldus*	Cholelithiasis, vomiting and constipation	Klimaczewski et al. (2014)
Caffeine	*Coffea arabica*	Neonatal apnoea and atopic dermatitis	Perrois et al. (2015)
Emetine	*Carapichea ipecacuanha*	Intestinal amoebiasis and expectorant	Smajlović and Dučić (2021)
Cocaine	*Erythroxylum coca*	Local anaesthetic	Fischman and Foltin (2021)
Colchicine	*Colchicum autumnale*	Amyloidosis treatment and acute gout	Gasparyan et al. (2015)
Ephedrine	*Ephedra vulgaris*	Nasal decongestant and bronchodilator	Parsaeimehr and Sargsyan (2013)
Ergotamine	*Claviceps purpurea*	Postpartum/postabortal haemorrhage	Smakosz et al. (2021)
Morphine	*Papaver somniferum*	Pain relief and diarrhoea	Mani and Dhawan (2011)
Narceine	*Papaver somniferum*	Cough suppressant	Lim (2013)
Nicotine	*Nicotiana tabacum*	Anti-smoking	Jing et al. (2016)
Papaverine	*Papaver somniferum*	Vasodilator and gastrointestinal disorders	Kang et al. (2018)
Noscapine	*Nicotiana tabacum*	Cough suppressant	Dang and Facchini (2014)
Reserpine	*Rauwolfia serpentina*	Hypertension and psychoses	Kass and Brown (1955)
Taxol	*Taxus brevifolia*	Anticancerous	Bhuju and Gauchan (2018)
Vinblastine	*Catharanthus roseus*	Hodgkin's disease, testicular cancer and blood disorders	Arora et al. (2010)
Vincristine	*Catharanthus roseus*	Burkitt's lymphoma	Alam et al. (2017)
Pilocarpine	*Pilocarpus pennatifolius*	Miotic in treatment of glaucoma and leprosy	Allevato et al. (2019)
Codeine	*Papaver somniferum*	Antitussive and analgesic	Mani and Dhawan (2011)

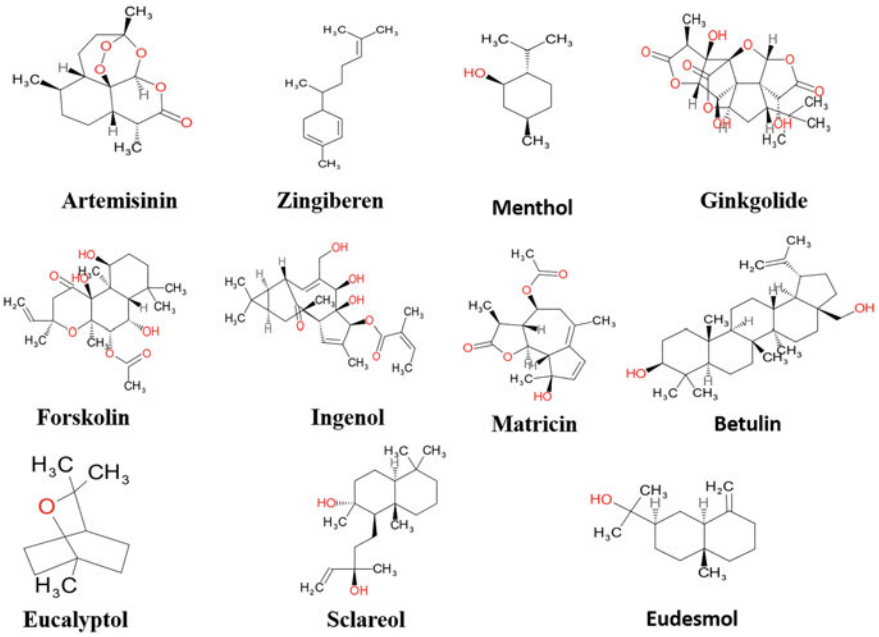

Fig. 1.2 Representative structures of terpenes. (Prepared using ChemDraw addon to MS Office)

insect repellents. One of the important anticancer compounds, namely, taxol, belongs to this group of secondary metabolites. In addition to this, other anticancer compounds act through various stages of cancer development like early start inhibition, differentiation of tumour cells, cell death and the invasion and suppression of metastasis and angiogenesis in the late stages through maintaining various intracellular signalling pathways (Ansari and Akhtar 2019).

3.2.1 Monoterpenes

They possess ten carbon atoms, that is, two units of isoprene with $C_{10}H_{16}$ as the molecular formula. Naturally, they are present in fixed and essential oils of plants among other sources. Monocyclic, bicyclic and acyclic type is the structural classification of monoterpenoids (Mabou and Yossa 2021). Secondary metabolites finding their place in this group generally have been used in pharmaceutical industries as they have a strong odour and aroma (Wojtunik-Kulesza et al. 2019). They are biologically active with strong antibacterial activity. Several researchers have documented that these compounds possess anticancer activity (Silva et al. 2021). The monoterpene composition present in essential oil in several species is linked with their antitumor activity (Sobral et al. 2014). 9-OH-isoegomaketone [(2*E*)-1-

(3-furanyl)-4-OH-4-Me-2-penten-1-one] is an important monoterpene and exhibits inhibitory activity on nitric oxide (Nam et al. 2017).

3.2.2 Sesquiterpenes

Sesquiterpenes have three isoprene units with the molecular formula $C_{15}H_{24}$. Sesquiterpenes have linear, cyclic, bicyclic and tricyclic forms as well as in the form of the lactone ring (Başer and Demirci 2007). Sesquiterpenes have anti-insecticidal and antibacterial activity (Jiang et al. 2021). Artemisinin is a sesquiterpene lactone, found in *Artemisia annua* roots and shoots with hyperactivity (Bisht et al. 2021). Some of the sesquiterpenes are arvestolides H and I, drimenin, chrysanthemulide A, santhemoidin A and artefreynic acids B, C and G.

3.2.3 Diterpenoids

Diterpenoids are a varied class of secondary metabolites having molecular formula $C_{20}H_{32}$ with four units of isoprene. They have tremendous biological activity like anticancer, anti-inflammatory, antifungal and antimicrobial activities (Topcu and Gören 2007). Diterpenes like grayanotoxin, forskolin, eleganolone, 14-deoxyandrographolide and marrubenol have cardiovascular activity (de Oliveira et al. 2008). Examples of diterpenoids include genkwanine P, laurifolioside A, cephinoids H, nudiflopenes F and I, drechmerin B, nicaeenin F, nicaeenin G and eupheliotriols F and L.

3.2.4 Sesterpenes

It has five isoprene units and $C_{25}H_{40}$ as the molecular formula. Besides this, it is found in marine organisms, fungi, insects, lichens and protective waxes (Negi et al. 2020). Sesterpenes have antimicrobial, anti-inflammatory and antifungal activity (Negi et al. 2020). Important sesterpenes include cybastacines A and B and scalarane sesterpenes.

3.2.5 Triterpenes

Triterpenes are formed from the squalene biosynthetic pathway and are an important category of secondary metabolites that usually contain 30 carbon atoms with 6 isoprene units (Santana-Molina et al. 2020). Triterpenes consist of several methyl groups, whose oxidation results in the production of aldehydes, alcohols and carboxylic acids. Saponins are produced from triterpenes through the glycosylation of many active sites. Few of the triterpenes are polyporenic acid B; pardinol B; pardinol E; pardinol F; xuedanencins G and H; cyclocariols A, B and H; etc.

3.2.6 Meroterpenes

Meroterpenes are compounds that are derived from mevalonic acid pathways with partial terpenoid skeleton. These are obtained from plants, animals, fungi, and bacteria (Chen et al. 2018). The biosynthesis of monoterpenes elaborates the diversity accessible to isoprenoid pathways and permits the assembly of natural products with exclusive structural properties. Meroterpenes include amestolkolide B, 6-OH-3-Me-8-phenylethylbenzo[b] oxepin-5-one, spiroapplanatumine G, etc. (Table 1.3).

3.3 Glycosides

The glycosides are made up of glucose entity attached to an aglycone. Therefore, glycone and aglycone are the two functionally and chemically independent parts of glycosides. Glycosidic bonds link the glycone and aglycone part in the glycoside (Bartnik and Facey 2017). A glycosidic bond is not that stable and is prone to hydrolysis via dilute acids and enzymes (β-glucosidases). Some of the plant secondary metabolites come about as glycosides naturally (Dembitsky 2004; Bruneton and Hatton 1999; Evans 2009). These glycosides from plants are mostly derived after postmodification done to secondary metabolites through the catalytic activity of plant enzymes such as glycosyltransferases. Besides this, other modifications done to these glycosides involve oxidation, acylation and degradation (Blanchard and Thorson 2006; Gantt et al. 2011; Yu et al. 2012). Still there exist gaps in understanding the physiological activities of these water-soluble metabolites in plants. It was found that these compounds are stored and transported inside plant cells and that they can play a role in cellular signalling, growth and development control, as well as allelopathy. They also participate in plants' defence against viruses and herbivores. Glycosides are often formed in response to environmental elements such as abiotic and biotic factors (Reichardt et al. 1988). Glycosidic linkage is of three different types O-, C- and S-glycosides relying upon the fact that glycosidic bond is via oxygen, carbon and sulphur linkages, respectively. These glycosides have different properties like O-glycosides and are found widely in plants. Linkage via carbon is very much prone to hydrolysis bonds in C-glycosides (Bartnik and Facey 2017) (Fig. 1.3).

It is a category of secondary metabolites that are extensively found in plants. Glycosides have tremendous medicinal properties and scope for pharmaceutical companies as antidepressants. A number of glycoside classes have been identified to date like coumarin, steviol, cyanogenic, phenolic, anthraquinone, iridoid, cardiac and saponins (Choudhary et al. 2021). Thoroughly researched glycosides in plants with biological effects on humans are anthranol, arbutin, antron, amygdalin, aucubin, catapol, digoxin, dhurrin, diosgenin, geniposidic acid, ginsenosides, hesperidin, loganin, rutin, lotaustralin, naringin, prunasin, rebaudioside, salicin, stevioside, sinigrin, sinalbin, theviridoside, etc. Some of the medicinal properties

Table 1.3 List of terpenes along with their source and biological activity

Terpenes	Plant source	Activity	References
Menthol	*Mentha piperita* L.	Antibacterial, antispasmodic, antiseptic and antiulcer activity	Oliveira et al. (2014), Kamatou et al. (2013), Freires et al. (2015)
Zingiberene	*Zingiber officinale* Roscoe	Component of ginger oil, used in the cosmetic industry and antiviral, antiulcer and anticancer agent	Millar (1998), Chrubasik et al. (2005), Shukla and Singh (2007)
Trans-cannabidiol	*Cannabis sativa* L.	Analgesic against asthma and spasms	Zhornitsky and Potvin (2012), Guy et al. (2004), Mechoulam et al. (2007)
Acanthoic acid	*Acanthopanax koreanum Nakai*	Anti-inflammatory agent, initiator of cell apoptosis and melanogenesis inhibitor	Kim et al. (1988, 2012), Traves et al. (2014)
Bisabolol	*Matricaria chamomilla* L.	Used in cosmetic products due to fine fragrance and antileishmaniasis	Oliveira et al. (2014), Kamatou and Viljoen (2010), Corpas-Lopez et al. (2015)
α-Santonin	*Artemisia maritima* L. ex. Hook. f.	Anthelmintic	Banerjee et al. (1993), Birladeanu (2004), Adekenov (2013)
Artemisinin	*Artemisia annua* Pall.	Antimalarial drug	Schafer (2014), Tang et al. (2014)
Ginkgolide	*Ginkgo biloba* L.	Drug for haemorrhagic stroke, Alzheimer's disease and ischaemic and anti-inflammatory activity	Xia and Fang (2007), Chi et al. (2015), Mohammad Nabavi et al. (2015)
Trigocherrin	*Trigonostemon cherrieri* Veillon	Drug against Chikungunya fever	Allard et al. (2012a, b), Bourjot et al. (2014)
Eucalyptol	*Eucalyptus globulus* Labill	Expectorant against asthma and antiulcer effect	Oliveira et al. (2014)
β-Caryophyllene	*Cannabis sativa* L.	Anticancerous activity	Nuutinen (2018)
Eudesmol	*Atractylodes lancea* (Thunb) DC.	Anti-dementia drug, antiangiogenic activity and induced apoptosis	Obara (2006), Tsuneki et al. (2005), Li et al. (2013)
Matricin	*Matricaria recutita* L.	Inflammatory skin and bowel diseases	Hitziger et al. (2003), Ramadan et al. (2006)
Sclareol	*Salvia sclarea* L.	Leukaemia, breast and colon cancer and fragrance ingredient	Dimas et al. (1999, 2006), Noori et al. (2010)
Forskolin	*Plectranthus barbatus* Andrews	Heart failure, autoimmune disorders, psoriasis and erectile dysfunction	Insel and Ostrom (2003), Alasbahi and Melzig (2012), Pavan et al. (2015)
Cucurbitacin B	*Cucurbitaceae* sp.	Anti-inflammatory, anticancer and hepatoprotective drug	Miro (1995), Setzer and Setzer (2003), Marostica et al. (2015)

(continued)

Table 1.3 (continued)

Terpenes	Plant source	Activity	References
Betulin	*Betula* sp. L.	Antibacterial, anticancer, antiviral and anti-HIV activity	Hayek et al. (1989), Alakurtti et al. (2006), Zhang et al. (2015)
Avicin D	*Acacia victoriae* Benth.	Anticancer agents and initiators of cell apoptosis	Haridas et al. (2001), Wang et al. (2010), Jayatilake et al. (2003)
Ingenol	*Euphorbia peplus* L.	Ingenol mebutate (Picato®) drug against actinic keratoses	Vasas et al. (2012), Bourjot et al. (2014)
Resiniferatoxin	*Euphorbia resinifera* O. Berg	Analgesic drug against cancer and inflammation. Urologic disorder agent	Appendino and Szallasi (1997), Wender et al. (1997), Elokely et al. (2016), Ladarola and Gonnella (2013)
Merrilactone	*Illicium merrillianum* A. C.Sm.	Parkinson's diseases and Alzheimer's disease	Huang et al. (2000), Inoue et al. (2003)
Myrcene	*Cannabis sativa* L.	Anti-inflammatory, anitooxidant and anti-nociceptive	Nuutinen (2018)
Thymol	*Thymus vulgaris* L.	Muscle swelling, insect bites and rheumatism	Guesmi et al. (2018)
Cis-carveol	*Mentha spicata* L.	Prophylaxis against prostate and breast cancer	Crowell et al. (1992), Chen et al. (2006)

of glycosides having been proven for pharmaceutical properties include antifungal, antiviral, anticancerous, analgesic, antioxidant and antidiabetic (Khan et al. 2018) (Table 1.4).

3.4 Phenolics

Phenolic chemicals are a wide category of bioactive secondary metabolites that are extremely important (Albuquerque et al. 2021; Cheynier 2012; Servili et al. 2013; Lin et al. 2016; Durrazo et al. 2019; Mark et al. 2019; Santos et al. 2021). Compounds with a phenol moiety are what they are called. Phenol is a benzene ring having a hydroxyl group substituted on it. As a result, its systematic name is hydroxybenzene. These chemicals have a diverse spectrum of biological effects. They are known to have antibacterial, antioxidant and anti-inflammatory activities (Ruiz-Ruiz et al. 2017). They may be found all over the place in nature. They are commonly found in different kinds of fruits such as banana, apple, mango, orange, peach, strawberry, papaya, pomegranate, pineapple and watermelon. The flavonol present in apple is myricetin, a hydroxybenzoic acid present in banana is gallic acid, anthocyanin reported in pomegranate are cyanidin and quercetin, hydroxycinnamic

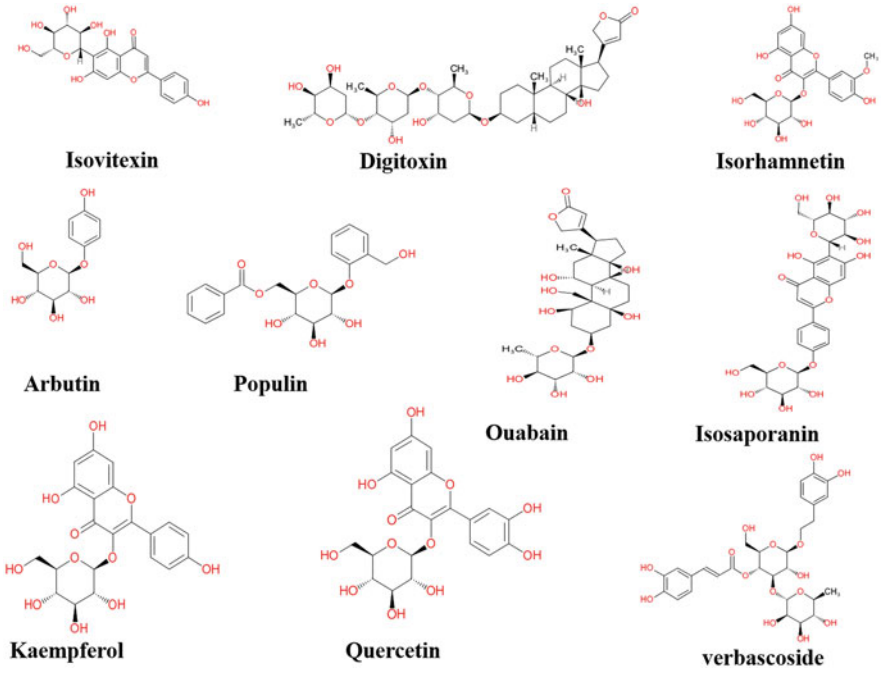

Fig. 1.3 Representative members of glycosides. (Structures drawn using ChemDraw addon to MS Word)

acid found in mango are ferulic acid and apigenin and flavone recorded in pineapple and watermelon is luteolin (Haminiuk et al. 2012).

The presence of an aromatic ring with at least one hydroxyl group is the significance of this category of secondary metabolites. Almost more than 8000 phenolic compounds have been documented to date (Harborne and Baxter 1993). It is quite significant to mention that more than 4000 phenolic compounds are flavonoids presenting as glycosides, aglycone and methylated derivatives. Major classes are isoflavone, anthocyanins, 4-isoflavonoids (flavonols and flavones) and flavan-3-ol derivatives (tannins and catechin). Phenolics and flavonoids were recorded as efficient in antioxidative, anticancer agent, cardioprotective, anti-inflammatory protection for the skin from UV radiations and antibacterial and medical applications (Tungmunnithum et al. 2018; Ullah et al. 2020). Simple and polyphenolic compounds are the two types of phenolic compounds (Harborne and Simmonds 1964; Vuolo et al. 2019; Tsimogiannis and Oreopoulou 2019; Vermerris and Nicholson 2009). Simple phenols are compounds with a single phenol ring with C_6 general skeleton representation. Resorcinol (1,3-dihydroxybenzene), c = catechol (1,2-dihydroxybenzene) and hydroquinone (1,4-dihydroxybenzene) are the examples of simple substituted hydroxy-phenols and dihydroxybenzenes (Fig. 1.4). Hydroxyquinol (1,2,4-trihydroxybenzene), pyrogallol (1,2,3-trihydroxybenzene)

Table 1.4 List of glycosides and their source and biological activity

Source plant	Glycosides	Activity	References
Stevia rebaudiana	Stevioside and Rebaudioside	Non-mutagenic, antimicrobial and antifungal	Yadav and Guleria (2012)
Calluna vulgaris	Arbutin	Skin whitening and prevents pigmentations	Lim et al. (2009)
Bergenia crassifolia	Hydroquinone and pyrogallol, 6-*O*-galloylarbuti	Antidiabetic, skin whitening and anti-inflammatory	Shikov et al. (2014)
Salix acmophylla	Acmophyllin A	Anticancerous	Shah et al. (2016)
Digitalis purpurea	Digitoxin and digoxin	Antitumour, anti-inflammatory and antimicrobial	Negi et al. (2012), Johan (2018)
Populus nigra	Salicin and populin	Anti-cough preparation, stimulant and expectorant	Si et al. (2009)
Filipendula ulmaria	Spirein and isosalicyn	Antimicrobial and antipyretic	Boeckler et al. (2011)
Urginea maritima	Proscillaridin A	Cytotoxic	El-Seedi et al. (2013)
Strophanthus gratus	Ouabain	Antimicrobial	Henneh (2013)
Citrullus colocynthis	Isosaponarin and isovitexin	Antioxidant	Delazar et al. (2006)
Betula papyrifera	Papyriferoside and platyphylloside	Anticancerous	Mshvildadze et al. (2007)
Antiaris toxicaria	Antiaroside P and β-antiarin	Anticancerous	Liu et al. (2013)
Lippia multiflora	Verbascoside, isoverbascoside, nuomioside A and isonuomioside A	Antihypertension, antimalarial and antioxidant	Arthur et al. (2011)
Wedelia calendulacea	19-α-Hydroxy-ursolic acid glucoside	Inhibition of renal tumour	Verma et al. (2017)
Desmidorchis flava	Desmiflavasides C	Cytotoxic	Raees et al. (2015)
Ipomea leptophylla	Jalapin and convolvulin	Antimicrobial	Barnes et al. (2003)
Asclepias subulata	Calotropin and desglucouzarin	Antiproliferative	Rascon Valenzuela et al. (2015)
Plantago psyllium	Verbascoside and plantamajoside	Anti-inflammatory, antibacterial and diuretic	Goncalves and Romano (2016)
Calotropis procera	Quercetin, kaempferol and isorhamnetin	Antimicrobial, anticancer and antiangiogenic	Nenaah (2013), Al-Snafi (2015)

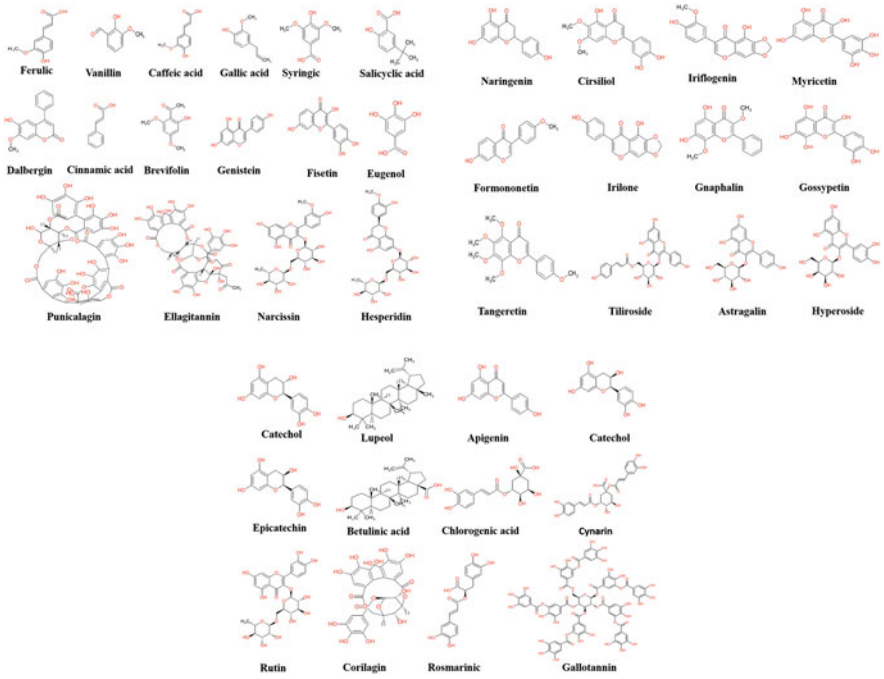

Fig. 1.4 Representative structure of flavonoids and phenolics. (Structures drawn in ChemDraw software addon to MS Office)

and phloroglucinol (1,3,5-trihydroxybenzene) are examples of simple trihydroxybenzene.

The phenols with carboxylic acid are called phenolic acids. Hydroxybenzoic acid is the compound with carboxylic acid directly linked with the phenyl ring. But the separation of carboxylic acid from phenyl ring by C=C bond results in the formation of hydroxycinnamic acids. Caffeic acids, cinnamic acids, ferulic acids and sinapic acids are common examples. Substitution of hydroxyl groups forms one of the benzoic acids called hydroxybenzoic acid. In another way, the substitution of hydroxyl groups with carboxylic acid can form phenolic acids. The substitution of two hydroxyl groups forms dihydroxybenzoic acids and three forms trihydroxybenzoic acids. 3,4,5-Trihydroxy benzoic acids and 2,4,6-trihydroxy benzoic acids are examples of this.

The phenolic compounds with multiple phenol units are considered polyphenols. C15 is the general structural skeleton of polyphenols. Polyphenolic compounds have a C15 general skeleton representation. Flavonoids are a family of naturally occurring polyphenolic chemicals that are distinguished by the flavan nucleus and are found abundantly in fruits, vegetables and plant-derived drinks. Flavonoids are thought to be responsible for the colourful appearance of leaves, flowers and fruits. UV light, diseases and herbivores are all protected by these chemicals in plants (Moon et al. 2006; Erdman et al. 2007). Flavonoids are dietary supplements that promote good

health and prevent illness. In epidemiological, animal-based and clinical studies, it was proved that flavonoids are capable of reducing the risk of several diseases such as cancer and cardiovascular disease. Antiviral, antibacterial and anti-inflammatory properties are also found in flavonoids. Flavonoid consumption is inversely associated with cardiovascular disease mortality, according to population study (Heim et al. 2002; Middleton et al. 2000; Nijveldt et al. 2001; Stangl et al. 2007). The markers of atherosclerosis measurement include blood platelet aggregation, lipoprotein oxidation and vascular reactivity. Flavonoids displayed the beneficial effects of these measures. Besides that, it was reported that the consumption of flavonoids reduced the mortality of cardiovascular disorder-related patients with the aid of its anti-inflammatory, antioxidant, antithrombotic and hypolipidemic activity (Arts and Hollman 2005). Flavonoids consist of pyrene and phenolic rings, and they are the derivatives of the benzo-γ-pyrone compound (Nijveldt et al. 2001). The fundamental structure of flavonoids encompasses the flavonoid skeleton, and various flavonoid compounds are formed by the combination of several methyls and hydroxyl groups on the skeleton. The skeleton contains three six-membered rings. The three rings are heterocyclic C-ring, aromatic B-ring and aromatic A-ring. The fused form of the C-ring and A-ring is bounded to B-ring with a C–C bond. The conjugation in the A- and B-rings and variation in the side group bonds are the major reasons behind flavonoid compound diversity. Flavones, flavanones, flavonols, anthocyanidins and isoflavones are the six categories of flavonoids (Table 1.5).

4 Conclusions and Future Directions

Plants are the storage basket of various metabolites including primary and secondary metabolites. They have various functional importance in the lifespan of both plants and humans. The biochemical and molecular regulations behind the physiological responses of the plant during normal conditions and stress conditions are strongly influenced by these phytochemicals. Among them, secondary metabolites play a key role in the production of flavours, dyes, fibres, glues, oils, waxes, pharmaceutical drugs and fragrances. Many of them were recognised by their pharmacological and pharmaceutical activity that resulted in the development of drugs, antibiotics, insecticides and herbicides from these compounds. Therefore high-throughput screening on the phytochemicals is carried out in the different corners of the world. It is important to understand the structural and functional properties of these compounds for the effective development of drugs and other commercial applications, because the activity of the compound depends on its structure and functional characteristics. Identification of plants and plant families with unique and diversified metabolites for the commercial application can help to develop the research based on them, thereby it can improve the conservation and cultivation status of the plants. With the aid of various metabolomic tools, it is possible to identify, isolate and purify the phytochemicals. The availability of data on whole useful plant species in the globe is very less; hence, it is important to conduct more specific, precise and accurate research on this field.

Table 1.5 Names of phenolics compounds with their source and biological activity

Plant source	Phenolic/flavonoids	Activity	References
Terminalia brownie	Gallic acid, ellagitannin, punicalagin, gallotannin and corilagin	Antibacterial activity	Salih et al. (2017)
Schinopsis brasiliensis	Gallic acid	Analgesic and anti-inflammatory activity	De Souza Santos et al. (2018)
Adiantum capillus	Rutin, quercetin and populnin	Anticancerous, anti-retroviral and antioxidant activity	Al-Snafi (2015)
Agrimonia eupatoria	Astragalin and tiliroside	Antidiabetic, anticancerous, anti-inflammatory, anticancerous, antioxidant, antiulcer and hepatoprotective activity	Riaz et al. (2018), Goto et al. (2012)
Canarium album	Brevifolin and ellagic acid	Hepatoprotective effect and antioxidant activity	Ito et al. (1990)
Cayratia pedata	Quercetin, *O*-coumaric acid and gallic acid	Antiviral, antioxidant activity and cytotoxic effect	Kumar et al. (2013)
Hugonia mystax	Catechol, gallic acid, caffeic acid, *p*-coumaric acid, vanillin and ferulic acid	Antioxidant, anti-inflammatory and anti-rheumatic activity	Pawar and Dasgupta (2018)
Alpinia officinarum	Galangin and alpinin	Aromatic stomachic, analgesic and antiemetic activity	Kaushik et al. (2011)
Althaea officinalis	Kaempferol, isoquercitrin and ferulic acid	Anti-inflammatory and antioxidant	Seifried et al. (2007)
Chloroxylon swietenia	Ferulic acid, quercetin and gallic acid	Antiviral, antioxidant and cytotoxic effect	Enkhtaivan et al. (2015)
Ammi species	Quercetin and kaempferol	Anti-inflammatory activity	Garcia-Mediavilla et al. (2007)
Lafoensia pacari	Ellagic acid	Gastroprotective effect and antiulcerative-gastric hypopoietic effect	Tamashiro et al. (2012)
Caraipa densifolia	Procyanidin trimer C1, procyanidin dimer B2, lupeol, epicatechin and betulinic acid	Chemoprevention	Da Silveira et al. (2010)
Anthemis nobilis	Apigenin, luteolin and quercetin	Antioxidant	Romanova et al. (2001)
Antirrhinum majus	Caffeic acid and chlorogenic acid	Antihypertensive and antioxidant properties	Espindola et al. (2019)
Apium graveolens	Epicatechin	Antihypertensive effect and antioxidant activity	Bernatova (2018)
Onopordum Illyricum	Cynarin	Antioxidant, antiradical and anticholinergic activity	Topal et al. (2016)
	Hyperoside	Induction of apoptosis	Wang et al. (2016)

(continued)

Table 1.5 (continued)

Plant source	Phenolic/flavonoids	Activity	References
Asparagus officinalis			
Cymbopogon schoenanthus	Hyperin	Antihyperglycaemic activity	Verma et al. (2013)
Astragalus hamosus	Astragalin	Antidiabetic, anticancerous, anti-inflammatory, anticancerous and antioxidant	Riaz et al. (2018)
Ballota nigra	Tangeretin	Antiproliferative, anti-invasive and antimetastatic antioxidant activities	Hirano et al. (1995), Martínez Conesa et al. (2005)
Bellis perennis	Apigenin, kaempferol, isorhamnetin, ferulic, sinapic, *p*-coumaric and salicylic acids	Expectorant, diuretic and anti-inflammatory activity	Schopke and Hiller (2006), Nazaruk and Gudej (2001), Grabias et al. (1995)
Cicer arietinum	Genistein	Anticancerous activity	Suthar et al. (2001)
Amburana cearensis	Vannilic	Anti-inflammatory activity	Khadem and Marles (2010)
Coriandrum sativum	Glycitin, caffeic acid and protocatechuic acid	Anti-inflammatory, anti-oxidant and anti-carcinogenic activity	Espindola et al. (2019), Kakkar and Bais (2014)
Cymbopogon schoenanthus	Tricin, luteferol and apigiferol	Antioxidant, acetylcholinesterase inhibitory activity and antimicrobial effect	Al-Snafi (2016)
Dalbergia sissoo	Biochanin, dalbergenone and dalbergin	Anti-osteoporotic, neuroprotective, antimicrobial, hepatoprotective and antibacterial activity	Kumar et al. (2014), Sarfraz et al. (2020)
Daphne mucronata	Cinnamic acid	Antidiabetic activity	Ruwizhi and Aderibigbe (2020)
Fraxinus ornus	Flavonoids and apigenin	Anticancerous and antidiabetic activity. Effective against Alzheimer's disease and depression	Salehi et al. (2019a, b)
Galium verum	Isorhamnetin and ferulic acid	Antioxidant, anti-inflammatory, anticancer, antimicrobial and antidiabetic activity	Zdunska et al. (2018), Antunes-Ricardo et al. (2015)
Hedera helix	Rosmarinic	Antioxidant, antiviral, anti-inflammatory, photoprotective, immunomodulatory and anti-Alzheimer's effect	Pérez-Tortosa et al. (2012), Swarup et al. (2007), Psotova et al. (2006), Alkam et al. (2007)
Ononis spinosa	Formononetin	Anticancerous activity	Tay et al. (2019)

(continued)

Table 1.5 (continued)

Plant source	Phenolic/flavonoids	Activity	References
Lippia nodiflora	Cirsiliol	Anticancerous activity	Prasad et al. (2019)
Lawsonia inermis	Apigenin and luteolin	Anticancerous activity	Liu et al. (2019), Lin et al. (2008)
Lallemantia iberica	Rosmarinic acid	Anti-ageing effects, antioxidant, antidepressant, antimicrobial and anti-inflammatory activity	Nadeem et al. (2019)
Juniperus communis	Naringenin	Cardioprotective effect, antitumor, antioxidant, antiviral, anti-inflammatory, antiadipogenic and antibacterial activity	Salehi et al. (2019a, b)
Juglans regia	Ellagic, syringic and *p*-coumaric	Anti-inflammatory, neuroprotective effect, anti-atherogenic and neuroprotective effects	Rios et al. (2018), Cao et al. (2016)
Iris pallid	Irilone and iriflogenin	Cancer chemopreventive activity	Wollenweber et al. (2003)
Hibiscus sabdariffa	Myricetin, eugenol and gossypetin	Antidiabetic, antioxidant, anticancerous, induction of apoptosis and ROS (reactive oxygen species) scavenger	Yi et al. (2015), Khan et al. (2013)
Hedera helix	Astragalin	Antidiabetic, anticancerous and anti-inflammatory	Riaz et al. (2018)
Gnaphalium luteoalbum/ *Artemisia* spp.	Jaceosidin and gnaphalin	Anticancerous and anti-proliferative	Nageen et al. (2021), Torrenegra Guerrero et al. (2018)
Glycyrrhiza glabra	Licoflavonol	Inhibitor of *Salmonella* T3SS	Guo et al. (2016)
Galium verum	Hesperidin, fisetin and chrysin	Antidiabetic, antioxidant, neuroprotective, anticancer and antidepressant activity	Ahmad et al. (2012), Yang et al. (2012), Sassi et al. (2017)
Allophylus africanus	Apigenin, luteolin, vitexin, apigetrin and cynaroside	Anti-inflammatory	Ferreres et al. (2018)
Trichilia catigua	Procyanidin, catechin, cinchonain I, epicatechin, apocynin e and 3-methoxybenzoylquinic acid	Neuroprotective, antioxidant, antidepressant and anti-inflammatory activity	Bernardo et al. (2018)
Anogeissus leiocarpa	Gallic acid, ellagitannin and ampelopsin	Antibacterial activity	Salih et al. (2017)

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Chapter 2
Phytochemically Rich Medicinally Important Plant Families

Himanshi Gangwar, Priya Kumari, and Vandana Jaiswal

1 Introduction

Phytochemicals are a vast variety of chemical entities found in plant parts such as seeds, leaves, stems and roots (Saxena et al. 2013; Forni et al. 2019). They play a protective role during stress caused by the environment while also contributing to the plant's colour, scent and flavour (Mathai 2000). The most important phytochemicals are saponins, alkaloids, phenolics, terpenoids and flavonoids (Velu et al. 2018). Plants have a natural ability to synthesize these phytochemicals, and their characterization has resulted in the discovery of novel, low-cost medications and great potential for therapeutic use (Ukwuani et al. 2013). They have a variety of biological activities, like antioxidant activity, anti-inflammatory activity, antimicrobial activity and anticancerous activity; phytochemicals are used therapeutically (Aye et al. 2019). Phenols are reported for various biological functions including scavenging activity, antiulcer, antidepressant activities and antitumour (Silva et al. 2007; Ghasemzadeh et al. 2010). Saponins possess antifungal activities and haemolytic, antiprotozoal and antiviral plant defence activities (Sarikahya et al. 2018). Alkaloids are known to have antimalarial, antioxidant, antimicrobial, antiplatelet aggregation, cytotoxic and anti-inflammatory activities (Chan et al. 2010; Bissim et al. 2019), whereas terpenoids have antibacterial, antiallergic, antioxidant, antimalarial, anticancer and antiviral activities (Yang et al. 2020). Some naturally occurring phytochemicals are reported as a potential inhibitor against coronavirus (SARS-CoV-2) including saikosaponin B2, silvestrol, lectins, tryptanthrin, quercetin and isobavachalcone (Mani et al. 2020). This book chapter includes information about phytochemical extraction, biosynthesis and their biological activities in some

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M. K. Swamy, A. Kumar (eds.), *Phytochemical Genomics*,
https://doi.org/10.1007/978-981-19-5779-6_2

important plant families including Amaranthaceae, Apiaceae, Cucurbitaceae, Lamiaceae and Lauraceae.

2 Extraction of Phytochemicals

The phytochemical extraction process includes tissue preparation and then extraction from leaves, barks, roots, etc. (Vongsak et al. 2013). The extraction of secondary metabolites from plant tissue utilizes different polar solvents (Pandey and Tripathi 2014). Conventional versus non-conventional extraction methods are used for this process. A conventional extraction method includes soxhlet extraction, maceration and hydrodistillation. A non-conventional extraction method includes supercritical fluid extraction, accelerated solvent extraction, ultrasound-assisted extraction and microwave-assisted extraction. A non-conventional method takes lesser time and is more advanced than the conventional method (Wen et al. 2018). After extraction, the next step is to be phytochemical screening that confirms the presence of different phytochemicals. The phytochemical screening process is conducted by both qualitative and quantitative techniques. Qualitative methods include a different chemical test for different phytochemicals such as Mayer's test, copper acetate test, alkaline reagent test and foam test for alkaloids, diterpenes, flavonoids and saponins, respectively. Quantitative techniques include different types of chromatography methods such as liquid, gas and thin layer chromatography for the determination of phytochemicals (De Silva et al. 2017). After the extraction and screening process, several spectroscopy techniques have been used for the recognition of phytochemicals (Ingle et al. 2017). The whole process of extraction is summarized in Fig. 2.1.

3 Biosynthesis of Phytochemicals

Primary and secondary metabolites are two types of compounds synthesized by plants. Primary metabolites (proteins, lipids, etc.) regulate the maturation of plants (Batra and Sharama 2013). Secondary metabolites are bioactive phytochemicals produced by the plant to protect itself. Since the plants are fixed to their roots and they cannot escape the stress episodes, therefore they adopt a few necessary ways to cope with the challenges like in response to specific environmental stresses. Secondary metabolites are produced both in the vegetative and reproductive tissues of the plant (Kennedy and Wightman 2011). Phytochemicals are of three types that include phenolic compounds, terpenes and alkaloids. The core component of such phytochemical comprises shikimate, acetyl coenzyme A and 1-deoxyxylulose-5-phosphate (Croteau et al. 2000). The first and the largest group of phytochemicals is a terpene, which is derived biosynthetically from units of isoprene. Isoprene units are divided into hemiterpenes, monoterpenes, sesquiterpenes and diterpenes based upon

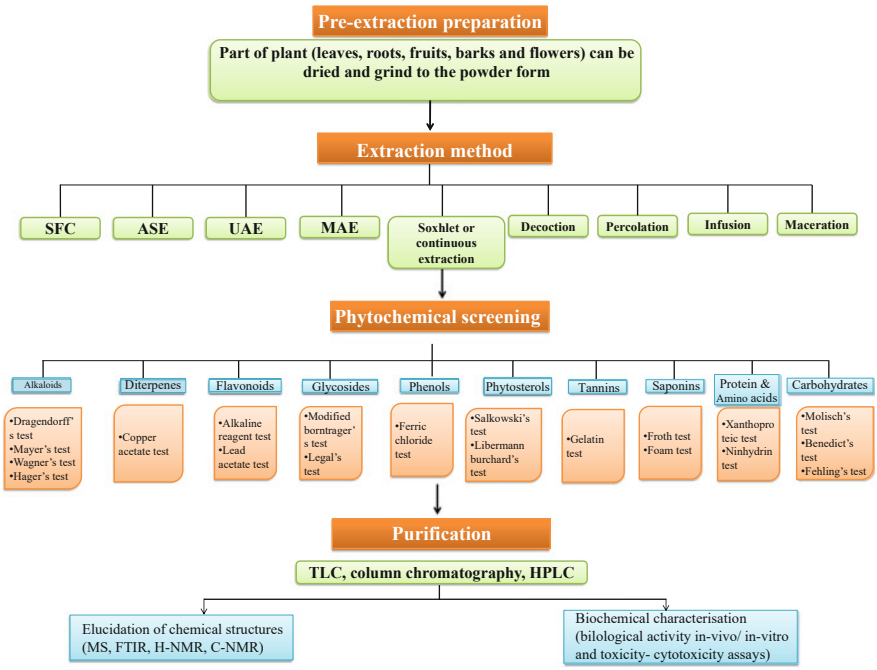

Fig. 2.1 Flow chart of extraction protocol of phytochemicals (*SFC* supercritical fluid extraction, *ASE* accelerated solvent extraction, *MAE* microwave-assisted extraction, *UAE* ultrasound-assisted extraction, *MS* mass spectrometry, *FTIR* Fourier-transform infrared spectroscopy, *NMR* nuclear magnetic resonance spectroscopy)

the addition of carbon atoms (Kennedy and Wightman 2011). Terpenes are made in two ways: the mevalonate pathway and the methylerythritol phosphate method. These two pathways are completed in two separate organelles, the cytosol and the plastid, respectively. The MEP (methylerythritol 4-phosphate) pathway produces monoterpenes, diterpenes and tetraterpenes, whereas the MVA pathway produces sesquiterpenes and triterpenes. The basic building blocks of these two processes—the high molecular weight (complex) terpenes, that is, isopentyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP)—are produced by the activity of enzyme prenyltransferase (Gutzeit and Ludwig 2014) (Fig. 2.2). Alkaloids are the second biggest group of phytochemicals. They include more than 15,000 secondary metabolites that are soluble in water, have at least one nitrogen atom in their structure and exhibit biological activities (Rodriguez-Garcia et al. 2017). The third major group is phenolic compounds; these compounds are synthesized through the shikimate pathway, and the precursor of this pathway is L-phenylalanine and L-tyrosine (Fresco et al. 2006). The above aromatic amino acids are responsible for synthesizing the major aromatic phenolic compounds that include lignans, coumarins, flavonoids, aromatic polyketides and so on (Cheynier et al. 2013).

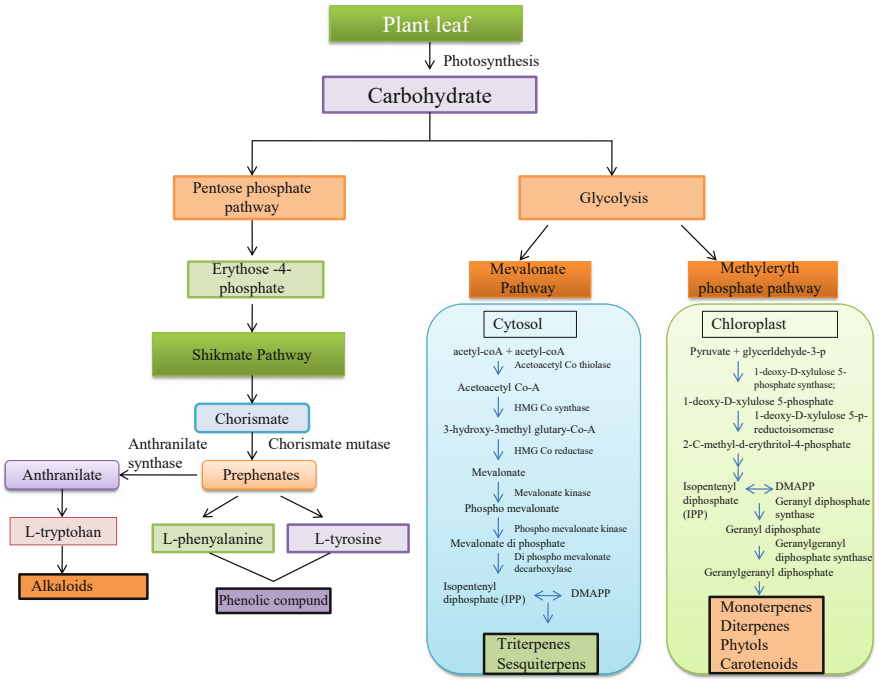

Fig. 2.2 Schematic view of phytochemical biosynthesis. (Modified after Dubey et al. (2003) and Gutzeit and Ludwig (2014))

4 Phytochemically Rich Plant Families and Their Important Biological Activities

A wide range of plant species have been well known to possess numerous medicinally valued bioactive compounds. The list of phytochemicals obtained from the different plants of the important families is summarized in Table 2.1, and the biological activities of the various plant species are described in Table 2.2.

4.1 Amaranthaceae

The Amaranthaceae family incorporates over 175 genera with 2000 species making it an enriched species lineage in Caryophyllales angiosperm order (Müller and Borsch 2005; Simpson 2010). This family includes annual and perennial plants and it includes majorly herbs and shrubs (Simpson 2010). This family is grown widely across the world (Kadereit et al. 2003). The phytochemicals of the Amaranthaceae family include terpenes, phenolic acids, flavonoids, betalains and

Table 2.1 List of phytochemicals of the following plant species

Plant family	Plant species	Plant tissue	Phytochemical	References
Amaranthaceae	*Chenopodium ambrosioides*	Leaves	Stigmasterol, β-sitosterol, octadecanoic acid, scopoletin, 1-piperoylpiperidine, *p*-coumaroyl pentoside acid, *p*-coumaroyl acid derivative, feruloyl pentoside acid, ferulic acid derivative, luteolin C-hexoside-*O*-pentoside, quercetin *O*-pentosyl-hexoside, kaempferol *O*-dirhamnosyl-hexoside, quercetin dirhamnoside, isorhamnetin dirhamnoside, isorhamnetin *O*-rhamnosyl-pentoside, quercetin *O*-rhamnosyl-glucuronide, kaempferol *O*-rhamnosyl-glucuronide and α-tocopherol	Shah and Khan (2017), Barros et al. (2013)
Apiaceae	*Angelica glauca*	Root	(*E*)-Butylidene phthalide, α-pinene, carvone, alloaromadendrene, camphene, (*E*)-ligustilide, spathulenol, linalool, *p*-cymene, (*Z*)-ligustilide, sabinene, b-bisabolene, (*Z*)-butylidene phthalide, limonene, b-elemene, *Cis*-ocimene, b-eudesmol, Myrcene, a-cadinol, terpinen-4-ol, carveol, α-phellandrene, c-terpene, borneol, b-caryophyllene, epi-a-cadinol and b-phellandrene	Rawat et al. (2018)
	Bunium persicum	Tubers	α-Thujene, *trans*-caryophyllene, *cis*-sabinene hydrate acetate, α-pinene, phellandral, sabinene, n-hexadecanoic acid, β-pinene, myrtenyl acetate, linalool, limonene oxide, β-myrcene, fenchone, α-terpinolene, α-thujone, α-bisabolol, 2-methyl-1-hepten-6-yn-3-ol, 3-cyclohexen-1-ol, α-terpinolene, 1-phellandrene, 3-menthene and γ -terpene	Chahota et al. (2017)

(continued)

Table 2.1 (continued)

Plant family	Plant species	Plant tissue	Phytochemical	References
	Centella asiatica	Leaves	Madecassoside, menthone, γ-curcumene, asiaticoside, germacrene A, neophytadiene, viridiflorol, β-pinene, chlorogenic acid, γ-terpinene, caryophyllene oxide, α-thujene, δ-cadinene, α-terpinene, linalool, 3-nonen-2-one, *p*-cymene, mintsulfide, β-elemene, phellandrene, madecassic acid, β-caryophyllene and α-pinene	Alqahtani et al. (2015), Oyedeji and Afolayan (2005)
Cucurbitaceae	*Momordica charantia*	Fruit and leaves	Campesterol, palmitic, β-sitosterol, stearic, stigmasterol, Δ5-avenasterol and 25,26-dihydroelasterol, 3-[(5-formyl-7β,25-dihydroxymethoxycucurbita-5,23-dien-3-yl)-oxy]-3-oxopropanoic acid, myricetin, β-sitosterol, catechin, 25-ξ-isopropenylchole-5,(6)-ene-3-O-β-D-glucopyranoside, caffeic and *p*-coumaric	de Oliveira et al. (2018)
	Citrullus lanatus	Fruit	Citrulline, protocatechuic acid glucoside I, phloroglucinol glucuronide, salicylic acid-*O*-hexoside I, vanillin hexoside I, tri-*O*-caffeoylshikimic acid I, ferulic acid hexoside I, 3-*O*-feruloylsucrose, kaempferol rhamnoside-hexoside I, isorhamnetin, quercetin 3-rutinoside, taxifolin dihexoside and luteolin 6-*C*-glucoside	Abu-Reidah et al. (2013)
Lauraceae	*Cinnamomum camphora*	Leaves	Oleanolic acid, β-sitosterol, daucosterol, tricosanoic acid, dimethylmatairesinol, luteolin, luteolin-7-*O*-β-D-glucoside and tricetin-7-methyl ether	Wu et al. (2019)

(continued)

Table 2.1 (continued)

Plant family	Plant species	Plant tissue	Phytochemical	References
	Cinnamomum glaucescens	Leaves	γ-Terpinene, α-pinene, linalool oxide, β-myrcene, α-thujene, linalool, sabinene, α-terpinolene, α-phellandrene, (*E*)-β-ocimene, δ-cadinene, allo-ocimene, α-muurolene, α-terpineol, α-gurjunene, α-amorphene, verbenone, selina-4(15), 7(11)-diene, calacorene, elemol, (*E*)-nerolidol, spathulenol, α-guaiol, β-oplopenone, caryophyllenol, β-eudesmol, α-cadinol, *cis*-a-santalol, valerenol, b-santalol, farnesol and benzyl benzoate	Chinh et al. (2017)
	Neolitsea pallens	Leaves, bark and fruit	α-Thujene, β-caryophyllene, β-phellandrene, α-pinene, 1-epi-cubenol, α-terpinene, γ-terpinene, myrcene, α-selinene, *p*-cymene, sabinene, germacrene D, α-phellandrene, *cis*-sabinene hydrate, β-pinene, δ-cadinene, camphene, bornyl acetate, α-ylangene, curcumenol and terpinolene	Padalia et al. (2007)
	Persea duthiei	Leaves	α-Bisabolol, *p*-cymene, α-pinene, limonene, epi-α-cadinol, myrcene, α-copaene, spathulenol, β-pinene, δ-elemene, camphene, β-eudesmol, terpinolene, caryophyllene oxide, linalool and α-terpineol β-selinene, α-Cubebene	Padalia et al. (2009)
	Persea odoratissima	Leaves	Heptanone-2-methyl-4,4-methyl pentanoic acid, Heptene-1-ol-2*E*, Sabinene, Decane-n, Terpinene-α, Methylheptane, terpinene-γ, pentylisobutanoate, mentha-2,4-diene, isopentyl butanoate, sabinene hydrate-*cis*, sabinene-*cis*, undecane, pinocamphone-*trans*, verbenyl acetate, decen-1-ol, pentyl butyrate, tridecane-n, tetradecane-n, α-copaene,	Pant et al. (2018)

(continued)

Table 2.1 (continued)

Plant family	Plant species	Plant tissue	Phytochemical	References
			germacrene-D, bisabolene-E, curcumene, hexenyl benzoate, sesquithuriferol, cubenol-1-epi, sesquilavandulol-E, β-selinene, geranyl valerate, bisabolol-n, heptadecane-n, sesquicineol-2-one, tridecanol acetate, bulnesol, rosifoliol, farnesol, bisabolol acetate, hexadecane, octadecane, isopropyl myristate, cryptomeridial, nonadecane, geranyl linalool, palmitic acid and phytol	
Lamiaceae	*Ocimum basilicum*	Leaves	Eugenol, β-pinene, α-humulene, α-bergamotene, spathulenol, limonene, terpinen-4-ol, γ-cadinene, caryophyllene, β-elemene, alloaromadendrene, α-cadinol, calamenene, linalool, carvone, bornyl acetate, methyl eugenol, α-amorphene, 1,8-cineole and α-bisabolene	Politeo et al. (2007)
	Origanum vulgare	Leaves	α-Thujene, β-pinene, δ-2-carene, β-phellandrene, terpinolene, *p*-cymene, caryophyllene, α-humulene, α-muurolene, β-farnesene, β-bisabolene, δ-cadinene, 1-octen-3-ol, bornyl acetate, terpinen-4-ol, methylthymol ether, 2-isopropyl-1-methoxy-4-methyl benzene, borneol, γ-terpinene, *p*-cymen-8-ol, thymol, carvacrol and sabinene	Jerković et al. (2001)
	Perovskia abrotanoides	Leaves	α-Pinene, sabinene, β-pinene, β-myrcene, 1,8-cineole, camphor, borneol, I-bornyl acetate, endo-bornyl acetate, δ-3-carene, *trans*-caryophyllene, α-humulene, δ-cadinene, α-cadinol and camphene	Ghaffari et al. (2018)
	Hyssopus officinalis	Leaves	Apigenin 7-*O*-β-D-glucuronide, myrtenyl acetate, camphor, germacrene, spathulenol, L-linalool, 1,8-cineole, *cis*-sabinol,	Fathiazad and Hamedeyazdan (2011), Tahir et al. (2018)

(continued)

Table 2.1 (continued)

Plant family	Plant species	Plant tissue	Phytochemical	References
			terpineol-4, myrtenal, bornyl acetate, α-cubebene, β-bourbonene, β-caryophyllene, α-caryophyllene, germacrene, cubenol, d-cadinene, nerodiols Z and E, caryophyllene oxide and elemol	
	Thymus linearis	Leaves	Sabinene, γ-cadinene, α-thujene, β-bourbonene, α-phellandrene, camphene, α-humulene, δ-3-carene, α-pinene, germacrene-D-4-ol, borneol, α-terpinene, carvacrol methyl ether, β-pinene, β-phellandrene, β-myrcene, α-terpinolene, α-terpinene, δ-cadinene, *p*-cymene and benzaldehyde	Verma et al. (2010)
	Thymus serpyllum	Leaves	α-pinene, caryophyllene, β-myrcene, α-humulene, Δ-cadinene, *p*-cymene, benzyl alcohol, thymol, γ-terpinene, carvacrol and α-terpinene	Kulisic et al. (2005)

essential oils. Amaranthaceae plants have a high concentration of triterpene saponins (Vincken et al. 2007).

4.1.1 Antioxidant Activity

Oxidation within the cell conducts normal and physiological processes. The free radicals produced by the oxidation step include superoxide and peroxide and then react with singlet electrons and form reactive oxygen species (ROS). The ROS are extremely reactive and unstable; those are well characterized to extract electrons from nucleic acids, proteins and fatty acids (Ishtiaq et al. 2014). The inactive ROS's conductive pathways include both enzymatic and non-enzymatic antioxidant systems.

Amaranth is considered as a viable antioxidant source (Lopez-Mejía et al. 2014). *Amaranthus spinosus*, often known as pigweed or spiny amaranth, are weeds grown widely in cultivated and fallow regions throughout India (Kumar et al. 2014). *A. spinosus* has been studied for its superoxide anion scavenging activity, ferric reducing antioxidant capacity and DPPH radical scavenging activity. Many studies have used contrasting sections of the plant and different extraction solvents to assess

Table 2.2 The biological activities several plant species belonging to diverse families

Biological activity	Plant family	Plant species	References
Antioxidant activity	Amaranthaceae	*Amaranthus spinosus*	Barku et al. (2013)
		Amaranthus viridis	Ahmed et al. (2013)
		Amaranthus graecizans	Ishtiaq et al. (2014)
		Amaranthus hybridus	Eseyin et al. (2015)
		Cornulaca monacantha	Mhiri et al. (2020)
		Amaranthus caudatus	Karamać et al. (2019)
		Amaranthus cruentus	Li et al. (2008)
		Amaranthus hypochondriacus	Lopez-Mejía et al. (2014)
		Amaranthus graecizans	Ishtiaq et al. (2014)
	Apiaceae	*Centella asiatica*	Kormin (2005)
		Meum athamanticum	Palá-Paúl et al. (2004)
		Cuminum cyminum and *Coriandrum sativum*	Demir and Korukluoglu (2020)
		Anthriscus cerefolium and *Anthriscus sylvestris*	Maleki Lajayer et al. (2020)
		Elaeosticta allioides, *Ferula clematidifolia*, *Hyalolaena intermedia* and *Elaeosticta polycarpa*	Jamalova et al. (2021)
		Angelica sylvestris and *Angelica pancicii*	Stanković et al. (2020)
	Cucurbitaceae	*Cucumis melo*	Vella et al. (2019)
		Benincasa hispida, *Momordica charantia*, *Trichosanthes cucumerina* and *Cucumis metuliferus*	Busuioc et al. (2020)
		Bryonia dioica	Bourhia et al. (2020)
		Lagenaria siceraria and *Luffa cylindrica*	Irshad et al. (2014)
		Cucurbita maxima, *Benincasa hispida*, *Citrullus lanatus* and *Cucumis melo*	Singh et al. (2016)
		Ibervillea sonorae	Estrada-Zúñiga et al. (2012)
		Cucurbita maxima, *Trichosanthes cucumerina*, *Lagenaria siceraria*, *Benincasa hispida*, *Momordica charantia* and *Luffa acutangula*	Sulaiman et al. (2013)
	Lamiaceae	*Satureja montana*	Zeljković et al. (2015)
		Thymus zygioides, *Origanum onites*, *Lamium purpureum*, *Salvia virgate* and *Salvia sclarea*	Ekin et al. (2019)

(continued)

Table 2.2 (continued)

Biological activity	Plant family	Plant species	References
		Plectranthus madagascariensis	Matias et al. (2019)
		Mentha × *rotundifolia*	Yahia et al. (2019)
		Satureja macrostema	Alonso-Carrillo et al. (2017)
		Premna latifolia	Ruwali and Negi (2019)
		Dracocephalum kotschyi	Khodaei et al. (2019)
		Lophanthus anisatus, *Satureja hortensis*, *Monarda fistulosa* and *Ocimum americanum*	Shanaida et al. (2018)
		Satureja thymbra	Tsimogiannis et al. (2017)
		Melissa officinalis, *Rosmarinus officinalis* and *Salvia officinalis*	Cocan et al. (2018)
		Marrubium astracanicum	Bursal et al. (2019)
	Lauraceae	*Ocotea notata*	Pereira et al. (2020)
		Cinnamomum triplinerve	Silva et al. (2019)
		Ocotea nutans	Betim et al. (2019)
		Aniba canelilla	Souza-Junior et al. (2020)
		Actinodaphne angustifolia	Uddin et al. (2020)
		Laurus nobilis	Guedri et al. (2020)
		Ocotea bicolor	Damasceno et al. (2017)
		Cinnamomum altissimum	Abdelwahab et al. (2017)
Anti-inflammatory	Amaranthaceae	*Cyathula prostrata*	Ibrahim et al. (2012)
		Alternanthera tenella	Biella et al. (2008)
		Gomphrena celosioides	Adeoti et al. (2016)
		Achyranthes aspera	Mengie et al. (2021)
		Climacoptera cristata	Wu et al. (2011)
		Anabasis setifera	Abdou et al. (2013)
		Climacoptera obtusifolia	Yeskaliyeva et al. (2006)
		Aerva monsoniae	Sandhya et al. (2012)
		Gomphrena virgata	Marinho et al. (2021)
	Apiaceae	*Bupleurum marginatum*	Ashour et al. (2018)
		Seseli resinosum, *Seseli gummiferum* and *Seseli petraeum*	Küpeli et al. (2006)
		Ridolfia segetum	Cabral et al. (2015)
		Alepidea amatymbica and *Alepidea natalensis*	Mulaudzi et al. (2009)

(continued)

Table 2.2 (continued)

Biological activity	Plant family	Plant species	References
		Pleurospermum candollei	Ali et al. (2021)
		Anthriscus sylvestris	Velescu et al. (2017)
		Hydrocotyle javanica	Krithika and Arumugasamy (2018)
	Cucurbitaceae	*Lagenaria breviflora*	Adedapo et al. (2013)
		Cucumis melo var. cantalupensis Cucumis melo var. reticulatus	Ezzat et al. (2019)
		Mukia maderaspatana	Salehi et al. (2019)
		Telfairia occidentalis	Eseyin et al. (2014)
		Trichosanthes cucumerina	Arawwawala et al. (2010)
		Zehneria scabra	Belay and Makonnen (2020)
		Momordica charantia	Bortolotti et al. (2019)
		Citrullus colocynthis	Sagar et al. (2021)
		Cucumis sativus	Nasrin et al. (2015)
		Momordica balsamina	Rajasree et al. (2016)
	Lamiaceae	*Salvia tiliifolia*	González-Chávez et al. (2018)
		Thymus vulgaris	Boukhatem et al. (2020)
		Glechoma hederacea	Chou et al. (2018)
		Melissa officinalis and *Origanum majorana*	Villalva et al. (2021)
		Salvia ceratophylla, *Salvia chloroleuca*, *Salvia fruticosa* and *Salvia chostachys*	Bonesi et al. (2017)
		Ocimum basilicum	Okoye-Festus et al. (2014)
		Vitex negundo	Dhanokar et al. (2020)
	Lauraceae	*Aniba riparia*	Vidal et al. (2020)
		Cinnamomum zeylanicum	Atsamo et al. (2021)
		Ocotea diospyrifolia	Silva et al. (2021)
		Nectandra megapotamica	Costa et al. (2019)
		Lindera sericea	Devkota et al. (2021)
		Persea americana	Ashande et al. (2019)

(continued)

Table 2.2 (continued)

Biological activity	Plant family	Plant species	References
Antimicrobial activity	Amaranthaceae	*Gomphrena globosa*	Arcanjo et al. (2011)
		Alternanthera brasiliana	Coutinho et al. (2018)
		Amaranthus caudatus	Jimoh et al. (2020)
		Amaranthus viridis	Ahmed et al. (2013)
		Digera muricata	Mathad and Mety (2010)
		Amaranthus spinosus, *Amaranthus hybridus* and *Amaranthus caudatus*	Maiyo et al. (2010)
	Apiaceae	*Prangos asperula*, *Smyrnium olusatrum* and *Daucus carota*	Khoury et al. (2018)
		Eryngium caucasicum and *Elaeosticta glaucescens*	Hamedi et al. (2019)
		Heracleum dulce, *Seseli devenyense* and *Seseli libanotis*	Widelski et al. (2021)
		Conium maculatum	Di Napoli et al. (2019)
		Coriandrum sativum	Handayani et al. (2019)
		Pituranthos scoparius	Ksouri et al. (2017)
		Ammodaucus leucotrichus	Naima et al. (2019)
		Ferula clematidifolia, *Elaeosticta allioides* and *Elaeosticta polycarpa*	Jamalova et al. (2021)
		Cuminum cyminum and *Ammodaucus leucotrichus*	Hajib et al. (2020)
		Ferula tingitana	Elghwaji et al. (2017)
	Cucurbitaceae	*Momordica charantia*, *Cucumis sativa*, *Praecitrullus fistulosus* and *Cucurbita pepo*	Sood et al. (2012)
		Coccinia grandis	Bhattacharya et al. (2010)
		Citrullus colocynthis	Gurudeeban et al. (2010)
		Trichosanthes cucumerina	Patel et al. (2013)
		Cucumis anguria	Francis et al. (2014)
		Cucurbita pepo	Noumedem et al. (2013)
		Cucurbita moschata	Abed El-Aziz and Abed El-Aziz (2011)
		Cucurbita maxima	Kabbashi et al. (2014)
		Cucumis metuliferus	Usman et al. (2014)

(continued)

Table 2.2 (continued)

Biological activity	Plant family	Plant species	References
	Lamiaceae	*Salvia sclarea*, *Monarda didyma*, *Thymus pulegioides*, *Thymus vulgaris* and *Thymus serpyllum*	Shanaida et al. (2021)
		Lavandula angustifolia, *Origanum majorana*, *Salvia officinalis* and *Thymus vulgaris*	Kot et al. (2019)
		Coridothymus capitatus	Marino et al. (2020)
		Micromeria frivaldszkyana	Mladenova et al. (2021)
		Hyptis suaveolens	Chung et al. (2020)
		Dracocephalum kotschyi	Ghavam et al. (2021)
		Mentha spicata	Bardaweel et al. (2018)
	Lauraceae	*Litsea glutinosa*	Lagudu and Owk (2018)
		Laurus nobilis	Guedri et al. (2020)
		Persea major	Volpato et al. (2017)
		Persia americana	Makopa et al. (2020)
		Aniba panurensis	da Silva et al. (2021)
		Ocotea zahamenensis	Nomentsoa et al. (2021)
		Ocotea comorensis	Ibrahim et al. (2021)
Anticancer activity	Amaranthaceae	*Aerva sanguinolenta*	Lalee et al. (2012)
		Celosia argentea	Rub et al. (2016)
		Salsola oppositifolia	Tundis et al. (2014)
		Amaranthus dubius, *Amaranthus spinosus*, *Amaranthus tricolor* and *Amaranthus viridis*	House et al. (2020)
		Pupalia lappacea	Ravi et al. (2012)
		Aerva lanata	Rajesh et al. (2011)
		Chenopodium album	Rana et al. (2020)
		Achyranthes aspera	Omidiani et al. (2020)
	Apiaceae	*Smyrnium olusatrum*	Maggi et al. (2012)
		Foeniculum vulgare, *Coriandrum sativum*, *Apium graveolens*, *Anethum graveolens* and *Petroselinum neapolitanum*	Gomaa et al. (2020)
		Ferula narthex	Alam et al. (2016)

(continued)

Table 2.2 (continued)

Biological activity	Plant family	Plant species	References
		Athamanta sicula	Di Stefano et al. (2011)
		Ammodaucus leucotrichus	Naima et al. (2019)
		Trachyspermum ammi	Abdel-Hameed et al. (2014)
		Ferula szowitziana	Aas et al. (2015)
	Cucurbitaceae	*Coccinia grandis*	Bhattacharya et al. (2011)
		Citrullus lanatus	Wehner and Maynard (2003)
		Cucurbita andreana	Jayaprakasam et al. (2003)
		Lagenaria siceraria	Kumar et al. (2012)
		Momordica charantia	Güneş et al. (2019)
		Cucumis melo	Ittiyavirah et al. (2013)
		Telfairia occidentalis	Eseyin et al. (2014)
		Trichosanthes tricuspidata	Saboo et al. (2013)

its antioxidant activity (Adegbola et al. 2020). The root tissues of *A. spinosus* contain high phenolic compounds that resulted in the antioxidant activity of this species (Barku et al. 2013). The leaves of *A. spinosus* were found to have antioxidant activity, and it was confirmed by an experiment by using different methanol, chloroform and ethyl acetate fractions, and the maximum antioxidant activity was found in the methanol fraction and the least antioxidant activity in the ethyl acetate fraction (Bulbul et al. 2011). Seeds of *A. spinosus* also have great antioxidant potential (Rjeibi et al. 2016).

4.1.2 Anti-inflammatory Activity

Amaranthaceae plants have a high concentration of triterpene saponins. The aglycone backbone of the saponins divided it into two major groups (Vincken et al. 2007). The most interesting from a medicinal standpoint are triterpenoid saponins, which are made up of a triterpenoid aglycone with a pentacyclic C_{30} structure. The use of saponins from the Amaranthaceae family could help treat a variety of disorders caused by high levels of nitric oxide, which promote inflammation, carcinogenesis and atherosclerosis. Quinoa saponin fractions have been reported to suppress the release of inflammatory chemicals and decreased the generation of inflammatory mediator nitric oxide (Yao et al. 2014). *Climacoptera cristata* seeds have an anti-inflammatory function due to the presence of celosin compounds that significantly inhibited nitric oxide production. Celosin G was discovered to be the

most active molecule against inhibition (Wu et al. 2011). *Anabasis setifera* Moq. was fractionated to assess its anti-inflammatory potential, which yielded α-amyrin 3-*O*-glucopyranoside and sophradiol as cyclooxygenase inhibitors. COX-1 and COX-2 were efficiently suppressed by *Anabasis setifera* oleanane saponin (Abdou et al. 2013).

4.1.3 Antiprotozoal Activity

In vitro and in vivo assays showed analgesic and antimicrobial properties in some species of genus *Alternanthera* (Trapp et al. 2015). Crude extracts of *A. littoralis* are highly rich in flavonoids, chemicals linked to antibacterial and scavenging activity (Salvador and Dias 2004). The ethanolic extract of *A. littoralis* leaves generated seven chemicals, including five novel alkaloids. Such extracts possess significant trypanocidal and leishmanicidal activity. Other alkaloids include hydroxytyrosol uridine and alternamide A (Koolen et al. 2017).

4.1.4 Anticancer Properties

The medicinal plant *Achyranthes aspera* is a popular anticancer agent that has been practised from the traditional medicine age to millennia (Bagavan et al. 2008). The anti-proliferative activities are reported in leaves. It was observed that when the cancer cell line is treated with methanol leaf extract, it reduced the expression of metalloproteases and VEGFs, and these two proteins play a major role in tumour formation which confirms its anticancer activity. These cancer lines treated with methanol leaf extract failed to develop colonies in cell survival and emphasizing its anti-proliferative activity (Subbarayan et al. 2010).

4.2 Apiaceae

The Apiaceae family contains approximately 3780 species in 434 genera predominantly. It is found in tropical and temperate regions (Sayed-Ahmad et al. 2017). Coumarins (Abd El Razek et al. 2001), terpenoids, polyacetylenes (Christensen and Brandt 2006), polyphenols, flavonoids and steroids (Derouich et al. 2020) are only a few of the phytochemicals and secondary metabolites found in this family.

4.2.1 Antioxidant Activity

Angelica sylvestris is rich in phytochemicals like α-pinene and limonene which give a strong antioxidant activity during DPPH (2,2-diphenyl-1-picryl-hydrazyl) scavenging assays. Another species of this genus, *Angelica pancicii*, also contains

α-pinene, α-phellandrene and β-phellandrene. These phytochemicals are extracted from the essential oils of both species. It is found that both species have shown antioxidant activity but *A. sylvestris* essential oil has stronger antioxidant activity than *A. pancicii* essential oil (Stanković et al. 2020).

4.2.2 Anti-inflammatory Activity

The family includes important plants like coriander, celery and parsley, some of which have therapeutic characteristics. The total polyphenols and flavonoids in hydromethanolic extracts from these three *Apiaceae* species were investigated, and their anti-inflammatory potential was checked. The Folin reagent test and aluminium chloride test were used to measure the number of phenols and flavonoids, respectively. Nitric oxide scavenging, protein denaturation inhibition and membrane stabilization assay were utilized to find anti-inflammatory properties (Derouich et al. 2020).

4.2.3 Antimicrobial Activity

The methanol extracts of *Smyrnium cordifolium*, *Falcaria vulgaris*, *Smyrniopsis munzurdagensis*, *Actinolema macrolema* and *Smyrniopsis aucheri* were examined for antimicrobial activity. Antimicrobial properties were tested in bacterial strains. These five species are rich in phenol and flavonoid phytochemicals. Reportedly, *Falcaria vulgaris* extract has the most potent inhibitory effect over bacteria among them (Zengin et al. 2019).

4.3 Cucurbitaceae

The family Cucurbitaceae comprises cucumbers and melons which sometimes are collectively called cucurbits. The family is considered to be medicinally and nutritionally important. It is cultivated widely within tropical and subtropical countries (Rajasree et al. 2016). These plants constitute some medicinal important phytochemicals including tannins, terpenoids, phytosterols, cardiac glycosides and resins (Sood et al. 2012).

4.3.1 Antioxidant Activity

The aqueous fruit extract of *Citrullus colocynthis* was measured for their antioxidant activity as well as the aqueous root extract of *Bryonia dioica*. Phytochemical analysis identified that the extracts from both species are rich in quinines, saponins, tannins and flavonoids. It was observed that polyphenols and flavonoids are richer in

B. dioica root extracts than *Citrullus colocynthis* fruits due to these phytoconstituents that showed the highest activity (Chekroun et al. 2015).

4.3.2 Anti-inflammatory Activity

Momordica charantia is an anti-inflammatory traditional medicine. It contains numerous phytochemicals, for example, alkaloids, steroidal glycosides, phenolics (Krawinkel and Keding 2006), lysophosphatidylcholines (Kobori et al. 2008), conjugated linolenic acid isomers (Chuang et al. 2006) and cucurbitane-type triterpenoids (Chang et al. 2011; Hsu et al. 2011). Oral administration of *Momordica charantia* in lipopolysaccharide (LPS)-injected mice showed the downregulation of pro-inflammatory cytokines and upregulation of anti-inflammatory cytokines (Chao et al. 2014). Cucurbitacins B and E in cucurbits display anti-inflammatory activity. Cucurbitacins inhibit the proinflammatory molecules such as COX-2 and TNF-alpha (Montesano et al. 2018).

4.3.3 Anticancer Activity

The phytochemicals isolated through fruit extract of *Cucurbita andreana* have the potential to inhibit tumour formation. Major phytochemicals present in *Cucurbita andreana* are cucurbitacins B and D (Jayaprakasam et al. 2003). *Hemsleya amabilis* contain cucurbitane triterpenes which are responsible for its anticancer activity and proved by in vitro studies with HCT-8, Hela and HepG-2 cell line (Feng et al. 2019).

4.3.4 Antimicrobial Activity

The ethanolic extracts of *Momordica charantia* have antibacterial and antifungal properties. A minimum concentration of the ethanolic extract was demonstrated to be efficient in killing 50% of parasites (Santos et al. 2012). The expert demonstrated a similar impact to metronidazole, suggesting that it offers a viable therapy option for candidiasis (Santos et al. 2012). *Momordica charantia* L. extract was also displaying antimicrobial activity against *Staphylococcus aureus* and *Escherichia coli*. The extracts were effective in inhibiting the development of all bacteria (Mada et al. 2013). Malaria is one of Africa's most lethal diseases. However, one of the primary issues that health practitioners face is parasite resistance to antimalarial medications. Bioactive chemicals from *M. charantia*, which could be a new supply of antimalarial medications, are one method to avoid resistance (Olasehinde et al. 2014). *M. charantia* also possesses antiprotozoal action, according to Pereira et al. (2016).

4.4 *Lamiaceae*

The Lamiaceae, sometimes known as Labiatae, is a flowering plant family with a worldwide distribution that includes about 236 genera and 6900–7200 species. Many of them are utilized as herbs and spices, as well as vegetables. The calming and relaxing properties are attributed to members of this family. In addition to this, it also strengthens and excites the body and has unique impacts on a specific organ or system. The active chemicals found in Lamiaceae plants have been shown to have natural antibacterial (Pop et al. 2013; Stanojević et al. 2010), antioxidant (Kamdem et al. 2013; Lin et al. 2012), antifungal (Stević et al. 2014) and anticancer (de Sousa et al. 2004; De et al. 2011) properties, implying that they could be viable alternatives to synthetic medicines in the treatment of many disorders.

4.4.1 Antioxidant Activity

The ethanol extracts of *Salvia officinalis* and *Melissa officinalis* were analysed for the antioxidant activity and total phenol content count. These extracts are rich in phenolic compounds such as ferulic acid, coumaric acid and rosmarinic acid. *Salvia officinalis* exhibited maximum phenol content with higher antioxidant activity. This result indicates that there is a strong correlation between the total phenolic compounds and antioxidant activity (Cocan et al. 2018).

4.4.2 Anti-inflammatory Activity

Salvia tiliifolia Vahl is used to relieve pain and inflammation in humans. *Salvia tiliifolia* is a predominant source of diterpenoid tilifodiolide (TFD) used for anti-inflammatory properties. The TFD value of anti-inflammation in vitro was evaluated by utilizing LPS-stimulated murine macrophages and calculating the levels of pro-inflammatory mediators for 48 h. The carrageenan-induced paw oedema test was used to investigate TFD's anti-inflammatory efficacy in vivo for 6 h. Result of in vitro anti-inflammatory tests showed that TFD exhibited concentration-dependent activity, and it inhibits TNF-alpha and IL-6 production as well as nitric oxide generation. Result of in vivo anti-inflammatory tests showed that TFD and IND (investigational new drug) (reference drug) significantly ($p < 0.05$) reduced ear oedema in TPA-induced ear oedema by 30% and 56%, respectively. In the carrageenan test, TFD inhibited paw oedema in a dose-dependent manner (González-Chávez et al. 2018).

4.4.3 Antimicrobial Activity

To analyse the antimicrobial activity, essential oil extract from the five different Lamiaceae plant aerial parts against multidrug-resistant *Staphylococcus aureus* was used. 1,8-Cineole and linalool were the primary components isolated from dried flowers of *Lavandula angustifolia*. Menthyl acetate, menthol and menthone were the primary components extracted from *Mentha* × *Piperita*. α-Terpinene, *γ*-terpinene, (*E*)-sabinene hydrate and terpinene-4-ol make up the majority extracted from *Origanum majorana* aerial parts. The primary components extracted from the *Salvia officinalis* were *p*-cymene, β-thujone, α-thujone, thymol, viridiflorol and camphor. γ-Terpinene were the most abundant constituents in *Thymus vulgaris*. *T. vulgaris* extraction displayed a strong inhibitory and bactericidal effect against *Staphylococcus aureus* (Kot et al. 2019).

4.5 Lauraceae

The Lauraceae family has 52 genera and 3000 species, most of which are found in tropical and warm subtropical parts of the world (Takaku et al. 2007). Several kinds of secondary metabolites, the majority of which are aromatic, are found in Lauraceae species and appear to be important for chemotaxonomic classification (Gottlieb 1972). The Lauraceae family is well known for producing essential oils. Many plants in the family that are vital for spice and flavour have a high amount of essential oils (Kumar Semwal and Badoni Semwal 2013). Secondary metabolites such as saponins, esters, lignans, neolignans, coumarins, butenolides, alkaloids, benzopyrans and steroids are found in the genus *Ocotea*, and many of them have anti-proliferative, antifungal, antiherpetic and antimicrobial activities (Salleh and Ahmad 2017).

4.5.1 Antioxidant Activity

The leaves, barks and fruits of *Cinnamomum triplinerve* have been analysed for phenols and flavonoids. The maximum level of phenols attains through bark extract, according to the results of the total phenol determination, and is most effective in the antioxidant capacity. This sample has the least amount of flavonoid but the most tannin. Leaf extract contained flavonoids, and total tannins had the lowest concentration of total phenols. Leaf extract showed very less antioxidant activity. The findings imply that *C. triplinerve*'s antioxidant activity is mostly due to the presence of phenolic substances such as flavonoids and tannins, which are free radical scavengers and effective in preventing oxidative processes (Silva et al. 2019).

4.5.2 Anti-inflammatory Activity

The subaveniumins A and B were isolated from the *Cinnamomum subavenium* for anti-inflammatory properties. These two isolated compounds showed an anti-inflammatory effect, and it was also observed that (−)-subaveniumins A had a significantly stronger inhibitory impact than (+)-subaveniumins A while compound (−)-subaveniumins B had a stronger inhibitory effect than (+)-subaveniumins B (Lai et al. 2015).

4.5.3 Antitumour Activity

Cinnamomum verum has previously been shown to have anticancer properties. 2-Methoxycinnamaldehyde (2-MCA) present in *Cinnamomum verum* holding anti-cancer properties were discovered. It decreased the cancer tissue size as well as showed an anti-proliferative effect. The extract has been effective against suppressing cell growth markers and increasing the level of pro-apoptotic molecules (Liu et al. 2017). Such work is also supported by the findings by Wong et al. (2016). Additionally, *Cinnamomum zeylanicum* has anticancer activities that were analysed by its ethanol extracts (Husain et al. 2018).

5 Conclusion and Future Perspectives

From the past few years, phytochemicals derived from plants have received immense attention from various researchers and technologists owing to their potent biological activities. The secondary metabolites are the major constituent, namely, phenolics, flavonoids, sterols, alkaloids, terpenoids, lignin, saponins, tannins and stilbenes responsible for various properties imperative for cell growth as nutritive, immune-modulative, prophylactic and therapeutic properties. This property is due to the production of reactive oxygen species (ROS) which is responsible for scavenging the free radicals and decreasing the oxidative stress in the cells leading to the reduction of various inflammatory signals responsible for reduced risk of various chronic diseases. The plants serve as potential raw material for the synthesis of drugs as they exhibit persuasive therapeutic efficiency, less or no side effects, and are cost-effective serving as an efficient and reasonable source for maintaining a balanced life. However, the inadequate supply of the raw material, lower stability, high cost of production, unidentified approach of action and deficiency of imperative regulatory system restrict the commercial application of plant-derived bioactive compounds as potential therapeutic drugs. In addition, a lot of research is still required to accomplish a complete understanding of these active ingredients and their derivatives concerning human health. This will include the complete understanding of the

complex cellular signalling along with the epigenetic mechanism of the cell in relation to dose and efficacy.

Acknowledgements This study was supported by the Council of Scientific and Industrial Research (CSIR) for providing funds (MLP-201). V.J. also thanks the Science and Engineering Research Board (SERB) for the Early Career Research Award and Department of Science and Technology (DST) for the INSPIRE faculty award. HG and PK thank CSIR for the Junior Research Fellowship. CSIR-IHBT communication number is 5025.

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Chapter 3
Bioprospecting of Plants for Phytochemicals: Important for Drugs

Kanwaljeet Singh, Ajay Kumar, Sushil Kumar, and Sumeet Gairola

1 Introduction

Biodiversity means the diversity of all living entities, such as plants, animals, and microbes as well as the genetic information and ecosystems that they create (CBD 1994). Biological diversity is becoming more economically important as a result of changes in globalization and increasing environmental challenges. Furthermore, as the rate of global change accelerates, the rising human population is compromising the Earth's life support system, particularly plant resources, which are becoming increasingly threatened (Balmford et al. 2003). Because natural resources are finite, it has become vital to protect genes, species, and ecosystems in order to maintain biodiversity (Dixit et al. 2021).

According to the *State of the World's Plants* report (Royal Botanic Gardens, Kew), 391,000 species of vascular plants exist globally, with 369,000 species being angiosperms. Approximately one-tenth of the world's plant species have been utilized for medical purposes, according to estimates (Chen et al. 2016). The demand for medicinal plants for health purposes is growing in developing and developed nations. This problem can be addressed by exploring the global diversity of medicinal plants in order to acquire information and resources as possible to meet the health requirements of the current generation (Balick et al. 1996). Plants are believed

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M. K. Swamy, A. Kumar (eds.), *Phytochemical Genomics*,
https://doi.org/10.1007/978-981-19-5779-6_3

to be the basis of about 25% of the prescriptions recommended for disease treatment around the world (Sahoo et al. 2017). Furthermore, it has been suggested that our current understanding of the chemical variability in plants constitutes only a small portion, implying that more research is needed. As a result of their vast metabolomic heterogeneity and expertise, humans will have more opportunities to use plants as a vital resource for bioprospecting (Krishnan et al. 2021).

2 Bioprospecting

The ultimate focus of bioprospecting is to investigate biodiversity in hopes to identify natural products and species that can benefit mankind. Reid et al. (1993) described the word "bioprospecting" as the process of screening biological life forms for new constituents with scientific and industrial value. The advantages of bioprospecting are frequently regarded to have a larger impact if they are based on local knowledge (Dhillion et al. 2002). However, due to the absence of an intellectual property rights regime, most of the developing nations are unable to fight for the protection of information on traditionally used plants (Homere 2003). This leads to the theft of local knowledge about the usage of plants, which is referred to as biopiracy (Mgbeoji 2001). Biopiracy is defined as the use of traditional knowledge in research activities without receiving consent, paying any cost, or acknowledging that the results are novel (Reid 2009). Biopiracy inflicts several upshots on local biodiversity such as overexploitation of endemic species, reduction in local niche of the species, and illicit privatization of biological samples, all of which have a negative impact on indigenous people's cultural, regional, and traditional identities and knowledge (Mgbeoji 2014). Biopiracy has a number of adverse effects for local biodiversity, including overexploitation of endemic biological material, reduction in biodiversity or local niche, and illegal privatization of biological material, all of which have a negative impact on indigenous people's cultural, regional, and traditional identities and knowledge. In this context, the Rio Declaration and the Convention on Biological Diversity in 1992 clarified indigenous people's and local communities' rights (Mackenzie and Jenkins 2001). These include equal and fair allocation of the benefits derived from the utilization of genetic resources under the Biological Diversity Act, 2002.

Beneficial natural products/organisms have helped indigenous populations and host countries since the 1992–1993 Convention on Biological Diversity. This will strengthen the cooperation between industry and local agency while also increasing job opportunities and improving socioeconomic positions. The manufacturing of medications and treatments is linked to plant variety (Mathur and Hoskins 2017). Plant-based drug development has expanded to a wide range of multidisciplinary fields and analytical techniques. A botanist, ethnobotanist, ethnopharmacologist, or plant ecologist gathers and identifies the plant of interest at the beginning of the procedure (Jachak and Saklani 2007). For locating and identifying bioactive chemicals from plants, ethnobotanically focused bioprospecting has grown more

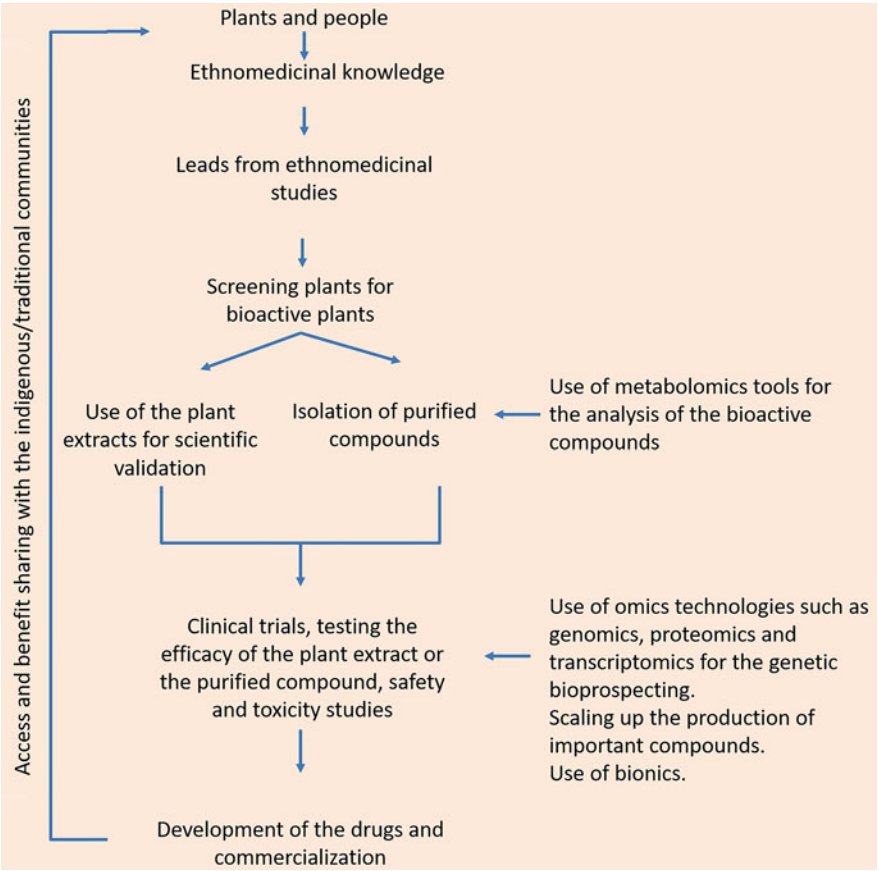

Fig. 3.1 Bioprospecting of medicinal plants for potential drugs and the use of various omics and computational technologies

effective than random testing. The prospecting of plants for drugs and pharmaceuticals from traditional knowledge is presented in Fig. 3.1.

Several examples of the well-known traditional medication leads produced using an ethnobotanical strategy are digitoxin and digoxin (*Digitalis purpurea* L.), cannabidiol and tetrahydrocannabinol (*Cannabis sativa* L.), aspirin (*Filipendula ulmaria* (L.) Maxim.), colchicine (*Colchicum autumnale* L.), vinblastine and vincristine (from *Catharanthus roseus* (L.), and codeine and papaverine (from *Papaver somniferum* L.) (Chadwick and Marsh 2008). In today's drug discovery technique, reverse pharmacology is equally significant. The three basic phases of this approach are (1) the experimental phase, which entails meticulous substantiation of direct clinical examination of a traditional drug; (2) the exploratory phase, which entails the evaluation of drug interactions, tolerance limits, and dose choices; and (3) the use of in vitro and in vivo strategies in preclinical studies to evaluate the target activity.

Experiments are carried out in the third phase to confirm the drug's efficacy and safety (Patwardhan and Mashelkar 2009).

Biodiversity research is surging in quest of new medications, crops, herbicides, pesticides, and other commercially relevant biological products. Biotechnologies have broadened the scope and effectiveness of bioprospecting to include all tasks related to the search strategy for genes, compounds, designs, and whole organisms with product development potential employing genetic, biochemical, and biophysical methods with damaging environment (Mateo et al. 2001). Although the physiologically active characteristics of plant/animal extracts have been used for a variety of reasons for several years, the use of bioprospecting for commercial and economic benefit was put in place and strengthened in the twentieth century. In today's world, it takes around 12 years from the development of a new medicine to its introduction into the clinic (Katiyar et al. 2012). Vinblastine and vincristine, two cancer treatment compounds, were discovered in the rose periwinkle plant in Madagascar in 1958 (Onaga 2001).

With input from local shamans and spiritual herbalists, Eli Lilly developed and synthesized these medicinal substances (Onaga 2001). In the pharmacy sector, for example, bioactive molecules derived from natural products include about a third of all small-molecule medications authorized by the US Food and Drug Administration (FDA) between 1981 and 2014. Bioprospecting has made a significant contribution to medication development for a wide range of infectious illnesses, cancers, and autoimmune disorders (Grabley and Thiericke 1998). In the agricultural industry, bioprospecting leads to the development of pesticides and herbicides, as well as bio-fertilizers (rhizobium) that boost plant growth, productivity, and resistance to harsh environmental circumstances (Kanchiswamy et al. 2015; Sahoo et al. 2017). Forests, protected areas, hotspots, and the ocean are all good sites to perform bioprospecting since they have a lot of biological diversity (Juan 2017). Bioprospecting projects are most typically conducted in the terrestrial environment. Artemisinin discovery by Tu Youyou, a Nobel Laureate, is a famous example (Tu 2016). Bioprospecting has resulted in the development of various medications that are employed to treat a plethora of illnesses and disorders (Table 3.1). The pharmaceutical market is perhaps the one that uses bioprospecting the most. According to conservative estimates, up to 25% of all prescription medications sold in the United States come from plant-based sources (Asher et al. 2017). Plants can be used to identify new chemicals in two ways, large-scale random collections and specialized collections that use phylogenetic and cultural clues, with a small number of samples having the best probability of success (McClatchey and Stevens 2001). Medicinal plant-based drug development has various benefits over traditional drug discovery based on synthesis. For instance, plant-based medications are inexpensive and nontoxic, have fewer side effects, have superior ADME (absorption, distribution, metabolism, and excretion) assent, and critically can prevent infections from developing drug resistance (Rout et al. 2009). A reasonable bioprospecting program must also incorporate novel techniques to upgrade the use of potential NPs in medicine, industry, and agriculture. Biodiversity provides three key sources of incentive for modern scientists: chemicals, genes and designs (Krishnan et al. 2021).

Table 3.1 List of drugs derived from plants and their medicinal importance

Drugs	Species name	Treatment	References
Andrographolide	*Andrographis paniculata* (Burm.f.) Nees	Colon cancer (both in vitro and in vivo)	Osman et al. (2015)
Aloin	*Aloe vera* (L.) Burm. f.	Cytotoxicity against human breast cancer cell lines	Esmat et al. (2006)
Artemisinin	*Artemisia annua* L.	Antimalarial	Liu et al. (1992)
Asiaticoside	*Centella asiatica* (L.) Urban	Antitumor, anti-inflammatory, immunomodulator and antioxidant	Siddique et al. (2008)
Atropine	*Atropa belladonna* L.	Anticholinergic	Rajput (2013)
Baccosoids	*Bacopa monnieri* (L.) Pennell.	Antidepressant, anticonvulsive, and antioxidant activity	Sairam et al. (2002)
Camptothecin	*Camptotheca acuminate* Decne.	Liver problem, digestive problem, and anticancerous effect	Kaur et al. (2011)
Colchicine	*Colchicum autumnale* L.	Anti-inflammatory and anticancer	Kurek et al. (2021), Lin et al. (2015)
Convallatoxin	*Convallaria majalis* L.	Anticancer	Zhang et al. (2020)
Digitoxin	*Digitalis lanata* Ehrh.	Anticancer	López-Lázaro et al. (2005)
Digoxin	*Digitalis purpurea* L.	Cardiotonic	David and Shetty (2021)
Galantamine	*Galanthus woronowii* Losinsk.	Alzheimer's disease	Heinrich and Teoh (2004)
Ginkgetin, ginkgolide A and B	*Ginkgo biloba* L.	Hepatocarcinoma, ovary, prostate, colon, and liver cancer	Xiong et al. (2016)
Harringtonine	*Cephalotaxus harringtonii* (Knight ex J.Forbes) K.Koch	Acute leukemia and lymphoma	Takemura et al. (1985)
Homoharringtonine		Anticancer and antiviral	Kantarjian et al. (2001), Dong et al. (2018)
Hyoscyamine	*Atropa belladonna* L.	Cholinergic	Teitel (1961)
Irinotecan	*Camptotheca acuminata* Decne	Anticancer	Bailly (2019)
Leptospermone	*Callistemon citrinus* (Curtis) Skeels	Antityrosinemia	Veeresham (2012)
Morphine	*Papaver somniferum* L.	Analgesic	Leppert et al. (2014)
Prostratin	*Homalanthus nutans* (G.Forst.) Guill.	Antiviral	Gulakowski et al. (1997)
		Antitumor	

(continued)

Table 3.1 (continued)

Drugs	Species name	Treatment	References
Psoralen and isopsoralen	*Psoralea corylifolia* L.		Wang et al. (2011)
Quinine	*Cinchona calisaya* Wedd.	Antimalarial and antiviral	Achan et al. (2011), Große et al. (2021)
Rivastigmine	*Physostigma venenosum* Balf.	Cholinesterase inhibitors to treat Alzheimer's disease	Kaasinen et al. (2002)
Scopolamine	*Datura stramonium* L.	Sedative, antiemetic, and amnestic	Renner et al. (2005)
Shatavarin	*Asparagus racemosus* Willd.	Anticancer	Bopana and Saxena (2007), Mitra et al. (2012)
Taxol	*Taxus brevifolia* Nutt.	Anticancer	De Furia (1997)
Theabrownin	*Camellia sinensis* (L.) Kuntze	Lung cancer (in vivo)	Wu et al. (2016)
Thymol	*Thymus vulgaris* L.	Anticancer	Islam et al. (2019)
Tinosporic acid and cordifoliosides	*Tinospora cordifolia* (Thunb.) Miersis	Hepatoprotective and antihyperglycemic activity	Singh et al. (2003)
Tylophorine	*Tylophora indica* R. Br.	Asthma, high blood pressure and bronchodilator	Mali and Dhake (2011)
Vasicine and vasicinone	*Adhatoda vasica* Nees.	Bronchodilator	Paramesh (2001)
Vinblastine and vincristine	*Catharanthus roseus* (L.) G.Don	Anticancer	Kruczynski and Hill (2001)
Withaferin A and D	*Withania somnifera* (L.) Dunal	Breast, cervix, prostate, and colon cancer (in vivo)	Lee and Choi (2016)
Gossypol	*Gossypium hirsutum* L.	Anticancer	Lan et al. (2015)
Theophylline	*Theobroma cacao* L.	Bronchodilator	Barnes (2013)
Galantamine	*Lycoris radiata* (L'Hér.) Herb.	Cholinesterase inhibitors to treat Alzheimer's disease	Haake et al. (2020)
Cannabidiol (CBD) and cannabidivarin (CBDV)	*Cannabis sativa* L.	Anti-inflammatory, anticonvulsive, neuroprotective, antitumor, and antiepileptic	Alves et al. (2020)

2.1 Chemical Prospecting

Biochemical prospecting entails looking for new, potentially beneficial compounds in living organisms. The advanced chemical screening and bioassay methods for identifying, extracting, and profiling new active chemicals from wild plants, animals, and microbes have paved the way for new breakthroughs in natural product research, as well as medicine and pharmaceutical innovation (Pushpangadan et al. 2018).

Industries related to agricultural chemistry, medicines, pharmaceuticals, cosmetics, and industrially significant chemical products such as proteins, enzymes, food additives and all others are benefiting from chemical prospecting of wild plant resources (Eisner 1997). The chemical techniques, namely, LC-NMR (liquid chromatography nuclear magnetic resonance), HPLC (high-pressure liquid chromatography), LC-MS (liquid chromatography-mass spectrometry), GC-MS (gas chromatography-mass spectrometry), FT-IR (Fourier transform infrared spectroscopy), UV-Vis (ultraviolet and visible) spectrometry, and LC-ToF-MS (liquid chromatography time-of-flight mass spectrometry), play a vital role in ensuring the quality of medicinal plants and finished herbal medications. HPLC involves the separation and detection of each compound based on the speed of the compound through the column. HPTLC, on the other hand, is a TLC-based analytical approach designed to improve the resolution of the compounds to be separated and allow quantitative analysis of the compounds (Schibli and Reich 2005). Likewise, mass spectrometry and high-field NMR allowed compounds to be identified in milligram quantities. In industrial labs, 500 MHz NMR spectrometers were commonly available, although early 600 MHz instruments were more limited. While mass spectrometry had sufficient sensitivity for analyzing sub-milligram quantities of material prior to the development of LC-MS in the early 1990s, its usefulness was greatly expanded. Scientists were able to obtain accurate mass spectra from nonhomogeneous samples with the introduction of LC-MS, allowing them to perform structural determinations of the samples that had not been totally purified, possibly saving the isolation time. Taxonomic connections and chemotaxonomy might predict the likelihood of a known drug being present in a new individual for particular groups of species. Many drugs have been discovered employing plant natural products, thanks to high-throughput assays using bioreactor and microfluidic devices. Opium and morphine are two of these natural products (Manglik et al. 2012).

Another strategy is biological activity-guided fractionation, which has been used to discover the lead drug candidate from any given phytochemical matrix. Its methodology, on the other hand, is not consistent. Two techniques to extraction design for bioactive guided fractionation leading to compound isolation as a lead compound could be used. The first is the parallel method, which is used when the plant's biological activity is known from its traditional use. The sequential technique is used when the biological activity of the subject plant is unknown and a random selection strategy is used to choose plants. Using hexane, chloroform, ethyl acetate, and butanol as solvents, extraction is done based on the polarity of the solvents, and fractions are obtained in a sequential procedure.

2.2 Gene Prospecting

It is useful in bioprospecting to find out which genes code for enzymes that catalyze the target molecule's metabolic pathways. Genomic technologies are essential for

developing a successful identification strategy for plants and natural product species (Buriani et al. 2012). The plant species used to make the natural product and attribute medicinal characteristics to it must be of excellent quality with correct identification and trustworthiness in order to be successful in new drug discovery (Thomford et al. 2018). Because different plant species contain different chemicals and quantities, using incorrect or different plant species will almost certainly influence the medicinal characteristics. Genomic approaches like DNA barcoding use short, standardized gene sections as internal species identifiers, allowing for quick, accurate, and automated species identification (Hebert and Gregory 2005). The use of DNA barcoding of natural goods has benefitted both biodiversity inventories (Meusnier et al. 2008) and herbal product authenticity (Newmaster et al. 2013). This strategy is fast gaining traction in manifesting the jurisdiction of IPRs (intellectual property of rights) of the developing nations over their own resources (Pushpangadan et al. 2018). Transcriptome analysis is another technique that gets information about genes that are expressed under specific conditions. Proteins, on the other hand, are an intriguing topic in gene prospecting because it involves the discovery and expression pattern of those genes which encode for a certain protein or enzyme of interest. Because proteins are the most common therapeutic targets, a drug lead's final usefulness in drug development is determined by its ability to bind to a target protein and alter the cell's metabolic processes (Maghembe et al. 2020). Advanced molecular technologies, such as DNA recombination and transgenics, have enabled the discovery, isolation, and introduction of gene of interest from one organism to another, bridging the biological gap. Transgenic methodologies are making remarkable progress by allowing desired traits to be transferred from one organism to bacteria, thereby making it potential chemical factories which finally produce products of interest. Bioengineered plants have developed protein treatments for humans and animals (Joshi and Lopez 2005).

Metabolomic research focuses on whole examination of small molecule metabolites utilizing liquid chromatography, mass spectrometry, nuclear magnetic resonance, etc. (Wishart 2008). In high-throughput screening procedures, MS-based metabolomics has demonstrated its utility in terms of increasing bioactive ingredient identification and giving molecular data for medicine development (Wang et al. 2019). Metabolomics is a commonly utilized method in the scientific community and is regularly used for medication discovery and development (Wishart 2008). This method together with genome-based characterization of gene products from ethnomedicinal plants is contributing in revealing new pathways for specific active metabolites (Garnatje et al. 2017).

2.3 Bionic Prospecting

New concepts, patterns, models, and processes are generated utilizing natural biodiversity as a reference point in bionic prospecting, a new realm. New sensing technologies, architecture, biotechnology, and bio-modeling, to mention a few, are

all part of bionic prospecting (Krishnan et al. 2021). Previously, the majority of bionic prospecting was done using biorational methods (Upadhyay and Singh 2021). The lotus flower's waxy covering, for example, is supposed to aid in its self-cleaning process. Similar strategies have been used to avoid dirt in buildings and autos, inspired by the flower (Krishnan et al. 2021). For identifying natural substances, biosensors are becoming increasingly relevant. These are devices that detect biological processes by using signals produced by the presence of a certain analyte.

Based on the detecting elements, biosensors are classified as DNA sensors, enzyme sensors, immunosensors, or aptasensors. Of these, enzyme-based biosensors are most often utilized as NP detectors in biological, environmental, pharmaceutical, and industrial sector. Biosensors offer the advantages of being sensitive, immediate, particular, and portable in contrast to typical analytical processes, which are costly and time-consuming (Rahimi and Joseph 2019). Diagnostics (Bohunicky and Mousa 2011), drug discovery (Salehabadi et al. 2018), and biomedicine all benefit from biosensors (Salehabadi et al. 2018; Wang et al. 2005). Biosensors quickly detect the natural products, particularly in composite media, and are the preferable detection methods when speed and efficient outputs are required (Piroozmand et al. 2020). Bioengineering tools include a wide range of technologies, ranging from outdated to cutting-edge technologies such as metabolomics, proteomics, and genomics and genetic engineering, all of which play a crucial part in the long-term viability of medications (Sarsaiya et al. 2019). Nanobiotechnology, on the other hand, is a cutting-edge field that entails using bioengineering methods to create pharmaceutically valuable nanomaterials or a plethora of nanocomponents to create devices with remarkable features (Kalia 2018).

3 Computational Approaches for Drug Discovery

The drug discovery process includes phases such as the identification and optimization of therapeutic target, the development of a target/lead into a drug molecule, the forecasting of ADME variables, and others. Many of these stages are quite complex and time-consuming if traditional drug discovery methods are used. This constraint can be overcome using a variety of computational approaches. In this approach, the physicochemical characteristics of bioactive compounds are compiled in a computerized system that can be searched for matches to complement the three-dimensional structure of a pharmaceutical target with the requisite activity. Computational approaches have greatly accelerated drug development (Singh and Dwivedi 2016). Analyzing the relationship between a compound's chemical makeup and its activity can be employed to design a predictive model.

Data mining, quantitative structure-activity relationship, machine-learning techniques for constructing computational models, and system biology are some of the key areas. Medical informatics, clinical bioinformatics, translational bioinformatics, and health informatics are all novel fields that can be incorporated into the discovery process (Katiyar et al. 2012). Some of the most common computational techniques

are genomics, chemoinformatics, bioinformatics, and system biology. Compared with conventional natural product discovery methods, these methods allow the rapid isolation, screening, and fingerprinting of natural products from milligram sample quantities (Skirycz et al. 2016).

Traditional and modern data can be merged to infer information using computational methods, making it simple to analyze and comprehend data generated by natural products. Using an in silico target-based method, the binding potential of several chemicals of *Pelargonium sidoides* and *Pelargonium reniforme* on *Mycobacterium tuberculosis* protein kinase G was predicted which has been proposed as a potential target for new antitubercular drugs. Flavonoids such as vitexin, populnin, and orientin all had a strong affinity potential for the enzyme in question. Coumaraldehyde, methyl gallate, coumaric acid, *p*-hydroxybenzyl alcohol, *p*-hydroxyphenyl acetic acid, and myricetin were among the other compounds with high efficiency indices (Qasaymeh et al. 2019).

4 Using Evolutionary Tool for Novel Bioactive Compound

Halse-Gramkow et al. (2016) have developed a complementary technique that employs phylogenetic-based analysis on historic applications or familiar chemistry to select lineages with the most likely to find desired attributes. A viable tool for highlighting lineages with desirable chemical or pharmacological qualities is to combine ethnodirected approaches with phylogenetic analysis (Wink 2003; Saslis-Lagoudakis et al. 2012).

5 Conclusion and Future Prospects

Bioprospection generates crucial leads for new product development, and many businesses are looking for new applications of biological species that have not been investigated previously. Biological diversity, as well as the chemical diversity of its constituents, has shown to be a valuable source of bioprospecting, resulting in the identification of key bioactive compounds. The benefits of medicinal plant bioprospecting to human society are numerous, particularly in terms of pharmaceutical development. The tremendous range of components found in medicinal plants has been identified using analytical techniques. The development of analytical instrumentation techniques, such as mass spectrum (GC-MS, LC-MS, LC-NMR, MS), high-performance liquid chromatography (HPLC), high-performance thin-layer chromatography (HPTLC), Fourier transform infrared spectroscopy (FT-IR), UV-Vis spectrometry, and liquid chromatography time-of-flight mass (LC-ToF-MS) spectrometry will accelerate the drug design and synthesis process. Many of the components have been discovered to be effective in human disease prevention, and many more are being researched for commercial use. The use of genetic techniques

is making a significant contribution to determining the true phytochemical diversity. Given the broad interest in and relevance of medicinal plants, research and development in the field should be encouraged in order to reap the most benefits.

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Chapter 4
Application of Phytochemicals in Therapeutic, Food, Flavor, and Cosmetic Industries

Anubhuti Kawatra, Shefali Gupta, Rakhi Dhankhar, Pratibha Singh, and Pooja Gulati

1 Introduction

Phytochemicals, the naturally occurring bioactive compounds, have been an integral part of human civilizations since ancient times due to their remarkable biological activities for nutrition, health, and food preservation (Ullah et al. 2020; Bhalla et al. 2021). These phytoconstituents are categorized into different groups based on their properties, chemical structure, and their role in a plant's metabolism. The structure-based classification includes alkaloids, phenolics, terpenoids, organosulfur compounds, and N-containing compounds, while the classification system based on their metabolic involvement categorizes phytochemicals as primary (amino acids, sugars, purines, pyrimidines, chlorophyll) and specialized metabolites (alkaloids, terpenes, flavonoids, organosulfur compounds, phenolics, glycosides, curcumins, saponins, etc.) (Campos-Vega and Oomah 2013; Pott et al. 2019). At present, the commercial production of phytochemicals has attained immense popularity owing to their benign nature and applications in various sectors. Further, extraction of these phytochemicals from wasted crops is also being widely employed for sustainable crop management and cultural economy. In recent years, numerous phytoconstituents have been isolated from wasted fruits/vegetables, and their molecular regulatory mechanisms have been identified for therapeutic/industrial purposes (Pinto et al. 2021a, b). In this context, waste generated from the plants could be

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M. K. Swamy, A. Kumar (eds.), *Phytochemical Genomics*,
https://doi.org/10.1007/978-981-19-5779-6_4

further employed to extract phytochemicals, thereby proving the potential value from wasted food reuse.

Traditionally, these phytochemicals have been used for ages in ethnomedicines and the practice is continuing till date (Ullah et al. 2020; Mitra et al. 2021). The renaissance in the plant-based medications has eventually also given us major clues to formulate novel therapeutic interventions listed in modern pharmacopeia including paclitaxel from plant *Taxus brevifolia* for cancer treatment, alkaloid galantamine from plant *Galanthus nivalis* for Alzheimer's disease, and apomorphine, a semisynthetic compound from *Papaver somniferum* used in Parkinson's disease (Veeresham 2012). The distinctive stereochemical profiles of phytochemicals, namely, flavonoids, alkaloids, phenolics, terpenoids, curcumins, and lectins, have been widely exploited to treat cancers, diabetes, cardiovascular diseases, inflammation, neural complications, and antimicrobial disorders including drug-resistant pathogens and novel corona virus strains (Wang et al. 2012; Shanak et al. 2019; Kawatra et al. 2021).

Besides, phytochemicals are being majorly employed to enhance the shelf-life, sensory, and nutritional value of food products at industrial scale due to their antioxidant, flavoring, enhancing, and stabilizing properties (Valverde 2013). They have been also advocated as high-valued cosmeceuticals (Ganesan and Choi 2016). Further, nanotechnology-driven modified drugs/delivery systems and other products are being developed in the market for improving drug solubility/stability, circulatory half-lives, concentrating drugs at target sites, lowering side effects in nontarget organs, and penetrating deep in the skin (Ganesan and Choi 2016; Khan and Gurav 2018; Ikram et al. 2021).

Even though the chemical and biotechnological significance of phytochemicals has been reviewed earlier, their major emphasis has been either on the therapeutic or the industrial applications of these bioactive entities.

Thus, in this chapter, we present a detailed survey of both industrial and therapeutic applications of phytochemicals. Furthermore, it highlights the major groups of phytochemicals and their relevance in circular economy, potent mechanism of action, and market/clinical status along with the recent research developments to develop an effective product/intervention.

2 Major Groups of Important Phytochemicals

Phytochemicals have been classified on the basis of their properties and chemical structure into alkaloids, phenolics, terpenoids, organosulfur compounds, and N-containing compounds. However, based on their role in metabolism of plants, phytochemicals have been classified as primary and secondary or specialized metabolites (Bellik et al. 2013). The sugar, proteins, amino acids, purines and pyrimidine of nucleic acids, and chlorophyll are considered primary metabolites. Remaining phytochemicals such as alkaloids, flavonoids, plant steroids, terpenes, lignans,

curcumines, saponins, phenolics, and glycosides are regarded as specialized metabolites (Campos-Vega and Oomah 2013; Pott et al. 2019).

Many important phytochemicals are alkaloids. Alkaloids are naturally basic compounds containing heterocyclic nitrogen. Based on the pharmacognosy, they have been classified majorly into pyrrolidines, indoles, isoquinolines, quinolines, pyridines, steroids, and tropanes (Kawatra et al. 2021). Alkaloids are important for the survival of a plant as they provide protection against microorganisms, insects, and herbivores. These can also protect a plant from other plants by allelopathy and hence could be used as weedicides (Macías et al. 2019). The plants containing alkaloids are commercially used in preparing dyes, spices, and drugs. Alkaloids also show a wide range of pharmacological activities like antihypertensive effects, antiarrhythmic effects, antimalarial effects, and antiviral and anticancer effects (Kawatra et al. 2021).

Phenolics are phytochemicals that contain hydroxyl group –OH bonded to the aromatic hydrocarbon. These are commonly found in various nuts, vegetables, and fruits. The phenolic compounds may be further classified into flavonoids, tannins, phenolic acids, coumarins, and stilbenes. Phenolics act as antioxidants by scavenging free radicals and modulating antioxidant enzymes (Halliwell 2008; Ziberna et al. 2010). Flavonoids are low molecular weight phenolic compounds consisting of 15-carbon phenylpropanoid core which is arranged into two aromatic rings linked by a heterocyclic pyran ring. Many flavonoids can be easily seen in flowering pigment in most angiosperm families. There have been over 6000 flavonoids contributing to colorful pigments of fruits, herbs, vegetables, and medicinal plants (Dixon and Pasinetti 2010). Flavonoids are necessary components in many different nutraceutical, medicinal, and cosmetic products. This is attributed to their biochemical properties like antioxidative, anti-inflammatory, antimutagenic, and anticarcinogenic properties coupled with their key cellular enzyme functions modulating capacity (Panche et al. 2016).

Among all, terpenes, also called as terpenoids or isoprenoids, are the biggest group of phytochemicals. Generally, the terpenoids are classified on the basis of total units of isoprenoid available in their structure. The largest categories consist of compounds monoterpenes (two units), sesquiterpenes (three units), diterpenes (four units), sesquiterpenes (five units), triterpenes (six units), and tetraterpenes (eight units) (Ashour et al. 2010). These are primarily involved in all the basic processes of plants, namely, growth, development, defense responses, and reproduction (Yang et al. 2012). Traditionally, they have been exploited for their pharmacological activity against cancers, malaria, ulcers, and viruses and other microbial disorders (Cox-Georgian et al. 2019). Carotenoid, a pigmented tetraterpenoid, has also been found rich in antioxidant properties (Krinsky and Yeum 2003). It further aids in maintaining eye health, healthy mucus membrane, and immunity.

Numerous foods contain plant-derived nitrogenous compounds that have antioxidant properties. These compounds include amino acids, amines, amides, pyrimidines, proteins, amino acids, and nucleic acids. Another important cyclic nitrogen-containing phytochemical is pyrazines which impart flavor to various

natural foods. Grapes and wine are rich source of nitrogen-containing compounds (Jackson 2008).

Sulfur-containing plant-derived compounds are known as organosulfur compounds. Plants can utilize inorganic sulfur and reduce them into sulfur-containing amino acids such as cystine, methionine, and cysteine. Sulfur-containing amino acids are the important components of enzymes, proteins, coenzymes, hormones, and tripeptide glutathione. Some vegetables such as onions, mushrooms, garlic, Brussels sprouts, broccoli, and cabbage are the rich source of organosulfur compounds. Many of the organosulfur compounds also provide flavor to cheese, coffee, chocolate, and wine (Qian et al. 2011). They also have medicinal properties such as anti-inflammatory, antiplatelet, antiaging, antioxidant, anticancer, and immunomodulator (Rajkapoor et al. 2005; Liu 2013).

Lectins, discovered in castor beans by Stillmark, are therapeutically important phytochemicals belonging to the family of carbohydrate-binding proteins (Mishra et al. 2019). Owing to their unique ability to bind analogously with the antibodies, lectins show minimal antigenic stimulation in vivo. Lectins possess antimicrobial activity and have been widely employed in the treatment of various fungal and viral infections (Bah et al. 2013). Early studies have also demonstrated the biological activity of lectins against metastatic cancers and inflammatory conditions (Mazalovska and Kouokam 2020).

3 Importance of Phytochemicals in Circular Economy and Sustainability

As per the recent FDA (Food and Drug Administration) statistics, food waste in the United States is estimated to be approximately 30–40% of the total food supply (https://www.fda.gov/food/consumers/food-loss-and-waste). Wasted fruits and vegetables represent one of the largest categories of solid material placed in landfills, and their decomposition produces toxic substances and greenhouse gasses that modulate the ecosystem as well as the quality of life of people. Recycling and utilization of food processing waste is a serious challenge for sustainable crop management. Hence, one of the promising strategies is to convert waste biomass to obtain phytochemicals for the formulation of new drugs and applications in industrial sectors (discussed in detail in Sect. 4) contributing to the concept of cultural economy. To do so, it is crucial to effectively analyze and characterize this waste and utilize different extraction/solvent methods. Cerulli and co-workers (2020) employed hydroalcoholic (7:3, v/v) extract solvents to recover polar bioactive compounds from *Castanea sativa* (chestnut) shells, an agronomical by-product generated during chestnut peeling. In similar attempts, a green extraction procedure, subcritical water extraction, has been also used to recover polyphenols with 6.7–9.2% yield from *C. sativa* shells (Pinto et al. 2021b). Sequential microwave-assisted extraction technique has been also employed to extract bioactives from peels

of *Citrus reticulata* (Kinnow). The recovered compounds showed a good extract yield (30.743%) with 69.887 mg CE/g flavonoids and 88.404 mg GAE/g total phenolics (Suri et al. 2022). Besides, solvent-free extraction has been employed to efficiently extract phytochemicals like phylloquinone, phenolics, and flavonoids from tomato leaf waste (Arab et al. 2019). The field waste of *Solanum melongena* L. (eggplant) has also been used to recover anthocyanins and glycoalkaloids (Mauro et al. 2020).

4 Applications of Phytochemicals in Various Sectors

Phytochemicals have been the mainstay of global healthcare systems since past centuries (Ullah et al. 2020; Bhalla et al. 2021). The stereochemical diversity of phytochemicals has been majorly exploited to ethnopharmacologically treat disorders like benign/malignant tumors, diabetes, antimicrobial diseases, cardiovascular complications, neural complications, and other chronic disorders (Lee et al. 2013; Borges et al. 2016; Gencoglu et al. 2017; Welcome 2020). On an industrial scale, phytochemicals can be used as nutraceuticals, ingredients, or additives in food (Valverde 2013). Further, they are being widely employed in cosmeceuticals designing due to their specific antioxidant properties (Ganesan and Choi 2016). Henceforth, phytochemicals as a broad group of natural compounds could be employed in diverse industrial and clinical applications. These applications are discussed in detail in the subsequent subsections.

4.1 Phytochemicals in Therapeutics

4.1.1 Phytochemicals in Cancer Treatment

Cancer, a complex multifactorial metabolic disorder, is currently one of the leading causes of increased mortality rate worldwide. As per the GLOBOCAN 2020 statistics, the disease alone claimed about ten million deaths in 2020 (Kawatra et al. 2022). Despite the research advances in its prognosis and treatment, an effective treatment of malignancies with a good therapeutic index is still undeveloped. Phytochemicals, in this regard, have appeared as a plausible option in novel anticancer drug discovery. Historically, significant scientific evidence has also elucidated the antitumor activity of phytochemicals (Khan et al. 2019; Issinger and Guerra 2021). In fact, estimates suggest that since 1940 about 50% of anticancer-approved drugs have originated from natural products, being administered either exclusively or synergistically with traditional therapeutics like chemotherapy and radiation therapy (Wang et al. 2012; Moraes et al. 2017; Cragg and Newman 2018). The four major classes of clinically exploited anticancer phytotherapeutics at present times include taxane diterpenoids (docetaxel, paclitaxel), camptothecin derivatives (camptothecin,

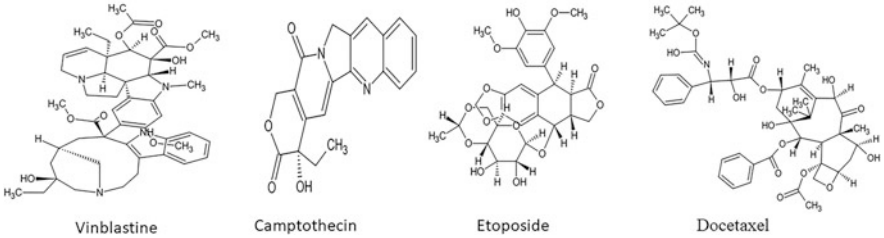

Fig. 4.1 Chemical structures of some important anticancer phytochemicals

irinotecan), vinca alkaloids (vindesine, vinblastine, vincristine), and epipodophyllotoxin (etoposide, teniposide) (Fig. 4.1).

Besides, phytochemicals like curcumin, genistein, tea polyphenols, resveratrol (RSV), epigallocatechin gallate (EGCG), gallic acid, isothiocyanates, silymarin, lycopene, apigenin, and gingerol have also demonstrated good anticancer activity in vitro and in animal models (Wang et al. 2012; Singh et al. 2016). The anticancer properties and mode of action of these various phytochemicals at multiple levels have aided in the regulation of tumor growth, survival, and metastasis making them interesting compounds for medical usage (Table 4.1). Phytochemicals exhibit these vast and complex set of anticancer moves against tumors via pathways of signal transduction including receptors on membrane (Deng et al. 2017), factors in transduction (Zhang et al. 2017), kinesis (Dou et al. 2018), microRNAs (miRNAs) (Cojocneanu Petric et al. 2015), downstream activator of tumor or inhibitor proteins (Adams et al. 2010), cyclins, and apoptotic caspases (Choudhari et al. 2020). These potent approaches aid in the suppression of tumors are represented in Fig. 4.2.

Su et al. (2013) have also elucidated suppression of melanogenesis by gallic acid. Another report showed that gallic acid inhibits bladder tumorigenesis through PI3K/AKT signaling suppression and mitochondrial dysfunction (ZENG 43). Genistein was shown to suppress hepatocellular carcinoma development. Chan and co-workers (2018) demonstrated that genistein decreases migration and proliferation of cancer cells via cell cycle arrest and apoptosis. Furthermore, a traditional Chinese medicine-derived compound, cantharidin, has been shown to inhibit the invasion of gastric cancer cells by suppressing the PI3K/AKT signaling pathway (Song et al. 2020).

However, poor water solubility, residual toxicity, and bioavailability are the major constraints limiting the large-scale manufacturing of these natural products to serve as first-line anticancer medicines. Therefore, the current research focus is toward eliminating the influence of these factors by following tenets of quality by design as well as employing nano-advances to enhance the pharmacological properties of these natural products. Recently, EGCG-loaded nanoformulation has been evaluated for its anticancer efficacy. Results showed that nanoformulation of EGCG maintained the activity of the phytocompound both as a proapoptotic and antiangiogenic agents (Siddiqui et al. 2009). In similar attempts, RSV has been loaded into solid lipid nanoparticles for overcoming the systemic toxicity and intracellular permeability barriers of the compound (Teskač and Kristl 2010).

Table 4.1 Important phytochemicals from various plants used as therapeutics

Plant name	Phytochemical	Diseases/therapeutic activity	References
Camellia sinensis	Epigallocatechin gallate	Anticancer, cardioprotective, antiviral, and antibacterial	Wang et al. (2012), Pagliaro et al. (2015), Kawatra et al. (2021)
	Catechin	Anti-inflammatory and diabetes	Sharma and Rao (2009), Naveed et al. (2018)
	Theaflavin	Diabetes	Naveed et al. (2018)
	Flavonol glycosides	Anti-inflammatory	Naveed et al. (2018)
Allium sativum	Allicin	Antiviral, anti-inflammatory, antioxidant, and neuroprotective	Borlinghaus et al. (2014)
	Quercetin	Antiviral, antibacterial, and cardioprotective	Michalska et al. (2010), Kawatra et al. (2021)
Vitis vinifera	Resveratrol	Anticancer, neuroprotective, cardioprotective, and anti-inflammatory	Teskač and Kristl (2010), Salehi et al. (2018)
Murraya paniculata	Coumarin	Antimicrobial, diabetes, and neuroprotective	Venugopala et al. (2013), Teoh and Das (2018), Kawatra et al. (2021)
Glycyrrhiza glabra	Glycyrrhizin	Antiviral, anti-inflammatory, and anticancer	Pastorino et al. (2018)
	Glabridin	Neuroprotective, anti-inflammatory, and anticancer	Pastorino et al. (2018)
Tinospora cordifolia	Berberine	Antiviral, diabetes, anticancer, and neuroprotective	Teoh and Das (2018), Saha and Ghosh (2012)
	Furano-lactone	Cardioprotective, anti-inflammatory, anticancer, and antimicrobial	Saha and Ghosh (2012)
Phyllanthus emblica	Gallic acid	Antimicrobial, anticancer, anti-inflammatory, and neuroprotective	Kahkeshani et al. (2019)
	Quercetin	Antimicrobial, cardioprotective, anticancer, neuroprotective, and diabetes	Gupta et al. (2016)
Aloe sp.	Anthraquinone	Diabetes, neuroprotective, antimicrobial, and anti-inflammatory	Semwal et al. (2021)
	Apigenin	Anticancer and anti-inflammatory	Kashyap et al. (2018)
	Lectin	Diabetes and antimicrobial	Bah et al. (2013), Mazalovska and Kouokam (2020)

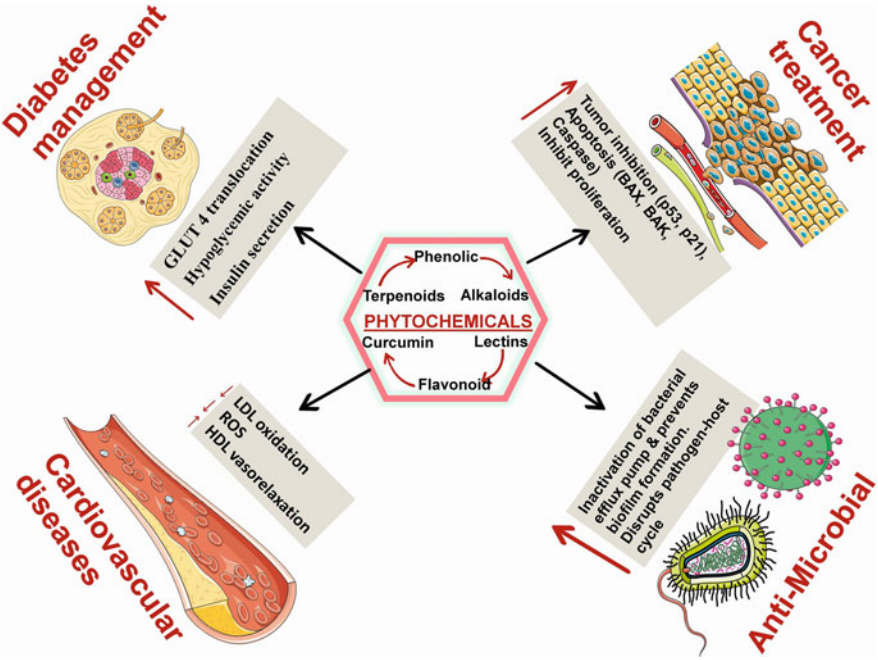

Fig. 4.2 Schematic representation of molecular mechanisms of phytochemicals against various disorders. (Some components of this image were created using the "Servier Medical Art" https://smart.servier.com/, licensed under creative commons attribution 3.0)

4.1.2 Phytochemicals in Cardiovascular Physiology Modification

The research evidence from several exhaustive in vivo and human subject experiments has implicated the role of high oxidative stress, circulatory cholesterol concentration, blood pressure, endothelial modification, and thrombotic tendency in the etiology of cardiovascular disorders (CVDs) (Senoner and Dichtl 2019). Therefore, to ameliorate the symptoms of cardiac damage, there has been a resurgence of interest in the usage of antioxidant and dietary fiber-rich phytochemicals like grapes, wine, curcumin, garlic, fenugreek seeds, green tea, products of cocoa, strawberry, blueberry, tomatoes, nuts, watermelon, and apricots (Ngugen and Schwartz 1999; Schini-Kerth et al. 2011; Zhang et al. 2015). The structures of major phytochemicals involved in the cardioprotective activity of phytochemicals are illustrated in Fig. 4.3. The epidemiological data of CVDs has further indicated the negative correlation between dietary intake of phytochemicals and mortality rate associated with CVDs.

Theobroma cacao (cocoa) is one of the best sources for biologically active flavonoids and oligomeric procyanidins available at present in the food industry. Significant induction of vasodilation has been noted by consumption of cocoa supplements; reverse is seen by infusing inhibitors of nitric oxide synthase (Ferri

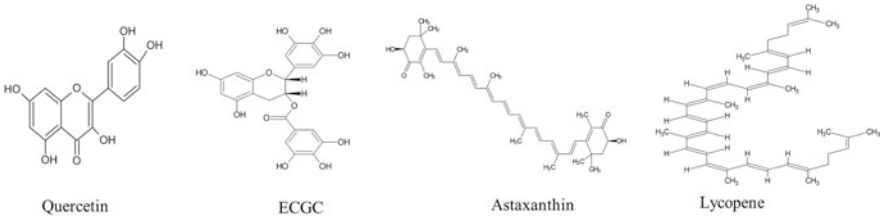

Fig. 4.3 Chemical structures of some cardioprotective phytochemicals

et al. 2015). Moreover, its efficacy has been also elucidated well in trials. Green tea, a widely consumed beverage, is also one of the main sources of biological active flavonoids like catechins, epicatechins, EGCG, etc. These bioactive compounds are potent scavengers, block NOS (nitric oxide synthase) induction, and thereby lower the chances of atherogenesis/atherosclerosis (Pagliaro et al. 2015) (Fig. 4.2).

Besides, polyphenolic compounds, acting at molecular level, have been shown to downregulate platelet aggregation and improve the functioning of endothelium via their anti-inflammatory, antithrombotic, and anti-aggregative properties. Among polyphenols, quercetin has been widely exploited to treat CVDs. It restricts ROS production, attenuates artheroscelrotic lesions, and blocks the platelet-reactive collagen receptor and PPAR-γ receptor (peroxisome proliferator-activated receptor gamma) which in turn lowers the susceptibility of LDL (low-density lipoprotein) oxidation (Michalska et al. 2010), thereby lowering the pathogenesis of cardiovascular disorders. Interestingly, the consumption of carotene-rich food comprising astaxanthin, lutein, lycopene, etc. has been also consistently associated with a decreased CVD risk (Bahonar et al. 2017; Mozos et al. 2018). Their CVD-protective activity has been attributed to their anti-inflammatory and HDL (high-density lipoprotein) vasodilation efficacy (Gencoglu et al. 2017). Phytochemicals involved majorly in cardiovascular disorder treatment along with their sources are summarized in Table 4.1.

4.1.3 Phytochemicals in Diabetes Treatment

Diabetes mellitus (DM) is a chronic-, noncommunicable-, lifestyle-/genetic-associated complex disorder that has been known to mankind for almost 2000 years (Bilous et al. 2021). The metabolic dysregulation of serum glucose levels in DM affects the pathophysiology of several organs, leading to extreme health complications including peripheral neuropathy and macrovascular and optic nerve damage (Nazarian-Samani et al. 2018). Although synthetic interventions offer greater effects in treatment, they, perhaps, also pose higher side effects in vivo including weight gain (with the exception of metformin), fluid retention in body, high chances of heart failure, gastrointestinal disturbances, and hypoglycemia (Maruthur et al. 2016). Henceforth, the search for safer and effective antidiabetic therapeutics is one of the most important areas of investigation. Several studies have now established that

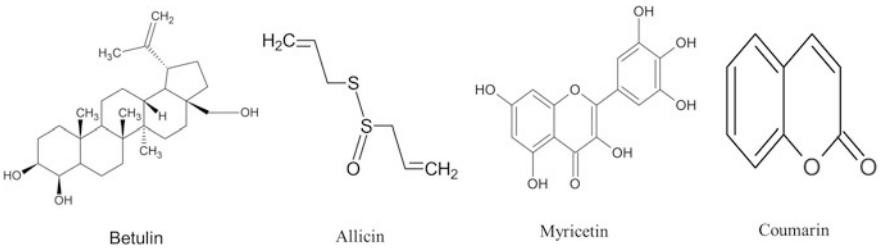

Fig. 4.4 Chemical structures of some antidiabetic phytochemicals

changes in diet have shown promising results in both prevention and management of diabetes.

Whole grain foods can play a role in delaying and preventing DM development. In general, whole grains can have many different phytochemical combinations (Liu 2007). The outer surface of grains, specifically the pericarp and aleurone layer, has very high content of phytochemicals like phenolic compounds, betaine, phytosterols, and folate contrary to the germ layers and the endosperm (Fardet 2010), acting as a natural therapy for DM. Besides, ancient Ayurveda, Unani, and Siddha findings have described the usage of a number of natural products such as harra, geloy, cinnamon, chirayita, fenugreek, black cumin, aloe vera, gymnema, bitter melon, nopal, ginseng, thistle tulsi, barberry, and amla and their extracts to control diabetes and its complications (Teoh et al. 2010; Singh 2011; Thent et al. 2012; Sakthiswary et al. 2014). These products mainly include phyto-derivatives of flavonoids, alkaloids, terpenoids, and phenolic compounds (Table 4.1) (Teoh and Das 2018).

Considering their high efficacy, these phytotherapeutics are being evaluated clinically to treat DM at present. These are known to improve glucose metabolism as well as provide health benefits to DM patients via their antioxidant status, capillary functioning, and lipid metabolism efficacy (Kumar et al. 2018). The structure of major antidiabetic phytochemicals is illustrated in Fig. 4.4. Research on the antidiabetic mechanisms of these plants has further shown that most of them exhibit hypoglycemic activity via augmenting PPARs (peroxisome-proliferator-activated receptors) (Rebhun et al. 2015), stimulating insulin secretion (Kalailingam et al. 2014), free radical scavenging/antioxidant activity (Baek et al. 2012), inhibiting α-amylase/α-glucosidase (Guo et al. 2015), upregulating GLUT-4 (glucose transporter type 4) translocation (Gautam et al. 2015), and preventing insulin resistance development (Perez-Gutierrez and Damian-Guzman 2012) (Fig. 4.2). In addition to these, several hypoglycemic phytochemicals like myricetin, betulonic acid, berberine, essential oils, triterpenoids and sterols, coumarins, alkaloids, carotenoids, flavonoids, saponins, tannins, bitter principles, and phenolic acids are being evaluated in vitro and in vivo for their antidiabetic potency (Teoh and Das 2018). Besides, ursolic acid, a common component of traditional Chinese medicine, has been shown to inhibit in vitro formation of Nε-(carboxymethyl) lysine and pentosidine, which have been implicated majorly in the pathogenesis of DM-related nephropathy and other complications (Kumar et al. 2018). Although

results seem highly promising, thorough investigations regarding their toxicity need to be conducted to develop an effective treatment strategy for DM.

4.1.4 Antimicrobial Properties of Phytochemicals

The prevalence, emergence, and reemergence of microbial diseases raise serious concern to the global public health domain. Several microbial infections such as Ebola, human immunodeficiency virus (HIV), drug-resistant tuberculosis (MDR-TB), chikungunya (CHIKV), influenza, and particularly the novel COVID-19 (SARS-CoV-2) outbreak have expedited the need to develop novel antimicrobial therapeutics with minimal side effects (Liu et al. 2017; Li and De Clercq 2020). Phytochemicals, in this context, have received significant attention due to their genotype-specific activity and high therapeutic index profile. Secondary metabolites extracted from different parts of plants, namely, stem, bark, seeds, flower, roots, leaves, and fruits, have been used ethnopharmacologically to treat microbial disorders for decades. Further, epidemiological data and other studies have documented the efficacy of flavonoids, terpenoids, tannins, coumarins, alkaloids, and lignans against a wide variety of microbes, namely, antibacterial (multidrug-resistant), MRSA (methicillin-resistant *Staphylococcus aureus*), MSSA (methicillin-susceptible *S. aureus*), *Mycobacterium*, nosocomial *Pseudomonas*, *Acinetobacter*, *Enterobacter* sp., biofilm producers (*S. epidermidis*, *P. aeruginosa*, etc.), and antiviral (dengue virus, hepatitis, *Zika virus*, SARS-CoV-1, SARS-CoV-2, HIV, CHIKV) (Borges et al. 2016; Kawatra et al. 2021) (Table 4.1). These major groups of phytochemicals being exploited as antimicrobial agents are represented in Fig. 4.5.

Notably, many of these bioactive compounds have already progressed toward clinical trials to form an effective treatment modality for these different disorders (Kumar et al. 2018; Kawatra et al. 2021). These majorly include extracts comprising polyphenols, cannabis, and ArtemiC (semi-synthetic curcumin-based formulation) against SARS-CoV-2, elderberry extract against influenza virus, *Artemisia annua* against HIV, *Allium cepa* against *H. pylori*, extracts of *Punica granatum* (pomegranate) in reductions of gingivitis, oral plaque, periodontitis, curcumins against many bacterial diseases, to name a few (Kumar et al. 2018; Kawatra et al. 2021). The

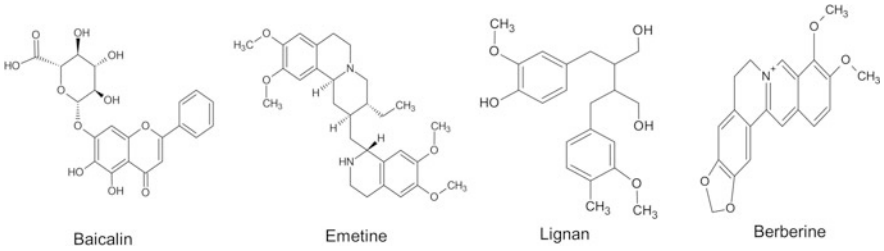

Fig. 4.5 Chemical structures of some antimicrobial phytochemicals

mode of action underlying their antimicrobial efficacy has been mainly attributed to disruption of the interaction with host cellular membrane proteins, proton motive force, replication/transcription enzyme inhibition, release of lipopolysaccharides, coagulation of the cell components, and biofilm inhibition (Fig. 4.2).

Besides, the synergistic aspects of these natural products with synthetic formulations are being widely appraised to target several disorders with better therapeutic profiles. Combination of phytocompound reserpine with antibiotic fluoroquinolones displayed promising activity against *S. aureus* via targeting the NorA efflux pump (Stavri et al. 2007). Similarly, corilagin/catechin and β-lactam antibiotic formulations have been shown to inhibit MRSA strains effectively (Shimizu et al. 2001; Shibata et al. 2005). In vitro analysis of the gallic acid-tetracycline combination also displayed promising results against several gram-positive and gram-negative pathogens (Saavedra et al. 2010). A synergistic formulation comprising *Phyllanthus urinaria* extract has been further approved for marketing to treat hepatitis B virus infection (Kawatra et al. 2021).

4.1.5 Other Therapeutic Applications of Phytochemicals

Besides, phytochemicals have been shown to modulate the functions of microglial cells, cerebral endothelial cells, and blood-brain barrier basal membrane components to attenuate inflammation and neural complications (Jin et al. 2019). Various classes of phytochemicals like polyphenols (resveratrol, salvianolic acid A, curcumin, baicalein, fisetin), alkaloids (berberine), carbohydrates, terpenoids (parthenolide, saponins), and sterols (ruscogenin) have been identified as anti-inflammatory agents (Welcome 2020). Also, phenol-rich natural products (drinks, food, herbs) have been found to exhibit neuroprotective effects via suppression of NF-κB and mitogen-activated protein kinase and JAK-STAT signaling pathway activation (Rangarajan et al. 2016; Spagnuolo et al. 2016; Rahimifard et al. 2017; Rose et al. 2021). These have been summarized in Table 4.1. Phytochemicals like lipoic acid, allyl isothiocyanate, and ginsenoside Rb1 have been also shown to alleviate inflammatory responses via their antioxidant activities against NADPH (nicotinamide adenine dinucleotide phosphate), oxidase, and NOS (nitric oxide synthase) (Welcome 2020). Therefore, these phytochemicals provide hope for further therapeutic advances in treating neuroinflammation and oxidative stress caused by different disease conditions.

4.2 Phytochemicals in Food and Flavor

Phytochemicals have been widely being employed in food and flavoring to enhance the nutritional, sensory, and storage profile of food products (Valverde 2013). Technically, only nontoxic substances can be used as additives in food at a recommended concentration (Nohmi 2018). The safety and regulation for their

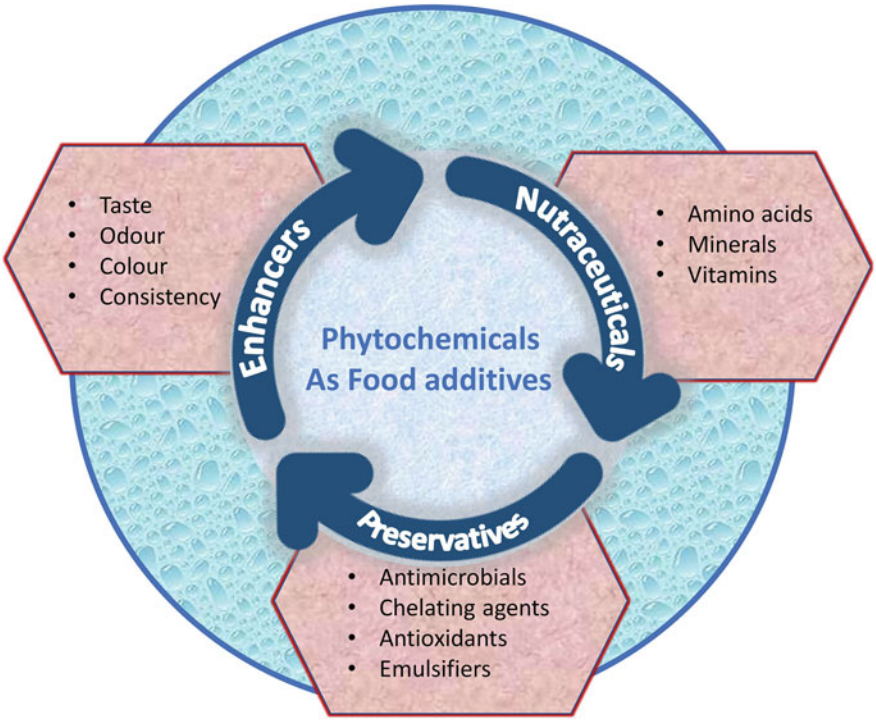

Fig. 4.6 Various aspects of the use of phytochemicals as food additives

commercial usage are generally governed by the food safety authorities. The United Nations Food and Agriculture Organization (FAO) and World Health Organization (WHO) have a joint committee of experts in food additives (JECFA) at international level (Valverde 2013). Food additives are generally classified on the basis of their technological usage or their biochemical characteristics (Fig. 4.6).

Phytochemicals employed in food processing are mainly derived from hydrocarbon esters pyranose, volatile sulfur compounds, pyrazines, carbonyl compounds, furanones, and terpenes (Valverde 2013). Further, to enhance the sensory value of food, many phytochemicals are used in food processing as coloring substances. During processing or storage, these coloring substances are used for adjusting or correcting food discoloration or color change. Anthocyanins, betaines, chlorophylls, and carotenoids are the four main classes of phytochemicals which are used as coloring additives (Valverde 2013).

Moreover, in order to extend the shelf-life of food products, phytochemicals rich in antioxidant (gingerol, catechins, carnosic acid), emulsifying/stabilizing (tocopherols, reduced glutathione, Kojic acid, etc.), and antimicrobial properties (allyl isothiocyanate, saponins, sulfur-rich garlic extract, etc.) are widely preferred as additives (Wang et al. 2010; Valverde 2013) (Table 4.2).

Table 4.2 Important phytochemicals from various plants used in food and flavoring

Plant name	Phytochemical	Food/flavoring property	References
Brassicaceae	Volatile sulfur compounds	Flavoring agent	Valverde (2013)
	Allyl isothiocyanate	Shelf-life	Wang et al. (2010)
	Zeaxanthin	Antioxidants	Xiao et al. (2019)
Allium sativum	Volatile sulfur compounds	Flavoring agent	Valverde (2013)
	Diallyl sulfide and diallyl disulfide	Shelf-life	Saladino et al. (2017)
Rosmarinus officinalis	β-Caryophyllene	Flavoring agent	Valverde (2013)
Stevia rebaudiana	Steviosides (diterpene glycosides)	Sweetening agent	Valverde (2013)
Glycyrrhiza glabra	Glycyrrhizin	Sweetening agent and shelf-life	Pastorino et al. (2018)
Daucus carota	Carotenes	Coloring agent and emulsifiers	Valverde (2013)
Camellia sinensis	Catechins	Antioxidants	Valverde (2013)
Zingiber officinale	Gingerol	Antioxidants	Si et al. (2018)
Dioscoreophyllum volkensii	Monellin	Sweetening agent	Valverde (2013)
Pentadiplandra brazzeana	Pentadin and brazzein	Sweetening agent	Valverde (2013)

Phytochemicals obtained from plants and fruits also have great sweetening properties, and interestingly many of these compounds used for sweetening are not sugars but proteins like monellin, pentadin, and brazzein. Pentadin and brazzein are one of the highly stable sweetening agents, exhibiting activity retention at even 98 °C (Valverde 2013). Besides, there are many other plant compounds that can be used as sweeteners, for example, monatin isolated from *Sclerochiton ilicifolius*, a South African plant (Abraham et al. 2005). Glycyrrhizin, a triterpenoid obtained from the root of liquorice, is another sweet tasting compound. These have been summarized in Table 4.2.

Furthermore, phytochemicals are being enormously exploited for their application as nutraceuticals. Several commercial food products like soft drinks/beverages, fermented milk, yoghurt, smoothies, condiments, ready meals, bakery, cheese, etc. have been fortified with phytoactive compounds for health benefits. The consumption of β-glucan-enriched oats to lower blood cholesterol levels has been approved by the EFSA (European Food Safety Authority) commercially (EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA) 2010). Phytosterol-rich food products have also shown good results in clinical trials for lowering blood cholesterol levels. Moreover, isoflavones (estrogens of plant origin) have been claimed to prevent CVDs, hormone-associated cancers, cognitive decline, and osteoporosis, as well as for treating menopausal symptoms (Valverde 2013). In order to target the consumers, some drink/beverage companies are also using polyphenols as

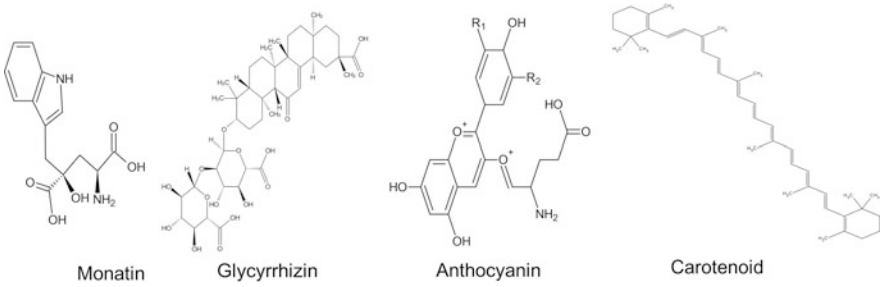

Fig. 4.7 Chemical structures of some food additive phytochemicals

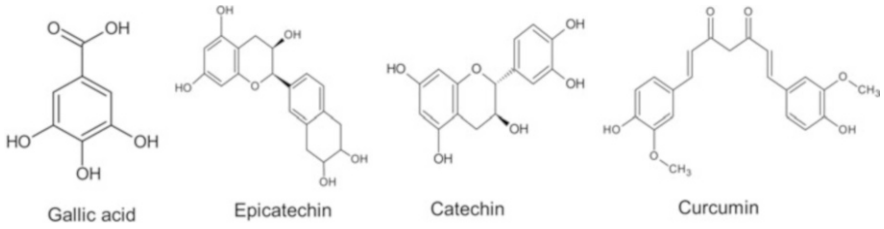

Fig. 4.8 Chemical structures of some phyto-cosmeceuticals

supplements in their existing products. For example, the "Diet Coke plus Antioxidants" has been successfully launched by the Coca-Cola company in many countries to attract the healthy products' market. The major group of phytochemicals employed in food processing and flavoring are represented in Fig. 4.7.

4.3 Phytochemicals as Cosmeceuticals

The cosmetic industry is a multibillion-dollar, evergreen industry with specialized products adding up almost every year. Phytoactive compounds, a treasure of mother nature, have been used in cosmetics and personal care regimes for decades. Different types of phytochemicals, namely, gallic acids, epicatechin, glabridin, catechin, curcumin, dynamic and hydroxybenzoic acids, carotenes, fatty acids, and complex polysaccharides have been categorized as high-value cosmeceutical ingredients (Valverde 2013; Ganesan and Choi 2016) (Fig. 4.8).

They have potential for application in beauty products like sunscreen, moisturizers, and antiaging cream and in many skin-based therapies (Cefali et al. 2016). These bioactive compounds improve the technical and sensory attributes of the cosmetic formulations and protect the skin from harmful ultraviolet radiations of the sun (Desam and Al-Rajab 2021). In addition, some of these phytocompounds like phenylethanoid glycosides are known to exhibit antiseborrheic (preventing seborrheic dermatitis) properties and thus are of great use in the cosmeceuticals for

Table 4.3 Important phytochemicals from various plants used in cosmetic industry

Plant name	Phytochemical	Cosmetic applications	References
Glycyrrhiza glabra	Licochalcone A	Depigmenting ability, hair care, and anti-acne	Pastorino et al. (2018), Cerulli et al. (2022)
	Glabridin	Depigmenting ability, UV protection, and anti-inflammatory	Veratti et al. (2011), Pastorino et al. (2018)
	Dehydroglyasperin C	Anti-wrinkle activity	Cerulli et al. (2022)
Curcuma longa	Curcumin	Antiaging, anti-wrinkle, and skin regenerative activity	Rafiee et al. (2019)
Camellia sinensis	Catechins	UV protection and antioxidant activity	Bae et al. (2020)
Rosa sp.	Gallic acid	Antiaging and antioxidant activity	Khan et al. (2018)
	Quercitrin	Antiaging, skin whitening, and antibacterial products	Li et al. (2021)
Solanum lycopersicum	Lycopene	UV protection and antioxidant activity	Islamian and Mehrali (2015)

the development of natural beauty products (Korkina 2007). The sources, properties, and applications of these phytochemicals in cosmetic industry are summarized in Table 4.3.

However, one of the major constraints of plant-based cosmetics is their low penetration and instability. In this context, nano-sized phyto-cosmeceuticals have also gained considerable significance as active skin care ingredients. Solid lipid nanoparticles, ethosomes, transfersomes, fullerenes, carbon nanotubes, and nano-structured lipid carriers are some of the emerging technologies currently being employed for enhancing the efficacy of phytochemicals as skin care products (Ganesan and Choi 2016). These nano-sized phytoconstituents enhance bioavailability of the compound to the skin and impart protection against aging-related issues (Ganesan and Choi 2016). So far, aloe vera, quercetin, vitamins C and E, resveratrol, and green tea nano-formulations have been successfully developed for applicability as lotions, gels, lip creams, and skin and hair care products for sustained effects. The results seem promising; however, further research on their exact mechanism of action is imperative to target site release from these nano-delivery agents.

5 Conclusion and Future Directions

Nature is truly a repository of countless bioactive compounds (phytochemicals) bestowed with distinctive chemical structures, giving rise to significant medical and industrial prospects. Phytochemicals like terpenes, caffeine, lectins, carotenoids, polyphenols, essential oils, etc. have been studied widely for their flavoring/additive properties. Meanwhile, certain derivatives of these natural products, like vinca

alkaloids, silymarin, quercetin, anthraquinones, curcumin, betulinic acid, resveratrol, etc., have already demonstrated notable pharmacological activity in treatment of cancers, cardiovascular diseases, diabetes, viral disorders, bacterial disorders, neural disorders, and inflammation individually as well as adjuvants of standard clinical formulations. These phytoconstituents are also being evaluated for their efficacy as natural beautifying agents. Although the prospects for application of phytochemicals have broadened, extensive research on their bioavailability, extraction, and toxicity, exact mode of action in cellular processes is the main challenge being faced for their commercialization and reach to clinics. The usage of novel extraction processes including recovery from wasted food, chemical analogs, synergistic therapies, and nanoparticle-based delivery mechanisms has aided in overcoming these bottlenecks to an extent. However, a comparative account on the relative in vivo interactions of phytochemicals, namely, phytochemical-phytochemical interaction and phytochemical-drug interaction, remains an important aspect which should be addressed in the near future to harness the biopotential of phytochemicals in biomedical sciences. Furthermore, employment of high-throughput computational tools like immunoinformatics, docking, and molecular dynamic simulation of nanoformulations could aid in designing novel biocompounds and take ahead the legacy of Siddha, Ayurveda, Unani/traditional formulations to treat different disorders via clinical validation in the near future and for production of high-value industrial products.

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Chapter 5
Technological Advancements for the Analysis of Phytochemical Diversity in Plants

Arun Kumar Kashyap, Thattantavide Anju, Sumit Kumar Dubey, Ajay Kumar, and Sushil Kumar

1 Introduction

Plants are a source of valuable products since time immemorial. Plants produce various molecules which are essential for their proper functioning, growth and development. Thousands of molecules, commonly known as phytochemicals, play a crucial role in the various functions of plants. Phytochemicals involve in the defence mechanism, cellular integrity and signal transduction (which act as signals) of the plants by controlling the gene expression, stability of proteins and metabolome flux (Tugizimana et al. 2013). The phytochemicals are also known to help the plants to fight against various environmental threats (Molyneux et al. 2007). The essential functions of the plants are carried out by the constant production of these phytochemicals inside the plant cell. Several phytochemicals were isolated and characterized from different vegetables, nuts, beans, fruits, seeds and grains (Mendoza and Silva 2018).

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M. K. Swamy, A. Kumar (eds.), *Phytochemical Genomics*,
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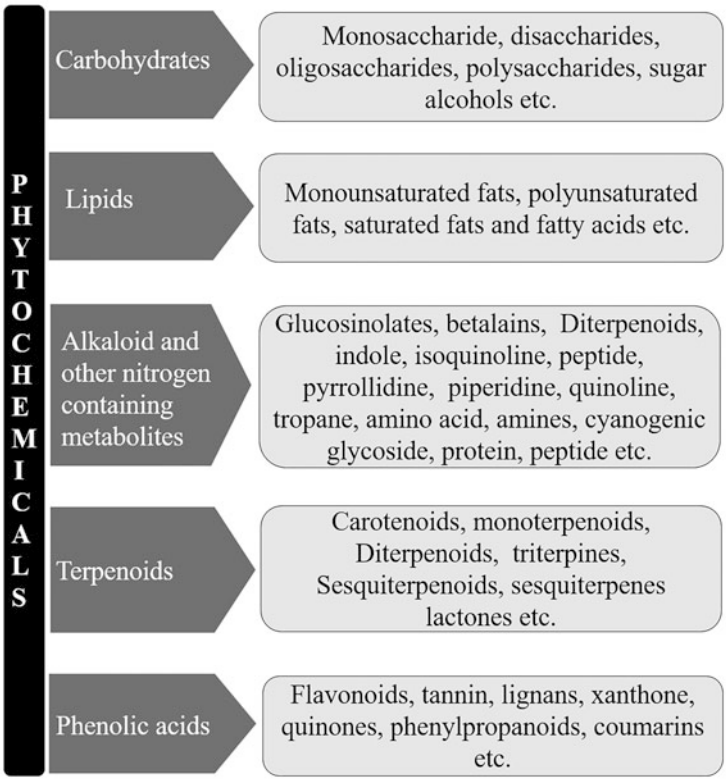

Fig. 5.1 Various categories of phytochemicals (Adapted and modified from Huang et al. (2016))

The plants produce many types of phytochemicals for various functions. Due to the diverse structures and forms of the phytochemicals, a concrete classification of phytochemicals has not been done. However, the phytochemicals are broadly divided into primary and secondary metabolites. Molecules, such as sugars and proteins, essential for growth, nutrition and other plant development, are considered as the primary metabolites. Molecules that take part in various defence responses are classified as secondary metabolites. Secondary metabolites consist of many types of molecules like alkaloids, terpenoids, phenolic compounds and flavonoids (Lampe and Messina 1998; Ramawat et al. 2009). The phytochemicals are classified into several further classes. The classification of the phytochemicals is given in Fig. 5.1 (Asfaw and Demissew 1994). These phytochemicals are not only beneficial to plants but also for human beings. Approximately, more than 20% of plants are known to play a major role in the health of humans by playing a crucial role in the treatment of chronic diseases such as cancer (Boots et al. 2008). Some studies prove that the phytochemicals found in plants can reduce the risk of cancer and other chronic diseases caused by the oxidative damage of the tissue (Lone and Lone 2012) because

compounds like vitamins A, C and E, phenolic compound, lignin, flavonoids and tannins act as excellent antioxidants (Suffredini et al. 2004). Besides that, phytochemicals work as a synergistic agent to help the body in the efficient intake of nutrients.

Phytochemical production varies depending upon the species and types of plants. In a few plants, excessive production of phytochemicals takes place whereas, in others, it produces in minimum quantity. Biapa et al. (2007) reported that more than 5000 phytochemicals are isolated and characterized with their possible health benefits. To assess the functional diversity of plant genera, the quantification of phytochemical diversity has seemed to be a hurdle towards the comprehensive understanding of the consequences associated with variation in phytochemicals across them. Phytochemical diversity explores the richness of particular biologically active ingredients, their relative abundance and the complexity of secondary metabolites at the molecular level (Richards et al. 2015). Sometimes, the phytochemical diversities of certain plant families and species are linked with the herbivores. A study was conducted with the piper (which belongs to the Piperaceae family) to assess the influence of phytochemical diversity on herbivore diversity (Richards et al. 2015). They recorded that the phytochemical diversity is positively linked with herbivores, and it reduces the herbivore damage as well. The literature also explained that the extensive diversity in phytochemicals attracts a more specialized crowd of herbivores that cascade the affirmative effect on herbivore opponents. Hence this represents positive associations between phytochemical and insect herbivore diversities in the ecosystem (Richards et al. 2015). The phytochemical diversity is closely connected with the ecology and taxonomic scales that can further be utilized for the conservation of taxonomic diversity (Sultan et al. 2008). The ecological perspective of phytochemical diversity between plants has been studied by chemical ecologists, but due to the lack of well-defined technologies for the characterization of molecules, the thorough study of the link between phytochemicals and ecology was still restricted (Nishida 2014). The phytochemical diversity is also an important trait to determine the functional roles of ecological flora in natural ecosystems and community-based natural resource management (Loranger et al. 2013). The measurement of phytochemical diversity is directly linked with various biodiversities (i.e., functional and trophic-level) (Firn and Jones 2003). The process of identification, characterization and separation of phytochemicals uses various techniques. It has been considered that the birth of phytochemical analysis was through the isolation of tartaric acid from the fruits of grapes in 1769 by Carl Wilhelm Scheele (a Swedish chemist) (Cordell 2011). Later, lot of research was conducted on the field of phytochemicals by the scientific community. The analysis of phytochemicals in the plants helps to understand the therapeutic potential, thereby giving an idea regarding the compound for semi-drug discovery (Pant et al. 2017). Besides that, it gives an idea regarding the overall cellular mechanism of the plants, nutritional significance and potential for the production of value-added products from the plants, a mechanism behind abiotic and biotic tolerance. The present book chapter gives a brief introduction to the various technologies used for phytochemical analysis.

2 Analysis of Phytochemical Diversity

Phytochemicals are natural compounds found in plants that take part in different responses to adverse environmental conditions. Since phytochemicals are compounds of prime importance and play a diverse role in various functions of plants, many studies deal with the analysis of the types and characteristics of phytochemicals. Still, the scientific community lacks complete information regarding the phytochemical diversity across ecosystems and species (Defossez et al. 2021). The emergence of metabolomic tools and other analytical methods fuelled the discoveries of phytochemical diversity (Wetzel et al. 2019). The major steps in the fundamental phytochemical analysis include the selection of plants, harvest, cleaning, drying, powdering and then extraction by employing different chemicals in combination with several techniques (Koparde et al. 2019). In the case of whole metabolomic analysis, it includes other advanced steps after the acquisition of data through analysis, that is, data mining and data integration (Vinay et al. 2021). Data mining is carried out by preprocessing of data, annotation of data and statistical analysis. The pathway or network analysis of the phytochemicals is derived through the data integration step (Vinay et al. 2021). Through metabolomic analysis, it is possible to gather ideas about all metabolites present in the sample. Various analytical techniques are applied to collect the data regarding metabolites in the plant which are further processed through the software. Various steps involved in metabolomic analysis and details about software and databases for data mining and data interpretation are given in Fig. 5.2. The techniques used in metabolomics are discussed in the upcoming sessions.

Different plants contain a wide array of phytochemicals. Hence, plant selection is the most crucial step for phytochemical analysis (Lahlou 2007). The plants with medicinal, nutritional, toxic properties and several other properties are selected according to the types of phytochemicals and purpose of the study for the analysis (Altemimi et al. 2017). The plant collection for the analysis could either be from the wild or the conservation areas like botanical gardens, nurseries or the garden. During plant collection, it is essential to select an appropriate plant and establish its identity and keep the record in the form of the herbarium. Proper cleaning of the samples to remove dust and other particles is essential before sample processing. For the long-term storage of the plants, water should be removed from the tissues (Jones and Case 1990). Different drying methods can be applied for this purpose such as naturally by placing the plant under sun rays or by drying through ovens or shade drying or by freeze-drying. Freeze-drying is an appropriate method for the analysis of compounds that degrade after harvest (Saifullah et al. 2019). Yuan et al. (2015) reported that drying could influence the quantity and quality of phytochemicals. After drying, grinding of samples is required to increase the surface of the reaction (Kim and Verpoorte 2010). The high surface of the plant samples results in high dense packing. Therefore, grinding the samples to fine powder with millers and blenders is ideal for analysis (Banu and Cathrine 2015).

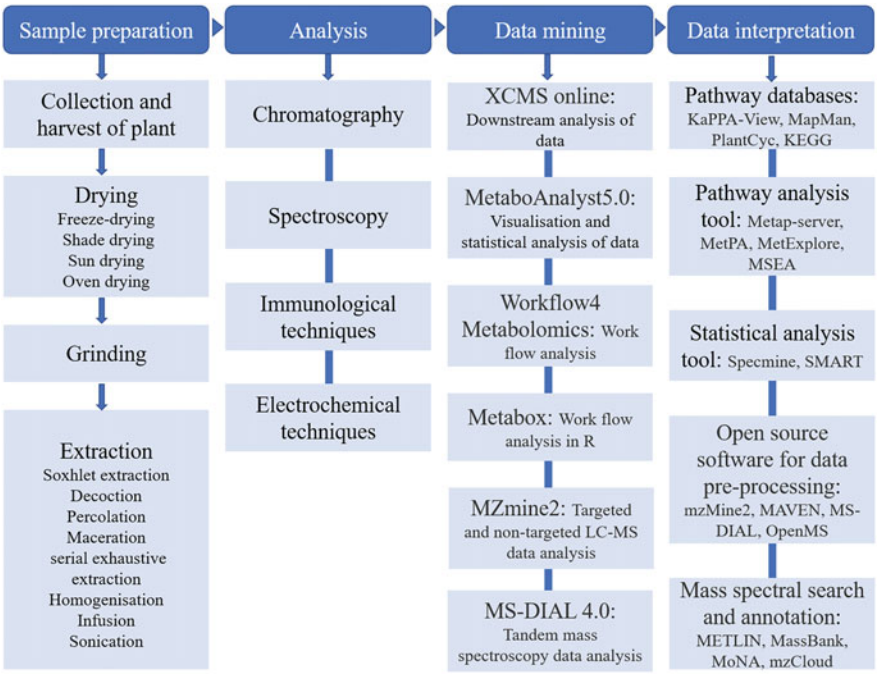

Fig. 5.2 Steps in metabolomic analysis (Adapted from Vinay et al. (2021).)

The grinding of the samples is followed by the extraction of the particular compound.

Extraction can be done by several methods like Soxhlet extraction, decoction, percolation, maceration or serial exhaustive extraction using different solvents, homogenization, infusion and sonication (Banu and Catherine 2015). The phytochemicals have specific biological activity and chemical properties; thus, making the choice of solvent is extremely important (Pandey and Tripathi 2014). The phytochemicals must not react and change properties after the addition of the solvents. An ideal solvent must evaporate with ease and be less toxic, should be able to dissolve a large number of compounds and preserve the compounds in their natural form (Ballard et al. 2010). Chloroform, acetone, water, alcohol and ether are commonly used solvents for different extraction purposes (Altemimi et al. 2017). After extraction, distinct techniques or technologies are employed to identify and characterize the phytochemicals. Ruan et al. (2008) found that methanol and acetone are effective in the extraction of phenolic compounds. Extraction of the bioactive compounds from plants by using solvents is dependent on the polarity of the solvent. Different combinations of solvents and methods are used in extraction to increase the efficiency of extraction (Jha and Sit 2022).

3 Technologies Used in Detection and Characterization of Phytochemicals

Several novel and advanced techniques for the isolation and purification of bioactive compounds (Altemimi et al. 2015, 2017) provide a faster method of isolation, purification and identification of bioactive compounds. (Mulinacci et al. 2004). To identify the bioactive compounds, generally, the in vitro methods are used than the in vivo methods because of the convenience of the in-vitro assay methods. The complexity in the properties and types of biomolecules present in different organs of the plants makes it difficult to optimize the technique and procedure for the identification and characterization of bioactive compounds (Adlercreutz et al. 1993). As mentioned above, the selected plants are subjected to extraction after sample collection by various techniques. Simple chromatography techniques such as column chromatography further fractionate the crude extracts. More advanced techniques like high-performance liquid chromatography (HPLC) provide an efficient and more convenient technique for the purification of bioactive compounds (Bradley and Desai 2000). Purification is the first step for the identification of bioactive compounds. Column chromatography and thin-layer chromatography (TLC) are used extensively for purification because of the diversity of the stationary phase and convenience (Zhang et al. 2005). Besides that, the detection of bioactive compounds can be achieved with various spectroscopic techniques like nuclear magnetic resonance (NMR), infrared (IR) and UV-visible and mass spectroscopy (Popova et al. 2009).

The phytochemical diversity has been precisely measured by the advanced modified chromatographic and spectroscopic tools in combination such as gas chromatography-mass spectroscopy (GC-MS), high-performance thin-layer chromatography (HPTLC), HPLC, optimum performance laminar chromatography (OPLC) and liquid chromatography-mass spectroscopy (LC-MS), UV spectroscopy, IR spectroscopy and NMR, namely, ^{1}H-NMR full spectra and ^{1}H-NMR downfield and X-ray diffraction (XRD) (Bernal et al. 2011). The aforementioned advanced technologies are widely used for the qualitative and quantitative analysis of phytochemicals. The words "analyte, compound, element, molecule, sample and substance" are interchangeably used to represent the phytochemicals characterized using these advanced analytical tools. Figure 5.3 represents a generalized outline of metabolomic workflow.

3.1 Chromatography

Chromatography is one of the prime techniques used for phytochemical analysis and used for the isolation of compounds present in relatively lesser quantities (Scott 2003). Ion exchange, paper chromatography, partition, size exclusion and surface adsorption and thin layer are the core methods that were further modified during the

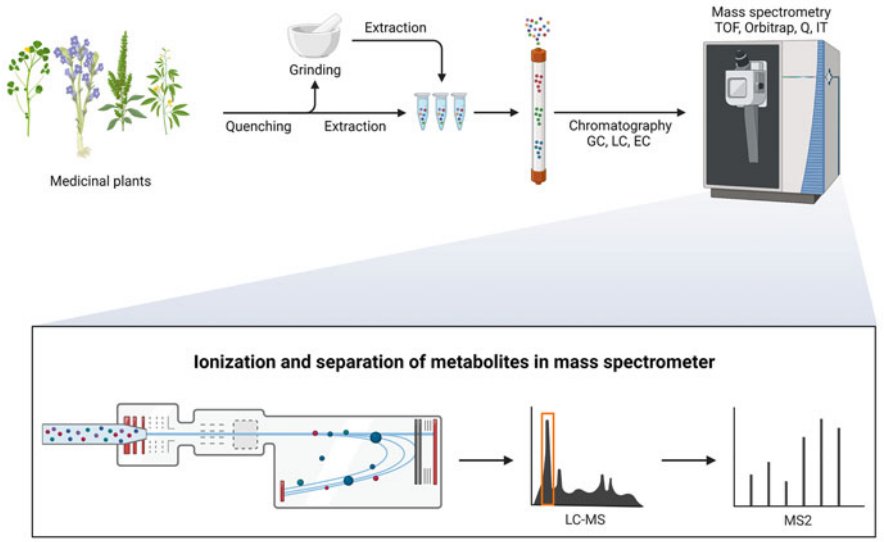

Fig. 5.3 General metabolomic workflow for the analysis of metabolites from medicinal plants (Created with BioRender.com)

advancement of techniques (Coskun 2016). The phytochemicals can qualitatively and quantitatively be analysed using advanced chromatographic techniques with mass spectroscopy (MS). The working principle of chromatography is the separation of compounds in a mixture on a surface of a stationary phase while moving through a mobile phase. The separation is based on the adsorption, partition, molecular weight difference and affinity properties (Coskun 2016). But gas chromatography has different principles. Photometric detector, refractometric detector, gas ionization detector system, polarographic detector, colorimetric detector and radiometric detector are the common types of detectors used in chromatography, and the selection of the detector is based on the character of the separated compound (Huber 1969). The GC-MS is based on the mass to charge ratio and apply to identifying and measuring volatile compounds (Janes et al. 2009). The GC quantifies the total amount of vapour generated by the analyte (sample) (Lisec et al. 2006). The GC-MS has used mainly two detectors, that is, thermal conductivity detector (TCD) and flame ionization detector (FID). The FID can ionize organic compounds by hydrogen-air flame, whereas TCD detects the differences in thermal conductivity between the carrier gas, for example, N_2 and Ar, and the target molecule to be detected (Tvrzická et al. 2002). Müller et al. (2002) developed a multiplex, sensitive, single-run GC-MS/MS method for the quantification of acidic phytohormones in the plants. Similarly, several analytical methods can be developed for the analysis of phytochemicals for obtaining precise information regarding the regulation of plant physiological responses. The major benefit of such advanced integrated techniques is their ability

to detect sensitive and relatively very small-sized compounds (Tugizimana et al. 2013).

Another popular method in chromatography is HPLC. Solvent-soluble compounds are analysed using the HPLC (Schmeisser et al. 2005). HPLC is used for the separation and detection of biochemicals that cannot be vaporized or decomposed under high temperatures (around 400 bars) (Luewisutthichat 2011). HPLC offers both quantitative and qualitative analyses simultaneously. A photodiode array (PDA) chip helps sense a wide range of wavelengths concurrently (Waksmundzka-Hajnos and Sherma 2010). C18 columns are preferably used because they can separate a wide range of hydrophobic ions and offer a high surface area during separation. HPLC tenders to the separation, identification and quantification of each component present in the sample (Czaplicki 2013). The other popular method in chromatography is HPTLC, which is an upgraded version of thin-layer chromatography (TLC). HPTLC has a pre-coated layer of sorbent with 150–200-μm thickness and 5–7-μm particle size because the reduction in thickness layer and particle size increase the separation efficiency of the TLC plate. HPTLC proposes qualitative and quantitative estimation and micro-preparative-based purification.

Overpressured-layer chromatography (OPLC) and high-speed counter-current chromatography (HSCCC) are also different types of chromatographic methods used in phytochemical analysis. OPLC is a combination of TLC and HPLC that simultaneously can process up to 4–8 samples (Tyihák et al. 2012). OPLC pump provides 50 bars of pressure for effective separation and purification of analytes. OPLC unit equipped with diode array detector to monitor the separation and purification of the analyte. HSCCC makes use of liquid stationary-phase thereby; it does not suffer from irretrievable adsorption (Khan and Liu 2018), which is the common issue raised with traditional chromatographic techniques. The ultra-high-performance liquid chromatography-mass spectroscopy (UHPLC-MS) method is used to estimate the drugs in bulk formulations. It is the same as HPLC, but due to the small particle size of sorbent used in the column, high pressure (6000 psi) is required for the analysis (Proch and Niedzielski 2021). In another technique, droplet counter-current chromatography (DCCC) does not use a solid stationary phase; thereby it prevents irreversible adsorption of analytes and it is most suitable to characterize polar compounds (Nahar et al. 2020). Therefore, the proper application of chromatographic methods for the phytochemicals can help to carry out the analytical research on pharmacology, drug development and nutritional screening.

3.2 Spectroscopy

The phytochemicals can be detected and identified using spectroscopic tools and techniques. Spectroscopic techniques majorly measure the spectra produced through the interaction of a compound with electromagnetic radiation or the emission of electromagnetic radiation by the compound (Chandarana et al. 2021). There exist different kinds of spectroscopic techniques based on the region of wavelength used,

nature of compound analysis and type of interaction involved (Kumirska et al. 2010). The techniques include atomic absorption spectroscopy (AAS), atomic emission spectroscopy (AES), UV-visible spectroscopy, infrared spectroscopy (IRS), mass spectroscopy (MS), fluorescence spectroscopy, nuclear magnetic resonance (NMR), surface plasmon resonance (SPR), Fourier transform infrared (FTIR), X-ray crystallography and X-ray diffraction (XRD).

The widely used method for the quantitative estimation of elements is AAS (especially metal ions) on the basis of absorbing patterns of optical radiation by free atoms. However, it is not much applicable in phytochemical analysis. In certain cases, the metal toxicity in the plants can be measurable by AAS (Anal and Chase 2016). AES is also a similar method that detects the emitted light intensity at a particular wavelength and can determine the concentration of the target element in a sample. Sometimes it is also called optical emission spectrometry (OES) (Hieftje 2000).

UV-visible spectroscopy techniques are popularly used for the identification of compounds of specific classes and for qualitative identification (Passos and Saraiva 2019). Plants contain a high amount of aromatic compounds which makes it suitable for the identification of classes of phytochemicals. The ultraviolet (UV) spectroscopic apparatus is able to detect conjugated *pi*-electrons and effectively characterize pigments and other phytochemicals (Lozada-Ramírez et al. 2021). UV spectroscopy efficiently estimates inorganic elements and compounds. It measures the absorbance by Beer-Lambert's law. The colorimetric methods rely on the complex formation, oxidation-reduction and catalytic effect during the sample processing and reaction. This is the most economic method for phytochemical analysis. Another technique fluorescence spectroscopy is based on the emission of fluorescence (luminescence) from the sample molecule upon excitation in a particular wavelength and generates a fluorescence peak (Romani et al. 2010). The limitation of this analytical technique is that the analyte must have fluorescent properties or possess some compatibility to produce fluorescent that can be detectable. Besides, it is a highly specific, sensitive and rapid analytical technique.

Infrared (IR) is most often used to mark out functional groups present in the sample on the basis of wavelength and intensity. Mid-infrared light excites molecules to higher energy levels, and the IR absorption band generates that characterizes the types of bonds present in the analyte (Van Eerdenbrugh and Taylor 2011). The molecules needed to analyse and absorb specific frequencies, and those frequencies are characteristic for their structure which is correlated in IR spectra. IR is most often utilized for the qualitative analysis of organometallic molecules/compounds (Utami and Siburian 2016). Fibre-optic probes are used in the near-infrared (NIR) analytical assay. Mid-infrared (MIR) is rarely used for the detection of functional groups. For this purpose, FTIR is used (Kadhim et al. 2016). FTIR spectrum is used for sensitive and rapid qualitative analysis. But, unlike XRD, it is a destructive method and CO_2 and H_2O sensitive (Simonescu 2012). FTIR is a dispersed method that is performed in a broad-spectrum frequency. FTIR assesses a wide range of wavelengths that are absorbed by analytes in the infrared region. The interferometer device integrated into FTIR identifies the analyte by generating optical signals in a wide range of IR

frequencies. The advantages of FTIR are spectral quality, rapidness and reproducibility. The potassium bromide (KBr) is used as a carrier due to its optical transparency for the sample under the IR spectrum, and hence it is not interfering with the absorbance of the sample/analyte. FTIR has limitations because of its relatively small-size chamber, and due to this, the mounted pieces can obstruct the IR beams. Attenuated total reflection-Fourier transform infrared (ATR-FTIR) is used for quantitative analysis over FTIR alone (Kadhim et al. 2016). ATR-FTIR offers non-destructive measurement of samples (Melucci et al. 2019).

Mass spectroscopy (MS) is an efficient method to determine the molecular weight of a sample (Baghel et al. 2017). MS serves as an important tool for the identification of biological compounds because it can identify and characterize, from small molecules to large complex protein molecules. MS is often integrated with GC for the accurate characterization of biological samples. MS spectrum is used to estimate the molecular weight of the analyte. Some modified MS can generate structural and chemical information. Capillary electrophoresis-mass spectrometry (CE-MS) has high resolving power and sensitivity (Lone and Lone 2012). It requires only a nano-litre volume of sample for analysis and also provides rapid analysis. Electrospray ionization is used to stimulate ion emission (Maxwell and Chen 2008). Certain modified CE-MS integrated with matrix-assisted laser desorption ionization (MALDI) is used for better molecular characterization (Zhang et al. 2005; Xu et al. 2017). MS is sometimes not considerable for nucleic acid and protein molecules, although it is most often used for phytochemical analysis and characterization. GC-MS technology has been widely used for the identification of compounds in several plants such as *Evolvulus alsinoides*, *Mentha piperita*, *Origanum dictamnus*, *Teucrium polium*, *Lavandula vera* and *Lippia triphylla* (Gomathi et al. 2015; Gherman et al. 2000; Proestos et al. 2006).

Nuclear magnetic resonance (NMR) provides a strong magnetic field to charged nuclei so that they shift to a higher energy state. ^{1}H NMR estimates the types and amount of hydrogen atoms residing in the sample molecule/compound, while carbon-13 NMR (^{13}C NMR) is used to find out the type and amount of carbon atoms in the sample (Pauli et al. 2014; Clendinen et al. 2014). NMR is able to characterize the physical, chemical and biological properties of the molecules. The simple molecular elucidation is carried out by one-dimensional NMR (1D NMR) spectroscopy, whereas the complex molecules are characterized using two-dimensional NMR (2D NMR) spectroscopy with wider frequency. Some 2D NMR spectroscopic methods are nuclear overhauser effect spectroscopy (NOESY) with ^{15}N-nuclear Overhauser effects (NOEs), correlation spectroscopy-92 (COSY^{92})/dynamic light scattering (DLS)/quasielastic light scattering (QELS), hetrocorelation spectroscopy (HETCOR) with ^{31}P cross-polarization magic angle spinning (CPMAS) spectra and total coherence transfer spectroscopy (TOCSY) with total spin coherence (TSC) (Claridge 2016). Other NMR methods such as ^{15}N-NMR and ^{31}P-NMR are also used to detect biological substances. Solid-state NMR spectroscopy is used to characterize the solids. Time-domain NMR spectroscopy measures the time required for excited nuclei to return to the equilibrium state. It is used for rapid analytical applications and highly reproducible but it has low

sensitivity (Alfattani et al. 2021). Surface plasmon resonance (SPR) is another highly sensitive technique and is based on the equilibrium dissociation constant (Kd); it can measure picomolar volume samples, but the expensiveness limits its applicability (Minunni and Bilia 2010). However, it measures the molecular interaction not specifically identification or detection.

Other popular techniques for spectroscopy are X-ray crystallography and X-ray diffraction (XRD); both are almost similar but XRD has some advanced features over X-ray crystallography (Srikanth et al. 2020). X-ray crystallography uses X-ray beams to find out the atomic structure of a crystal, whereas X-ray diffraction (XRD) provides detailed information about the crystallographic structure and chemical composition of the molecules. X-ray crystallography based on the diffraction pattern generated from X-ray beam. The crystallographic data of atoms, molecules and other biological compounds are maintained by the Commission on Crystallographic Nomenclature (CCN). X-ray diffraction is used for the determination of the chemical composition of the molecules based on crystallographic data. This technique is highly reliable for proteins and other biological molecules (Srikanth et al. 2020).

3.3 Immunological Techniques

There are several immunological techniques used in plant metabolite analysis. Enzyme-linked immunosorbent assay (ELISA) is used to quantify biological molecules on the basis of antigen-antibody interaction which is visualized by chromophore (mostly enzyme) that produce the measurable coloured products (Clark and Engvall 1980). Similarly, Western blotting is used to detect proteins of interest from the complex mixture of proteins, and it requires only nanograms of the sample to characterize, but it is an expensive, time-consuming method and not capable to quantify the number of proteins in the samples (Mishra et al. 2017). A radioimmunoassay (RIA) is a very sensitive immunoassay that is used to measure substance concentrations in biological samples using radiolabelled molecules (Grange et al. 2014). Other immunological methods such as capillary electrophoresis immunoassay, chemiluminescence immunoassay, cloned enzyme donor immunoassay, flow injection immunoassay, fluoroimmunoassay and liposome immunoassay are documented for pharmaceutical drug identification and characterization (Uto 2014).

3.4 Electrochemical Techniques

The diversified phytochemicals in plants have also huge pharmacological applications despite their ecological and evolutionary importance. Electrochemical techniques, namely, voltammetry (potential difference), polarography (electrolysis, oxidation-reduction), amperometry (potentially generated between polarizable and nonpolarizable electrodes) and potentiometry (potential between two electrodes), are

majorly used in the drug assay (Aboul-Enein and Ozkan 2012). A review published on analytical techniques in pharmaceuticals mentioned the routine analytical methods prescribed for assaying bulk drug materials. The routine analytical methods include AAS, argentometry, complexometry, fluorimetry, GC, gravimetry, HPLC, indicators, IR, microbiological assay (antibiotics), NMR, polarimetry, polarography, potentiometric redox (i.e., iodometry and nitritometry), titration and UV-vis spectrophotometry (Siddiqui et al. 2017). The discovery and advancement of various analytical tools provide an opportunity to understand the phytochemical composition of the plants. In the case of whole metabolomic analysis, the whole data should be processed with various software and identification of metabolites from libraries. Later, various statistical tools are implemented to derive biologically relevant information from the analysis and pathway interpretation of the metabolite (Booth et al. 2011).

4 Major Challenges in Phytochemical Analysis

The complexity of metabolites that exist in the plant tissues is acting as a major hindrance for the analysis, separation, purification and characterization of phytochemicals (Vinay et al. 2021). The diversity among plants also demands more specific modification in extraction and separation techniques, which act as another barrier in the research on phytochemicals (Yoo et al. 2007). Another challenge is the requirement to carry out the analysis in very few quantities which can affect the accuracy of the results (Belani and Kaur 2018). In the case of metabolomics, the constant change in the metabolite synthesis and accumulation inside the plant cell due to ecological changes, nutritional availability and abiotic and biotic stress results in non-equilibrium of the metabolome. Therefore, the quantity and quality of the phytochemicals analysed are dependent on the ecological and climate factors of the place and time of collection which reduce the reproducibility of the results (Khakimov et al. 2014). The expensive analytical tools are also a major challenge for the researchers who work in developing and developed countries (Khakimov et al. 2014). Therefore, it is essential to develop a comprehensive statistical and analytical method by understanding the multivariate property of the biological system and gaining more experience. To overcome these challenges, innovations in the detection, quantification and isolation techniques of the phytochemicals are needed.

5 Conclusion and Future Perspectives

Plants have been used for different purposes for a long time. The properties of different plant extracts or bioactive fractioned were utilized for human benefits without knowing the physical and chemical characteristics of its constituents. The

extraction and analytical techniques provide significant development for the advancement and quality control of different plant-based products. With the discovery of advanced analytical platforms, the identification and characterization of the molecules have become easier. The rapid growth in the field of metabolomics is providing more promise in phytochemical analysis. With the advancement in technologies, it has become more convenient to identify and utilize bioactive compounds on the basis of their chemical and physical properties. Due to the challenges such as the non-equilibrium of metabolites inside the cells, the diversity of phytochemicals from different species is demanding the integration of various analytical tools for the specific analysis of each plant sample. Rapid, precise and cost-effective methods should be developed to understand the therapeutic, nutritional, stress tolerance and value-added product synthesis capability of the plants.

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Chapter 6
Safflower (*Carthamus tinctorius*) Metabolites and Their Pharmacological Uses

Vimalraj Mani, Karthi Natesan, Jae-Won Choi, Mallappa Kumara Swamy, and Bala Murali Krishna Vasamsetti

1 Introduction

Carthamus is a complex genus of plants in the Compositae or Asteraceae family, comprising 15 different species of east Mediterranean origin. However, most of the species were classified under diploid, which includes three polyploidy species (*C. creticus* L., *C. lanatus* L., and *C. turkestanicus*) (Mani et al. 2020). From the ancient civilization, safflower has been mainly cultivated for its seed as it is considered the main source of edible oil and birdseed (Furuya et al. 1987). This crop has traditionally been produced for its blooms, which have commercial applications as dyes/natural colors and food flavors. The flowers used in a larger amount in these industries were being harvested by handpicking and used for the commercialization of synthetic aniline dyes. Safflower is grown in 60 countries throughout

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M. K. Swamy, A. Kumar (eds.), *Phytochemical Genomics*,
https://doi.org/10.1007/978-981-19-5779-6_6

the world, including Kazakhstan, followed by India, the United States, Mexico, Argentina, Turkey, and others (https://www.worldatlas.com/articles/world-s-top-safflower-producing-countries.html). Considering the socioeconomic importance, the commercial value of these particular oilseeds has been significantly increased in recent years; thereby its cultivation has increased to meet the high demand for oilseed crops to compensate for the lack of nutritional oil (Ghaderi et al. 2011). This chapter summarizes the metabolomic studies conducted on safflowers. Next to classical metabolomic studies, this chapter also focuses on several metabolomic-based working techniques, which were used to characterize different germplasm metabolomes to find preferred quality or at least to produce valuable medicinal products.

2 Factors Influencing Safflower Cultivation

2.1 Biotic Stress

Along with the growing world populace, there are more than a few enlightenments to consider about the future worldwide production of food and other products from crops. Meanwhile, the availability of terrestrial land across the world was decreasing due to global climate changes, soil erosion, lack of agricultural farming, and pathogens (Rosegrant and Cline 2003; Lobell et al. 2008). Safflower, on the other hand, is particularly susceptible to infections, such as bacteria, fungi, viruses, and nematodes. As far as we know, there is not much extensive research on understanding stress tolerance and disease resistance mechanisms in safflower (Ghaderi et al. 2011). The two most common and serious diseases affecting safflower plants are the fungal *Alternaria* (*Alternaria carthami*) leaf spot and bacterial blight (*Pseudomonas syringae*), often found on the same plant. Both diseases share the same symptoms of outsized, reddish to brown colored unequal lesions having yellowish to green edges on foliage and flower bracts, which turn to colorless with the time. Above-average rainfall is another factor that has resulted in a major loss of yield, and extended periods of high humidity may have contributed to a significant disease outbreak.

2.2 Abiotic Stress

The prime and severe abiotic stresses include salinity and drought stresses, and they are considered as a menace to crop yield, globally (Guo et al. 2014). These abiotic stresses also cause a major problem in producing and managing safflower in several parts of the ecosphere. To overcome water scarcity and to conserve freshwater sources, non-potable water, including reprocessed, effluent, or domestic water, could turn into a key resource of irrigation for safflower, predominantly in semidry and dry regions (Tuck et al. 2006).

2.3 Other Diseases

Other diseases occasionally cause problems with safflower plants, although they tend to be less serious. These include leaf spot disease (*A. carthami*) and safflower rust, caused by *Puccinia carthami* and *verticillium* and *Fusarium wilts*. These diseases are found primarily among commercial safflower crops and are seed-borne or airborne. Rust of safflower is caused by the fungus *Puccinia calcitrapae* var. centaureae (syn. =*P. carthami*). The life cycle of rust is complex and involves five different spore stages. The rust pathogen may overwinter and reappear the next spring, when teliospores undergo germination and form basidiospores, which then cause infection in safflower. Infected plants produce pycnia (yellow blisters on the superior part of foliage) and aecia (white cups) on the inferior part of foliage or stem nearby the soil line. Aeciospores move with the wind currents and infect newly planted safflower, resulting in summer spore production. Summer spores are produced in repeating cycles of progressive disease development throughout the summer. Summer spores are moved long distances (regionally) by the wind.

3 Omic Approaches

3.1 Genomics

Safflower is mostly a self-pollinating dicot crop with an estimated haploid genome size of 1.14 GB, and the crop is suffered from lacking technologically advanced genetic sources (Jhajharia et al. 2013). About 285 nucleotide sequences and 41,000 expressed sequence tags (ESTs) are testified in safflower via the subtractive genomic library and Compositae database. The genome size of safflower is 2.70 pg (2C value) (García-Moreno et al. 2010; Hamdan et al. 2011). SemBioSys has a program on safflower genomics for generating safflower bacterial artificial chromosome (BAC) library and EST library, isolation, and characterization of seed-specific promoters, oleosin, and other seed storage protein genes and genes involved in lipid metabolism. There is a need to develop microsatellite markers for use in variety identification, DNA fingerprinting, and genetic mapping studies. Microsatellites or simple sequence repeats (SSRs) are single-locus markers and are characterized by their hypervariability, abundance, uniform distribution throughout the genome, codominant inheritance, reproducibility, ability to automate assays, and transferability (Naresh et al. 2009). Isolation of SSR markers is a laborious, a time-consuming, and an expensive process. However, with the availability of ESTs for safflower, it is possible to identify genic SSRs enabling the mapping of genes of known function. The vast genomic resources from other Compositae members, namely, sunflower and lettuce, provide a potentially valuable source for mining SSR markers. The transfer success of *Helianthus* SSRs to safflower was 13% (http://cgpdb.ucdavis.edu/).

3.2 Transcriptomics

With the advancement of the high-throughput next-generation sequencing (NGS) tool, RNA-Seq (RNA sequencing) has been widely used to provide new candidate genes and validate and refine genetic simulations for biological and metabolic pathways in animals, plants, fish, and microbes (Hale et al. 2009; Bellin et al. 2009; Tang et al. 2011; Vasamsetti et al. 2021; Park et al. 2021). RNA-Seq-derived transcript sequences have helped in the annotation of functional genes involved in life processes (Novaes et al. 2008; Monnier et al. 2010). In recent times, certain sequencing by synthesis approaches, such as the Illumina Genome Analyzer, the Roche/454 Genome Sequencer FLX Instrument, and the ABI SOLiD System, is being developed by using NGS platforms (Li et al. 2012). Of these, the Roche/454-pyrosequencing platform is the effective sequencing approach for transcriptome research of unacquainted plant genomes (Rothberg and Leamon 2008; Sun et al. 2013). Also, Solexa/Illumina platform was used to sequence the safflower genome (Li et al. 2012). As a milestone, it has produced outsized clean sequencing reads that were utilized for de novo assembling and functional annotation of unigenes, that is, major genes unique to about-to-flower stages. The NGS data also provided about 1,140,594 clean reads, that is, 562,930 and 577,664 clean reads in complete and initial flowering phase samples, respectively. By de novo assembly, nearly 40,139 unigenes were assembled. The study results also showed that the sequenced reads can be related to those reads acquired via pyrosequencing in *Podophyllum hexandrum* (Deng et al. 2014) and *Lonicera japonica* Thunb (Muir et al. 2001). This omic study also includes that data of 51,591 mapped unigenes to 281 KEGG pathways and can be categorized into 43 key functional groups. This research data mentioned above would provide a basis for more investigations on secondary metabolic activities in safflower.

3.3 Metabolomics

The word “metabolome” describes the noticeable metabolites that occur within whole tissues. The metabolome of a plant is profoundly diverse and constitutes more than 200,000 metabolites (Fiehn 2002). Identifying and categorizing these metabolites are crucial for studying plants’ development and their response to ecological deviations. Metabolomics is an impending technique that mainly includes the identification and enumeration of metabolite levels across various biological species and different tissues. It is exceptionally treasured in discovering biomarkers and improving crop value and yield (Alseekh et al. 2018). Earlier investigations focused on various uses of metabolomics to crop researches that were based on several analytical techniques, such as mass spectroscopy and nuclear magnetic resonance (NMR). For a quantitative metabolomic study, mass spectrometry and NMR combined with gas chromatography-mass spectrometry (GC-MS) or liquid

chromatography-mass spectrometry (LC-MS) are a major choice (Lei et al. 2011). Nevertheless, at present, Fourier transform-ion cyclotron resonance-mass spectrometry (FT-ICR-MS) is widely employed as it offers the greatest potential of instantaneously detecting and identifying thousands of plant metabolites in precise high-throughput tests. Further, it provides an extreme resolution and ultrahigh mass precision effectively utilized in plant metabolomic studies (Aliferis and Jabaji 2012; Adrian et al. 2017; Maia et al. 2019).

4 Metabolomic Approach in Safflower

Metabolic studies of the chemical constituents, excluding pigments, reveal the occurrence of free sugars, phytosterols, organic acids, minerals, fatty acids, and polyphenols (Park and Park 2003; Liu et al. 2005). Early studies have revealed the intraspecific variation among the Chinese safflowers by using the molecular marker, amplified fragment length polymorphic (Zhang et al. 2006). Thus, safflowers can exhibit chemo-profile differences. Over 200 chemical constituents have been sequestered from safflower (*C. tinctorius*), and the most common ones include fatty acid, coumarins, flavonoids, and polysaccharides (Zhou et al. 2009). A research study has identified primary and secondary metabolites by using high-performance liquid chromatography/Q-Orbitrap mass spectrometry (UHPLC/Q-Orbitrap MS) from the florets of safflower, and the structure of hydrophilic and lipophilic constituents was recognized from cultivated *C. tinctorius* and wild *C. oxyacantha* safflower seed oils through LC-HRMS/MS (Chakradhari et al. 2020).

Oilseeds are a key source of vegetable oils, and their fatty acid structure, which varies greatly depending on the plant species, influences their applications, that is, whether they are employed for nutrition, industry, or medicine. Safflower oil contains two main unsaturated fatty acids. The unsaturated fatty acids oleic (18:1) and linoleic acid (18:2) make up around 90% of the total fatty acids in safflower oil, while saturated fatty acids like palmitic (16:0) and stearic acid make up the remaining 10% (18:0) (Weiss 2000). However, in many other studies, the fatty acid composition of safflower seeds has shown great variability (Velasco and Fernandez-Martinez 2001). Overall the safflower oil is used for medicinal as well as dietetic purposes (Han et al. 2009). Early studies reported the fatty acid compositions of 200 safflower accessions originating from 37 countries, indicating that linoleic acid and oleic acid have a remarkable range of differences, that is, between 3.9% and 88.8% and between 3.1% and 90.60%, respectively (Fernandez-Martinez et al. 1993). The fatty acid compositions among oils from cultivated and wild species were not very different, indicating that the seed oil of the wild safflower may be suitable for human consumption and industrial purposes. The oil content was in the range of 29.20–34.00%, 20.04–30.80%, and 15.30–20.80% in *C. tinctorius*, *C. oxyacantha* Bieb., and *C. lanatus* L., respectively (Sabzalian et al. 2008). Due to the high content of unsaturated fatty acids, countries, such as India, the United States, Mexico, Spain, and Australia, use them as cooking oil (Gecgel et al. 2007).

Fatty acid composition of the different vegetable oils and blended oils will be determined using multiple chromatography techniques, including GC-MS, LC-MS, etc. Earlier studies showed 14 fatty acid components that were analyzed by using high-performance liquid chromatography (HPLC) (Katkade et al. 2018).

5 Genetic Characteristics Related to the Fatty Acids

Knowles and Hill (1964) reported that the main gene locus, ol, governing the quantities of oleic and linoleic acid with the genotype *olol* leads to 72–80% oleic acid in the safflower seed oils and the genotype OLOL resulting in 72–80% linoleic acid, whereas the genotype ol'ol' or OLol1 has about equal amounts (45%) of each acid. In 1989, they reported that the genotypes OLOL and olol were more stable with regard to temperature changes, in contrast to the gene ol1. At the highest temperature, in the genotypes ol1ol1 and OLol1, the linoleic acid content was slightly decreased, and oleic acid was increased by the corresponding amount. A new gene locus (li)—different from st (governing the levels of oleic and stearic acid) or ol—controlling for high levels of linoleic acid, was found in the genotype from Portugal (Portugal 253568) with very high levels of linoleic acid (87–89%) and very low oleic acid content (3–7%). Hamdan et al. (2011) reported on the inheritance of high content of linoleic acid in safflower and its link with nuclear male sterility. The results showed a linkage of five random amplified polymorphic DNA (RAPD) bands to the Li (controlling for the very high linoleic acid content) and Ms (controlling for nuclear male sterility, NMS) gene loci. The RAPD fragments were improved to obtain sequence-characterized amplified region (SCAR) markers. A linkage map constituting the Li and Ms gene loci and SCAR markers was created. The SCAR markers flanked the two loci at the lowest distances of 15.7 cM from the Li locus and 3.7 cM from the Ms locus.

5.1 Flavonoid

Flavonoids such as quercetin, shannesol, hydroxysafflor yellow A, safflower yellow A, rutin, apigenin, and myricetin are important constituents of safflower (Ji et al. 2018; Li et al. 2012). Flavonoids possess wide range of antioxidative pharmacological activities. The extracts of safflower exhibit a cardioprotective effect and thus can help in improving myocardial ischemia, improve the heart rate, reduce the area of myocardial infarction, and supply oxygen to the myocardium (Bao et al. 1984). *C. tinctorius* extracts inhibited the platelet aggregation, persuaded by ADP, and possess recognizable depolymerization effects on ADP-aggregated platelets (Yang and Ma 2010). *C. tinctorius*-derived total flavones showed a hypotensive effect on the investigational animals (Maneesai et al. 2016).

5.2 Other Metabolites

Earlier reports have revealed the variations in other metabolites, for example, very high oleic acid content (>85%) and high palmitic acid content (>10%) (Fernandez-Martinez et al. 1993). Unlike fatty acids, lower inconsistency in tocopherol profile was observed in safflower germplasms. Johnson et al. (2007) observed no discrepancy of tocopherol profile, while Velasco and Fernandez-Martinez (2001) noticed trivial disparity of increased gamma-tocopherol content. Nonetheless, recently identified natural mutant of *C. oxyacantha* produced seeds with high gamma-tocopherol content (>90%) instead of the standard high alpha-tocopherol content (>90%). As the mutant showed introgression of *C. tinctorius*, simultaneous selection for high gamma-tocopherol content and morphological traits produced a high gamma-tocopherol safflower line designated IASC-1 (Velasco et al. 2005).

6 Part-Wise Safflower Pharmacological Compounds and Uses

Safflower is a medicinal plant that contains numerous valuable pharmacological compounds present in every part of the plant. In Pakistan and India, each part of the plant was traditionally used to increase sexual desire (Knowles 1989). Many studies found that there have been numerous data in support of the safflower medicines for cardiovascular disease, women's menstrual problems, bone pain, and swelling in trauma cases (Table 6.1).

6.1 Seed

The safflower seed contains major chemical compounds such as fatty acids, vitamin E, carotenoids, and flavonoid. Whole safflower seeds include 38–48% oil, 15–22% protein, and 11–22% fiber. The hull makes up 18–59% of the seed weight (Aydeniz et al. 2014). Specifically, the oil contains 70% of polyunsaturated fatty acid, linoleic acid, and 10% monounsaturated oleic acid (Knowles and Ashri 1995).

6.2 Flower

The safflower petals (flower) contain major chemical compounds such as safflower yellow carthamidin, a flavonoid (Adamska and Biernacka 2021). It contains two main pigments (yellow and red), and these sources have been used for food and

Table 6.1 Pharmacological effects of safflower extracts

Part	Chemical compound extract	Model	Effects	References
Flower	Water extract	Mice	Improve skin condition in mice and reduce immune inflammatory response	Fan et al. (2019)
Seed	Dried safflower extract	Mice	Against oxidative stress	Park et al. (2019)
Flower	Hydroxysafflor yellow A (HSYA)	MRC-5 Cell culture	Tumor necrosis factor (*TNF*) proliferation and inflammatory response	Liu et al. (2019)
Seed powder	Fatty acid	Male Sprague-Dawley rats	Against nonalcoholic fatty liver disease (NAFLD)	Mohamed et al. (2019)
Extract	Lipopolysaccharide	Rat cardiomyoblast cell H9c2	Inhibit cardiac hypertrophy	Tung et al. (2020)
Flower	Safflower yellow injection	Male wistar rats	Anti-myocardial ischemia reperfusion injury (MIRI)	Li et al. (2019)
Flower	Carthamin yellow (CY)	Male rats	Reduce ischemia-reperfusion injury	Lu et al. (2019)
Flower	Danhong injection	Rat	Blood stasis syndrome	Bi et al. (2019)
Flower	Hydroxysafflor yellow A	HBSMC cell	Activating PAF and inhibit the HBSMCs	Guo et al. (2019)
Seed	Scopolamine	Male mice	Inhibition of cholinergic dysfunction and oxidative stress	Kim et al. (2019)
Flower	Hydroxysafflor yellow B (HSYB)	MCF-7 cells	Effect on proliferation of cancer cells	Qu et al. (2019)
Flower	Hydroxysafflor yellow A	Guinea pigs	Effect on OVA (ovalbumin)-induced asthma	Zheng et al. (2019)
Seed oil	Linoleic acid	Mice	Effects in apolipoprotein and hepatic fatty acid metabolism	Ide et al. (2017)
Flower	Hydroxysafflor yellow A	Male mice	Inhibit tumor growth without detrimental effects	Ma et al. (2019)
Flower	Hydroxysafflor yellow B (HSYB)	Rat	Protect brain ischemia/reperfusion injury	Du et al. (2019)
Flower	Safflower yellow (SY)	Transgenic mouse model	Anti-inflammatory effects of Alzheimer's disease	Zhang et al. (2019)
Flower	Hydroxysafflor yellow A	Mice	Anticancer agent for human hepatocellular carcinoma (HCC)	Zhang et al. (2019)

textiles to coloring. Recently, these pigments used for cosmetic colorings like face and hair cream, shampoo, and body lotion. Flowers are used for important medicinal purposes like cardiovascular, cerebrovascular, gynecological disease, coronary heart disease, angina pectorius, and hypertension (Adamska and Biernacka 2021). The safflower petals can produce a range of colors yellow, red, and white based on the variety.

7 Pharmacological Importance of Safflower Metabolites

The most often used methods for measuring safflower quality are qualitative identification using thin-layer chromatography (TLC) and content determination via high-performance liquid chromatography (HPLC). To control the quality of safflower, hydroxysafflor yellow A and kaempferide are used as marker components. Some of the pharmacological effects of safflower extracts and their isolated compounds are shown in Tables 6.1 and 6.2. Several antinutritional factors (ANF) are commonly distributed in the oilseeds. In safflower, ANFs such as tannins, luteolin, acacetin, and serotonin derivatives are reported. Safflower containing ANF compounds are used for various medical and pharmaceutical applications such as antioxidant, anti-inflammatory, antibacterial, and anticoagulant effects (Huang et al. 1999; Duarte et al. 2001; Dajas et al. 2003; Benavente-Garcia and Castillo 2008; Lin et al. 2008). Earlier studies reported that ANFs reduce blood glucose, plasma cholesterol, and cancer risks. In case of high concentration intake, it causes adverse physiological effect (Singhal et al. 2018).

8 Conclusions

Safflower contains alkaloids, quinochalcones, flavonoids, polyacetylenes, alkanediol, fatty acids, lignans, steroids, and other chemical components. Among them, flavonoids and quinochalcones are the distinctive and bioactive chemoconstituents of safflower. However, prior studies have primarily relied on chemical analysis, with an emphasis on secondary metabolites. The changes in the concentration of such main metabolites among different sections of safflower samples must be investigated using newly developed analytical techniques. The application of existing and emerging methodologies in safflower metabolomic research in the near future can contribute to its productivity and quality.

Table 6.2 Some of the pharmacological effects of safflower metabolites

Parts	Metabolite group	Compounds	Function/medicine	References
Flower (color)	Carthamin (hydroxyethyl carthamin)	Hydroxyl carthamin A	Vascular disease, cerebrovascular disease, coronary heart disease, angiitis, hypercholesterolemia inhibitors, hyperlipidemia inhibitors, anti-obesity agents, acne, and dermatitis	Meselhy et al. (1993), Zhou et al. (2015), Fan et al. (2014)
	Safflower yellow	Safflower yellow A and B	Cardiovascular effect, neuroprotection, liver and lung protection, antitumor activity, metabolism regulation, and endothelium cell protection	An et al. (1990), Meselhy et al. (1993), Liu et al. (1992), Zhu et al. (2003), Love (1999), Wang et al. (2007), Yu et al. (2007), Asgarpanah and Kazemivash (2013)
Plant	Polysaccharide	Rhamnose, arabinose, xylose, mannose, glucose, and galactose	Role of immunoregulatory, antitumor effect, and proliferation and metastasis in breast cancer	Zhou et al. (2018), Wakabayashi et al. (1997), Ando et al. (2002), Shi et al. (2010), Luo et al. (2015)
	Flavone	Kaempferol-3-0-rutinoside	Vascular disease, improved blood circulation, and pain killer	Liu et al. (2005)
Seed	Fatty acid	Linoleic acid (ω-6-fatty acids)	Hormonal agents, drug stabilization, osteoporosis, hyperlipidemia, antidiabetic, decrease fat accumulation in rat, decrease body weight, and insulin resistance	Fernandez-Martinez et al. (1993), Shimomura et al. (1990), Norris et al. (2009), and Neschen et al. (2002)
	Carotenoids	β-Carotene, β-cryptoxanthin, and lycopene lutein	Antioxidant nutrients	Serani and Piacenti (1992), Aparicio et al. (1999), and Luterotti et al. (2002)
	Vitamin E	α-Tocopherol, β-tocopherol, γ-tocopherol, and δ-tocopherol	Antioxidant activity against oxidation	Vosoughkia et al. (2011), Khalid et al. (2017)
	Serotonin	*N*-Feruloylserotonin and *N*-(*p*-coumaroyl) serotonin	Antioxidation, anti-inflammation, anticancer, and antiaging and improve blood circulation	Sakamura et al. (1978), Roh et al. (2004), Kang et al. (1999), Bae et al. (2002), Kim et al. (2004), Hotta et al. (2002), Takimoto et al. (2011)

(continued)

Table 6.2 (continued)

Parts	Metabolite group	Compounds	Function/medicine	References
	Lignans	Matairesinol, and 8′-hydroxyarctigenin	Fracture and osteoporosis	Kuehnl et al. (2013)
	Flavonoid	Tilianine	Fracture and osteoporosis	
		Acacetin	Anti-inflammatory, antioxidation, blood vessel expansion, arrhythmia inhibition, antiplatelet aggregation, and antitumor activities	Hattori et al. (1992), Guler et al. (2011), Chiyomaru et al. (2012)
		Cosmosiin	Aging skin, whitening, and wrinkles	Prasad et al. (2012), Chen et al. (2013)
		Luteolin	Antioxidative properties, neuroprotection, antidiabetic, antihypertensive, and cancer prevention effects	Huang et al. (1999), Duarte et al. (2001), Dajas et al. (2003), Benavente-Garcia and Castillo (2008), Lin et al. (2008)
Young leaf	Lignans	8′-Hydroxyarctigenin	Whitening and wrinkle	Kuehnl et al. (2013)

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Chapter 7
A Perspective on Therapeutic Potential of an Invasive Weed, *Lantana camara*

Monika and Neelima Dhingra

1 Introduction

Nature, the mother of craftsman of molecules has provided an inexhaustible array of small molecules (Thomford et al. 2018). Natural products (NPs) hold enormous potential and remained at the core of the drug discovery. Secondary metabolites are being structurally optimized with evolution to regulate the endogenous defense mechanisms against other organisms or external environment and explain the strong correlation of NPs with cancer and infectious diseases (Lachance et al. 2012; Atanasov et al. 2015). NP libraries demonstrate structural and qualitative activity information for around 470,000 NPs, but ~4000 NPs with only experimental values, indicating untapped profile of natural products to a greater extent (Li and Weng 2017; Calixto 2019). Utilization of natural products for therapeutic reason is traced back to the early lifetime of human in the history. Plant used as a curative agent, is portrayed in the paintings, discovered in the Lascaux caves in France, which is believed to be a period between 13,000 and 25,000 BC (Berger 2006).

Firstly, isolated molecule from the plant, poppy (*Papaver somniferum*), was a morphine by Friedrich Wilhelm Serturner (1783–1841), and it was an introduction as a therapeutic agent by Merck in 1826, which had sparked and hastened the young scientist to discover new molecules from the natural resources (Krishnamurti and Rao 2016). Since the immemorial time, natural product stands as a foundation of

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M. K. Swamy, A. Kumar (eds.), *Phytochemical Genomics*,
https://doi.org/10.1007/978-981-19-5779-6_7

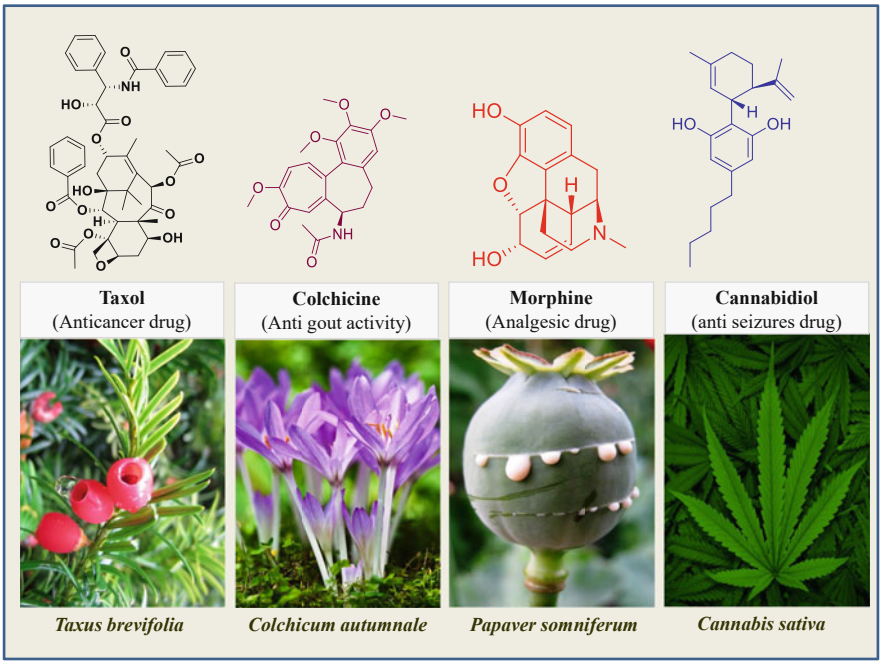

Fig. 7.1 Successful examples of natural products and their uses in human diseases

traditional system of medicine as blockbuster in drug discovery and an active area of research. The trend of isolation of natural products, secondary metabolites, and their semisynthetic modifications has continued and intensified to further improve their pharmacokinetics and pharmacological profile (Newman and Cragg 2020). Moreover, individuals are nowadays drifting back to the customary medications on the account of side effects associated with long-term usage of allopathic drugs (Bhat et al. 2019). The history of medication is filled with noteworthy stories of natural products' discovery, which has greatly impacted on the advancements in biomedicine and pharmacotherapy (Shen 2015). Few examples of drugs that are established for treating several types of human diseases are given in Fig. 7.1.

Plants encompass a diverse chemical space with tremendous medicinal compounds that have become a topic of global importance for the drug development. Especially, plant-derived metabolites played an important role to treat a myriad of maladies (Hussein and El-Anssary 2018). The potential of these secondary metabolites can be attributed to their unique structural scaffolds and high complexity which in turn generates biological screening database. Besides being attractive drug leads, high degree of stereochemistry and wide range of pharmacophores increase receptor binding selectivity, allowing these natural scaffolds as a source of new drug. These striking characteristics and advancement in sophisticated analytical tools have created lively interest of the researchers toward natural products (Harvey et al. 2015; Rodrigues 2017).

Table 7.1 Weed plants with pharmaceutical importance Stepp (2004)

Weed plants	Drug(s)
Adonis vernalis	Adoniside
Agrimonia eupatoria	Agrimophol
Ammi visnaga	Khellin
Anabasis aphylla	Anabasine
Andrographis paniculata	Andrographolide and neoandrographolide
Artemisia annua	Artemisinin
Atropa belladonna	Atropine
Berberis vulgaris	Berberine
Brassica nigra	Allyl isothiocyanate
Cannabis sativa	Epidiolex
Cassia senna	Sennosides A and B
Cassia spp.	Danthron
Catharanthus roseus	Vinblastine and vincristine
Centella asiatica	Asiaticoside
Cissampelos pareira	Cissampeline
Colchicum autumnale	Colchiceine, colchiceine, and demecolcine
Datura metel	Scopolamine
Digitalis purpurea	Digitalin, digitoxin, and ditalin
Dioscorea spp.	Diosgenin
Glycyrrhiza glabra	Glycyrrhizin
Hyoscyamus niger	Hyoscyamine
Lobelia inflata	Lobeline
Mentha spicata	Menthol
Papaver somniferum	Codeine, morphine, papaverine, and noscapine
Ricinus communis	Castor oil
Silybum marianum	Silymarin
Symphytum officinale	Allantoin
Urginea maritima	Scillaren A
Valeriana officinalis	Valepotriates
Vinca minor	Vincamine

As far as plants are concerned, the importance of weeds in the pharmacopeia is being unnoticed, in spite of noteworthy evidences that especially, weeds are a vital resource of medicines for indigenous peoples and have high significance in ethnic pharmacopoeias in relation to other plant species (Stepp and Moerman 2001). There are a number of evidences that weeds are relatively possessing greater quantities of bioactive secondary compounds and are thus likely to hold promise for the drug discovery (Ekwealor et al. 2019). Few examples of weed plants as pharmaceutical agents are summarized in Table 7.1. One such significant weed is *L. camara* L. (Verbenaceae) (Fig. 7.2) and has attracted a lot of interest among scientists in the last two decades (Hussain et al. 2011). Linnaeus, in 1753, described the genus *Lantana*, and later on, the subgenus *Camara* was defined by Chamisso in 1832 (Santos 2002).

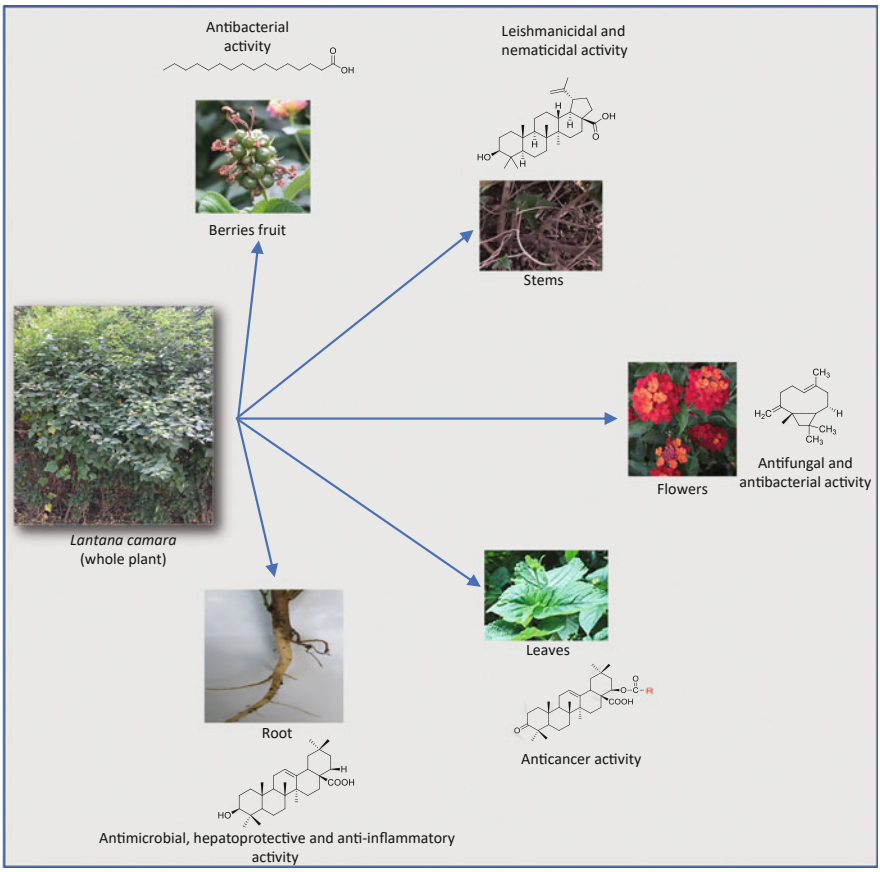

Fig. 7.2 *Lantana camara* and its components with biological activities

L. camara, commonly known as *Lantana*, wild or red sage, is regarded as one of the ten most noxious weeds in the world (Bhagwat et al. 2012). *Lantana* is native to subtropical and tropical America, but a few taxa are indigenous to tropical Asia and Africa (Munir 1996). It is the most widespread species of this genus and has spread over in approximately 60 countries with recorded 650 varieties. The weed is found to grow widely at altitudes from sea level to 2000 m in tropical, subtropical, and temperate regions and can thrive very well under rainfall ranging from 750 to 5000 mm per annum. The plant is susceptible to frosts and low temperatures, saline soils, boggy or hydromorphic soils, low rainfall, coralline soils with poor water-holding capacities, and high incidence of tropical hurricanes (Taylor et al. 2012).

The spread of *Lantana* across the globe started as early as the 1690s. In India, it was first introduced in 1807, in Kolkata, during the introduction of various plants in the botanical gardens (Kannan et al. 2013; Kohli et al. 2006). Modern system of medicine is starting to recognize the beneficial effects of phytotherapy, including *Lantana*, which is widely studied for the antiulcer, antimalarial, antimicrobial,

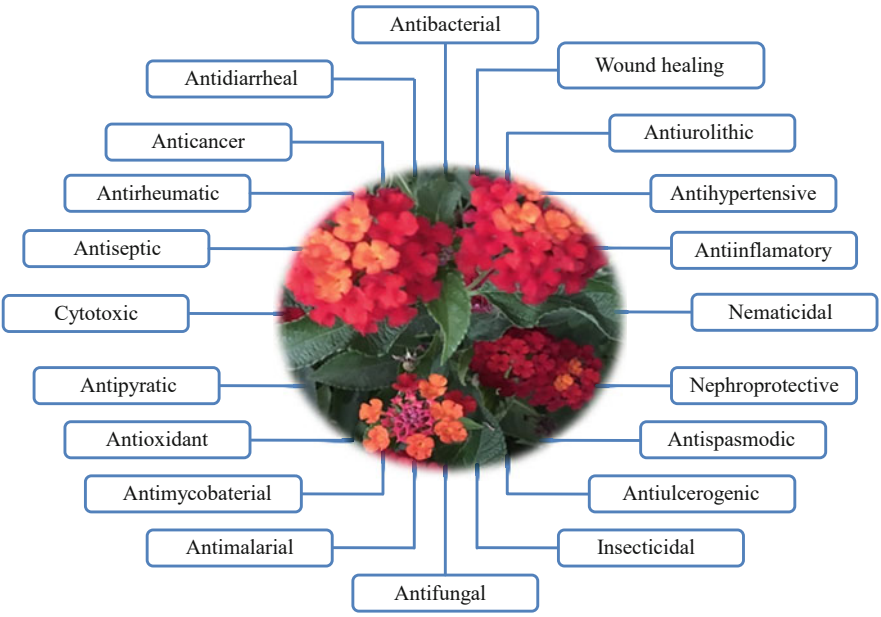

Fig. 7.3 Biological activities of *Lantana*

nematicidal, anticancer, antihyperglycemic, wound healing potentials and antihypertensive, etc. (Fig. 7.3) (Begum et al. 1995; Suthar et al. 2014a; Ahmed et al. 1972; Ghisalberti 2000; Rajashekar et al. 2014a). In this chapter, authors have made an attempt to bring together the phytochemistry, biological activities, toxicity, and pharmaceutical-based formulations from different parts of the *Lantana camara* that would help researchers to explore its latent and potent potential.

2 Principal Constituents of *Lantana*

The phytochemistry of *Lantana* has attracted considerable interest, mainly due to its vast and versatile medicinal effects oriented toward its secondary metabolites. Innumerability of triterpenoids, flavonoids, iridoid glycosides, oligosaccharides, phenylpropanoid glycosides, naphtha quinines, etc. has been isolated with their striking features from the leaves, flowers, berries, stem, roots, and whole part of *Lantana* which are indicated in Table 7.2.

Leaves, the most extensively explored part of *Lantana*, have been reported with pentacyclic triterpenoids from the oleane series, a very few to the ursane and lupane series, along with ones having an oxide bridge from C-3 to C-25. Among all, lantadenes A and B are the first recognized toxic and major components of *Lantana*, whereas lantadenes C and D are other components with suspected toxicity (Barton et al. 1956; Barre et al. 1997). Various flavonoids and iridoid glycosides have been

Table 7.2 Phytoconstituents from *L. camara* species and their therapeutic potential

Phytochemical	Plant parts	Biological activity	References
22-β-Acetoxylantic acid	Leaves	Antibacterial and antifungal activity	Barre et al. (1997)
Betulinic acid	Stem and aerial parts	Leishmanicidal, cytotoxic, and nematicidal activity	Srivastava et al. (2010)
Bicyclogermacrene	Leaves	Antibacterial activity	Seth et al. (2012)
Camarinic acid	Aerial parts, leaves, and stems	Nematicidal activity	Begum et al. (2000)
Camaric acid	Stem and aerial parts	Nematicidal activity, leishmanicidal activity, and antimycobacterial activity	Qamar et al. (2005), Begum et al. (2014), and Saleh et al. (1999)
Camarinin	Stem and aerial parts	Nematicidal activity	Begum et al. (2015) and Begum et al. (2000)
1,8-Cineole	Leaves	Inhibiting the growth of plant and antibacterial activity	Satyal et al. (2016)
E-Caryophyllene	Leaves	Antifungal, antibacterial, antimicrobial, and cytotoxic activity	Satyal et al. (2016) and Passos et al. (2012)
Coumaran	Leaves	Acetylcholinesterase inhibitor, and insecticidal activity	Rajashekar et al. (2014a)
Euphane triterpene lactones	Leaves	Thrombin inhibitory activity	O'Neill et al. (1998)
Gautin	Leaves	Antibacterial and antifungal activity	Patil et al. (2015)
Geniposide	Root	Hypolipidemic activity	Ghisalberti (2000)
11α-Hydroxy-3-oxours-12-en-28-oic acid	Aerial parts	Nematicidal activity	Begum et al. (2015)
Lancamarinic acid	Aerial parts	Antibacterial activity	Ayub et al. (2019)
Lancamarinin	Aerial parts	Antibacterial activity	Ayub et al. (2019)
Lancamarone	Leaves	Cardiotonic property	Sharma and Kaul (1959)
Lantic acid	Leaves and stems	Antibacterial activity	Saleh et al. (1999)
Lantadene A	Leaves, stem, and roots	Hepatotoxicity, antimicrobial, antiviral, antitumor, antitubercular, allelopathy, cytotoxic, antitumor, leishmanicidal, and nematicidal activity	Sharma et al. (2007b), Begum et al. (2014), Heikel et al. (1960), Brown and Rimington (1964), Verma et al. (1997), and Sharma et al. (1991)
Lantadene B	Leaves and stem	Hepatotoxicity, antimicrobial, antiviral, antitumor, allelopathy, and nematicidal activity	Inada et al. (1995), Barton et al. (1954), Begum et al. (2014), Brown and Rimington (1964), Sharma et al. (1987), Kong et al. (2006), and Suthar et al. (2014b)

(continued)

Table 7.2 (continued)

Phytochemical	Plant parts	Biological activity	References
Lantadene C	Leaves and stem	Hepatotoxicity and antiviral activity	Johns et al. (1983), Sharma et al. (1992), and Inada et al. (1995)
Lantadene D	Stem	Anticancer activity	Sharma et al. (2007b)
Lantanilic acid	Leaves, stem, and roots	Leishmanicidal, nematicidal activity, and antibacterial activity	Qamar et al. (2005), Begum et al. (2014), Begum et al. (2015), Saleh et al. (1999), Barua (1975), and Qamar et al. (2005)
Lantanolic acid	Roots and aerial part	Leishmanicidal activity	Begum et al. (2014) and Barua (1975)
Linaroside	Leaves and aerial parts	Nematocidal activity and antimycobacterial activity	Begum et al. (2000)
Lancamarone	Leaves	Cardiotonic	Sharma and Kaul (1959)
Lantanoside	Leaves and aerial parts	Antimicrobial, antimycobacterial, and nematicidal activity	Begum et al. (2000)
Lantic acid	Leaves	Antibacterial activity	Saleh et al. (1999)
Lantoic acid	Aerial parts	Leishmanicidal activity nematicidal activity	Begum et al. (2008) and Begum et al. (2014)
Lantaninilic acid	Aerial parts	Leishmanicidal	Begum et al. (2014)
Lantamine	Stem and roots	Antipyretic activity antispasmodic properties	Sastri (1962)
Lutenolin-7-*O*-β-galacturonyl-(2→1)-*O*-β-galacturonide	Flowers	Antioxidant activity hepatoprotective activities	Abou El-Kassem et al. (2012)
Oleanolic acid	Leaves, stem, root, and aerial parts	Antimicrobial, hepatoprotective, anti-inflammatory, antifertility antihyperlipidemic, antimicrobial, nematicidal, antiulcer, activity antiurolithiatic activities	Srivastava et al. (2010), Qamar et al. (2005), Siddiqui et al. (1995), and Sharma (1989)
Oleanolic acid	Leaves and aerial parts	Anti-inflammatory, nematicidal, anticancer, inhibitors of human leucocyte elastase, inhibit leukotriene synthesis, and leishmanicidal activity	Giner-Larza et al. (2001), Qamar et al. (2005), and Begum et al. (2014)
Oleane-12-en-3β-ol-28-oic acid 3β-D-glucopyranoside (OAG)	Leaves	Antiulcer activity	Kazmi et al. (2018)
Pectolinarin	Leaves	Larvicide, acetyl cholinesterase, antioxidant, and cytotoxicity activities	Fonseca et al. (2019)

(continued)

Table 7.2 (continued)

Phytochemical	Plant parts	Biological activity	References
Reduced lantadene A	Leaves	Hepatotoxicity, antiviral, anti-cancer, and cytotoxic	Tailor et al. (2013), Inada et al. (1995), and Sharma et al. (2007b)
Reduced lantadene B	Leaves	Cytotoxic and anticancer activity	Kumar et al. (2013b) and Sharma et al. (2007c)
Ursolic acid stearoyl glucoside	Leaves	Anxiolytic-like effect	Kazmi et al. (2013) and Srivastava et al. (2010)
Urs-12-en-3β-ol-28-oic acid 3β-D-glucopyranosyl-4′-octadecanoate	Leaves	Antidiabetic potential	Kazmi et al. (2012)
Verbascoside	Leaves and stem	Cardiotonic, vasodilatory agent, antihypertensive, anti-fungal protein kinase C inhibitor, antitumor, anti-inflammatory, and immune disorders	Mahato et al. (1994), Oyourou et al. (2013), Herbert et al. (1991), and Molnar et al. (1989)
Vanillic acid	Leaves	Allelopathy	Singh et al. (1989)

reported from stems of *Lantana*. Typical steroids like β-sitosterol, campesterol, and stigmasterol including β-sitosterol glucoside have also been found to be present in the stems of *Lantana* (Lai et al. 1998). Rootlets and root bark of *Lantana* are considered as a good source of a plentiful triterpenoids called oleanolic acid. Additional components isolated from the root of *Lantana* include iridoid glycosides and oligosaccharides like the viridoside, geniposide, 8-epiloganin, lamiridosides, verbascotetraose, etc. (Misra et al. 1997; Pan et al. 1993). Different researchers across the globe have reported majority of mono- and sesquiterpenes from the flowers of *Lantana* including β-curcumene, *E*-nuciferal, *Z*-nuciferol, g-curcumene, curcumene, davanone, *E*-nerolidol, *E*-farnesene, β-caryophyllene, etc. as major constituents (Khan et al. 2016a). Despite of a myriad of phytoconstituents in the different parts of *Lantana*, a number of reports have also mentioned the effect of season, geographical location, and developmental stage of the plant on existence of the particular chemical constituent (Liu et al. 2016). Few of the promising secondary metabolites of *Lantana* are exemplified in Fig. 7.4.

3 Pharmacological Activities of *Lantana*

Nature is a rich source of many medicines that need to be explored (Piper et al. 2018). *Lantana* extracts and their secondary metabolites (Fig. 7.4) are proven for their pharmacological activities. The possible mechanism of actions of its different valuable active compounds is summarized in Fig. 7.5. Various pharmacological activities of *Lantana* are discussed in the following subheadings.

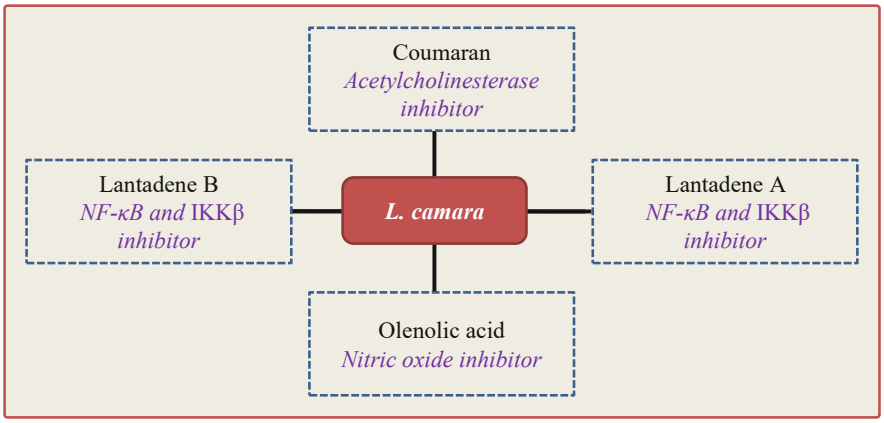

Fig. 7.4 Common phytoconstituents of *Lantana*

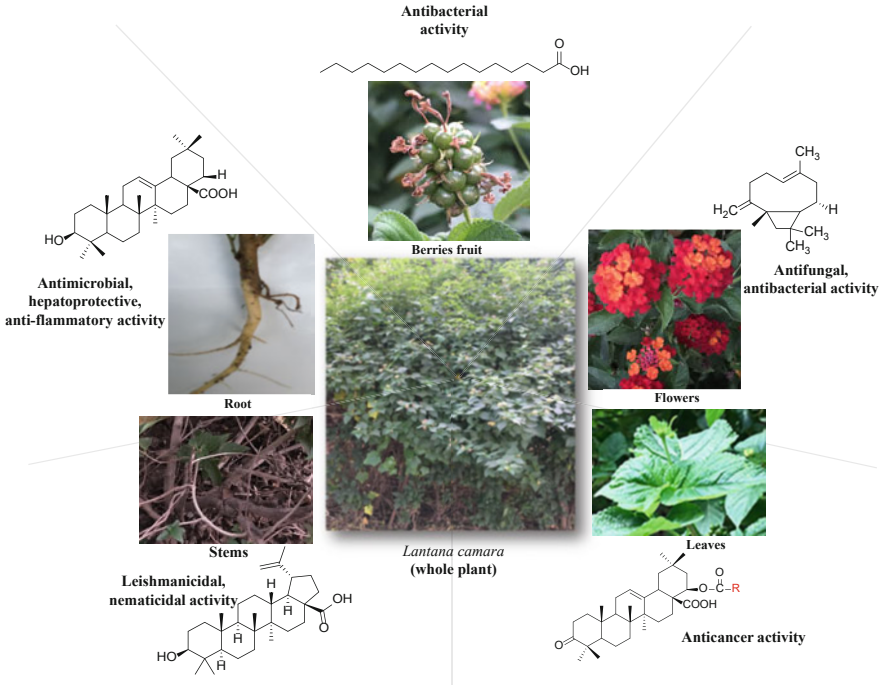

Fig. 7.5 Possible mechanisms of active constituents of *Lantana*

3.1 Antibacterial Activity

The increasing incidences of drug-resistant pathogens have drawn the attention of the pharmaceutical and scientific communities toward studies to explore the antimicrobial activity of untapped plant-derived chemotypes (Savoia 2012). Seth et al. explored the antibacterial activity of extracts (benzene, chloroform, methanol, water) and essential oils of *Lantana* leaves against *Bacillus cereus*, *Staphylococcus aureus*, *Micrococcus luteus*, *Escherichia coli*, and *Pseudomonas aeruginosa* strains (Seth et al. 2012). In respect of antibacterial activity, essential oil showed high degree of sensitivity against all the strains except *P. aeruginosa*. Further, in contrast to chloroform extract with low antimicrobial activity, petroleum ether, benzene, and water and methanolic extracts afforded good to moderate activity against all tested bacterial strains. Research group concluded that high antibacterial potential of essential oil may be because of the presence of sesquiterpene hydrocarbons as major constituents that include *E*-caryophyllene, bicyclo-germacrene, α-curcumene, and germacrene. Sabinene, α-pinene, and γ-terpinene along with (*E*)- and (*Z*)-citral and 1,8-cineole are some of the monoterpene hydrocarbons and oxygenated monoterpenes observed in the extract with antibacterial activity.

A survey of the literature indicates that composition of the plant constituents is strongly shaped by contemporary environment, such as climate and geographical location. Considering this, Satyal et al. (2016) explored the chemical diversity and antibacterial effect of *Lantana* sample from different geographical locations: Cuba, Nepal, and Yemen. Around 112 compounds have been reported by the researchers in the essential oil of *Lantana* from Cuba, constituting 91.2% of the total oil composition, and some of the important constituents are (*E*)-nerolidol, (*E*)-farnesene, β-caryophyllene, germacrene D, and 1,8-cineole. Essential oil from this region was found inactive against *E. coli* and *Candida albicans*, with high IC_{50} values >64 μg/mL, but reasonably good against *S. aureus* with low IC_{50} values (12.13 μg/mL) (Satyal et al. 2016).

Samples from Nepal indicated a total of seventy-seven (77) compounds of *Lantana* oil, with 92.4% of the composition containing oxygenated sesquiterpenoids, davanone, and *E*-nerolidol and monoterpenoids as major constituents. In comparison to antibacterial activity of Cuba sample, *Lantana* oil from Nepal is found more active against *S. aureus* (Pino et al. 2004).

Furthermore, sample collection from Yemen reported a total of seventy-one (71) compounds, comprising 89.4% of total composition, with sabinene and β-caryophyllene as major components. Oil from Yemen showed antimicrobial potential against *S. aureus*, *B. subtilis*, and *C. albicans* with inhibition zones ranging from 26 ± 2.8 to 38 ± 3.6 mm. Outcomes of the research were also found in agreement with Kasali et al.'s (2004) reports indicating the role of β-caryophyllene and (*E*)-nerolidol in antibacterial activity (Fig. 7.4).

Another study performed by Saleh et al. (1999) indicated strong antimicrobial potential of lantic acid (from leaves and stem of *Lantana*) against *E. coli* and *B. cereus*. Efforts have been made to investigate the antibacterial potential of isolated

novel compounds. Marginal antibacterial activity of isolated lancamarinic acid and lancamarinin (Fig. 7.5) from the aerial part of *Lantana* was observed against gram-positive and gram-negative bacteria (Ayub et al. 2019). Deena and Thoppil (2000) observed the potential antibacterial effect of *Lantana* essential oil against *P. aeruginosa* on an account of the presence of β-caryophyllene, geranyl acetate, terpinyl acetate, bornyl acetate, and D-limonene as promising constituents. Patil et al. (2015) isolated novel flavone glycoside with unique aglycone moiety called gluten (Fig. 7.4), which expressed highest antibacterial activity against *E. coli* and antifungal activity against *Aspergillus niger* fungi. Another study also indicated the antibacterial potential of oleanolic and ursolic acids from *Lantana* (Hart et al. 1976).

3.2 Antifungal Activity

Fungal diseases and emerging infection remained a unique problem due to evolving epidemiology of invasive fungal infection and compel the discovery of new antifungal compounds (Roemer and Krysan 2014). Plants with a wide variety of bioactive secondary metabolites such as saponins, tannins, terpenoids, alkaloids, flavonoids, etc. represent the rich source of antifungal agents (Arif et al. 2009).

Alternaria spp. is a fungal pathogen known to attack older plants and responsible for different diseases in vegetable plants (Singh 2015). Singh and co-workers evaluated antifungal potential of *Lantana* using methanolic, ethanolic, and acetonic leaf extracts in comparison to standard drug griseofulvin. Reports indicated the approximately 50–60% inhibition of fungal infection in potatoes and tomatoes by methanolic extract, while ethanolic and acetonic extract showed excellent results with 100% inhibition in comparison to standard drug griseofulvin (69%) (Singh and Srivastava 2012). Deena and co-workers observed remarkable antifungal activity of essential oil from aerial parts of *Lantana* over nystatin against *A. niger*, *C. albicans*, *Fusarium solani*, and *P. aeruginosa* fungi and accounted β-caryophyllene, geranyl acetate, terpinyl acetate, bornyl acetate, and D-limonene as its major composition (Deena and Thoppil 2000). Another study by Passos et al. emphasized and correlated the dose-dependent antifungal activity of *Lantana* oil within its major germacrene-D and *E*-caryophyllene against *Corynespora cassiicola* fungi (Passos et al. 2012).

3.3 Insecticidal Activity

Insects are responsible for the transmission of many diseases affecting crop plants, domestic animals, and humans. Development of resistance to synthetic pesticides, high operational cost, and environmental pollution generated the need for developing naturally and environmentally friendly alternative approaches to control vector-borne disease (Lounibos 2002; Cantrell et al. 2012). Bioinsecticidal potential of *Lantana* was first indicated, while accessing the antimicrobial properties of hydro-

distillation of extracted essential oils from its leaves. Cumulative mortalities observed with essential oil against *Culex pipiens* were related to some of the reported major constituents like β-caryophyllene, caryophyllene oxide, germacrene-D, curcumene, bicycle sesqui-phellandrene, cadinene, α-pinene, and limonene. *Lantana* holds the potential to serve an alternative to the currently used insecticides on account of the presence of its bioactive molecules and their ability to kill destructive pests especially with tendency to attack stored grains like *Sitophilus oryzae*, *Callosobruchus chinensis*, and *Tribolium castaneum*. Rajashekar et al. (2014b) further evaluated its insecticidal potential using leaf extract in different solvents (methanol, ethyl acetate, hexane, and acetone) against *S. oryzae*, *C. chinensis*, and *T. castaneum*. Fumigant and contact toxicity was expressed by methanol extract against all these bacteria with 4H-1-benzopyran-4-one, dihydro-1, 3-oxathiole, coumaran, 3-methoxy-4,5,7-trihydroxyflavone, propionic acid, 5-oxymethylfurfurole, 2,6-dimethoxy phenol, *p*-hydroxy benzaldehyde, 2-hydrozinopyridine, 2-methoxy-5 vinyl phenol, and phytol volatiles as major constituents. These observations were found to be in agreement with earlier published reports indicating the fumigant and biopesticide potential of coumaran (Scharf et al. 2006; Barakat 2011).

3.4 Antimalarial Activity

Mosquitoes represent a huge threat for humans and animals worldwide, by acting as vectors for important parasites and pathogens that include malaria and filariasis along with arboviruses, such as dengue, West Nile virus, and Zika virus (Tolle 2009). And the continual use of clinically approved chloroquine to prevent and treat falciparum malaria resulted in the widespread appearance of chloroquine-resistant parasites in Kenya and other tropical countries (El-Kamali and Khalid 1996; Milliken 1997). On other hand, worsening economic situation of the sub-Saharan African countries made it tough for health authorities to expand their modern health services, which required an effective and low-cost delivery medical system. Further, the escalating costs of non-chloroquine drugs pushed the local people to switch to traditional remedies for the management of this menace (Hostettmann et al. 2000). The use of *Lantana* herbal medicine in the management of malaria has been documented in various researches (Njoroge and Bussmann 2006; Tabuti 2008). Reports by Clarkson et al. claimed the in vitro anti-plasmodial activity of dichloromethane/methanol (1:1) leaf/twig extract against a chloroquine-sensitive strain (D10) with a IC_{50} value of 11 g/mL (Clarkson et al. 2004), whereas reports by Weenen et al. (1990) showed high activity of root bark nonpolar extract against multidrug-resistant K1 strain.

Another study was made by Jonville et al. to evaluate the effect of dichloromethane and methanol extracts of *Lantana* against the 3D7 and W2 strains of *Plasmodium falciparum*, wherein dichloromethane fraction showed promising antimalarial potential against W2 strains (Jonville et al. 2008). Outcomes of another

study concluded that ethanolic extract of *Lantana* leaves displayed good activity (IC_{50} <17.5 μg/mL) against *P. falciparum* (Celine et al. 2009). Experiment conducted by researchers found the insecticidal activity against important vectors of malaria, dengue, and chikungunya on an account of caryophyllene, eucalyptol, α-humulene, and germacrene from the essential oil of *Lantana* leaves (Dua et al. 2010). Another group of researchers from Africa indicated the mosquito repellent and insecticidal potential of aromatic substance of *Lantana* (Pavela and Benelli 2016; Rattan 2010; Dickens and Bohbot 2013).

3.5 Antiulcerogenic Activity

A number of reports have highlighted the potential of medicinal plants and their bioactive molecules as a major source for the treatment of peptic ulcer (Sharifi-Rad et al. 2018). Encouraging findings with antiulcerogenic effect by methanolic extract of *Lantana* leaves (LCME) in aspirin-induced gastric ulcerogenesis in pyloric-ligated rat model strengthened the medicinal importance of natural resources. The studies revealed the significant increase in the gastric pH after the oral administration of LCME at 500 mg/kg doses in comparison to standard famotidine. Inhibition of ulcer index, reduction in lipid peroxidation, and increase in reduced glutathione levels were also some of the additional parameters. A comparable protective effect (68.90%) to that of standard drug famotidine was also observed in cysteamine-induced duodenal ulcer models. Claimed protective effect of the extract was attributed to a number of factors like strengthening of duodenal mucosa or increased gastric and duodenal alkaline secretion or increased luminal prostaglandin levels (Sathish et al. 2011).

3.6 Antimycobacterial Activity

The upsurge of drug-resistant strains of malaria, viruses, and bacteria is a major health threat worldwide, and the search for alternative therapeutics from the nature remained a key area of interest for alternative therapeutics (Willcox and Bodeker 2004). Begum et al. isolated and evaluated flavonoids, linaroside, and lantanoside from the aerial parts of *Lantana* after preparing semisynthetic linaroside acetyl derivative. All the compounds exhibited the 30%, 37%, and 98% inhibition against *Mycobacterium tuberculosis* H37Rv strain, at 6.25 μg/mL concentration. A team of investigators have indicated a remarkable inhibitory activity by acetylated analogue than its parent molecule (Begum et al. 2008).

On the other hand, Kirimuhuzya et al. reported the antimycobacterial activity using methanol and chloroform extracts of *Lantana* against three strains of *M. tuberculosis* H37Rv, rifampicin-resistant TMC-331, and a nonresistant wild strain (28–25,271). But methanolic extract exhibited the highest activity against all

the three strains in comparison to used chloroform extract. On grounds of existing multidrug-resistant strain, their outcomes became a landmark discovery, especially when extracts were found to be effective against the rifampicin-resistant strains of *M. tuberculosis* (Kirimuhuzya et al. 2009). The burden of HIV/AIDS and TB coinfection prompted Dibua et al. to screen the antitubercular effect of leaves of *Lantana* against mycobacterial isolates from people living with HIV/AIDS. The observed activity of the *Lantana* extracts is consistent with their use in traditional medicine for the treatment of *Mycobacterium* species (Dibua et al. 2010).

3.7 Wound Healing Activity

The use of *Lantana* herbal medicine in the treatment of skin itches, leprosy, scabies, and antiseptic for wounds has also been highlighted in various reports by Saxena and Sharma (1999) and Day et al. (2015). Further, Nayak et al. claimed the wound healing property after topical application of ethanolic extract of *Lantana* leaves on Sprague Dawley rat model. The outcomes indicated the significant enhancement in the wound contraction rate, collagen synthesis, and decreased wound healing time at the practical dose of 100 mg/kg/day. It has been postulated that the reduction in wound area might be due to triterpenoids in leaf extract. The presence of flavonoids with potential to minimize oxidative damage to the excision wound tissue could have added and promoted wound healing capacity (Nayak et al. 2009).

3.8 Antidiarrheal Activity

Diarrhea is one of the most prevalent human disorders, and understandably its remedy occupies a special place in the annals of medicine (Bateman and McGahey 2002). A study performed by Sagar et al. to evaluate the effect of LCME on neostigmine-induced gastrointestinal transit in mice indicated its remarkable antimotility action and confirmed that LCME might possess certain components with anticholinergic effects (antisecretory properties) and may account for diarrhea potential (Sagar et al. 2005). Another study by Tadesse al. found the significant antidiarrheal activity by its aqueous stem extract in castor oil-induced diarrhea model. Considerable changes were produced on the measured parameters like diarrhea onset, decreased defecation frequency, and decline in the weight of faces. Authors added that tannins present in plant precipitated the proteins in the intestinal mucosa by forming the protein tannates and made the intestinal mucosa more resistance to chemical alteration followed by reduced peristaltic movements and intestinal secretions (Tadesse et al. 2017).

3.9 Anticancer and Antiproliferative Activity

Despite the great success of targeted cancer immunotherapies, the development of drug resistance and disease relapse always remained a huge burden in cancer patient treatment (Ventola 2017). Natural products (secondary metabolites) have long been investigated as invaluable sources by the medicinal chemist for drug design, with particular effectiveness in cancerous and infectious diseases (Rodrigues et al. 2016). This has fueled renewed interest in NP discovery to identify new pharmacophores for innovative cancer drug development (Hassannia et al. 2020).

Chemopreventive effect of leaves of LCME has been tested in Swiss albino mice model by Sharma et al. using 7,12-dimethylbenz[*a*]anthracene (DMBA) as skin cancer inducer. Significant decrease in incidence of skin papilloma with slight weight gain in mice and reduced death rate were observed for treatment group in comparison to disease control (DMBA) group. Further, histopathological studies showed hyperplastic papillomatous lesions without the evidence of infiltration or cytological atypia indicating that LCME may reverse the depletion of skin Langerhans cells and local immunosuppression (Sharma et al. 2007a).

Oleanolic acid isolated from the methanol extract of *Lantana* has been found to exhibit promising cytotoxicity against A375 cells. Further detailed mechanistic studies indicated its antiproliferative potential, through NO• (nitric oxide radical) inhibition via downregulation of inhibitor of nitric oxide synthases (iNOS) protein (Ghosh et al. 2010). Methanolic extract showed the presence of lantadenes A, B, and C and icterogenin, but lantadene B exhibited highest anticancer activity. Lantadene B was found to block the G1/S transitions after inducing MCF-7 cell cycle arrest in G1. Anticancer potential of *Lantana* was further enhanced, on account of their radical scavenging activity indicated in 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay and observations agreed with earlier reports indicating DPPH scavenging activity in the range of 54–62% (Shamsee et al. 2019; Bhakta and Ganjewala 2009).

Tumor necrosis factor (TNF) is a major inflammatory cytokine involved in the pathological process of autoimmune disorders, chronic inflammation, and malignant disease (Balkwill 1992). Sharma et al.'s group indicated anticancer potential of lantadenes A and B and suggested the inhibition of nuclear factor-kappa B (NF-κB) and nuclear factor kappa-B kinase subunit beta (IKKβ) as possible mechanism (Chauhan et al. 2014). Investigation made by Srivastava et al. indicated the cytotoxic potential of betulinic acid (BA), oleanolic acid (OA), and ursolic acid (UA) from *Lantana*, against cancerous HeLa cells via induction of apoptosis in DNA laddering assay (Srivastava et al. 2010).

Studies performed by Han et al. on lantadene A indicated cell death features such as shrinking, chromatin compression, bulging of the cell membrane, and nucleic acid disintegration using MCF-7 cancer cell line (Han et al. 2015). In addition to terpenoids, other isolated phytoconstituents like alkaloids like camerine, isocamerine, micranine, and lantanine have also shown anticancer potential (Prakash et al. 2013). Badakasn et al. claimed that apoptosis could also be the possible mechanism for the antiproliferative activity of *Lantana* leaves and root extract

while using human Jurkat leukemia cells (Badakhshan et al. 2009). Other reports also indicated cytotoxic potential of crude *Lantana* extract against HeLa cells and human WI-38 fibroblasts (Srivastava et al. 2010).

3.10 Antiurolithiatic Activity

Urolithiasis represents stone formation in any location of urinary tract including the kidneys and bladder (Khan et al. 2016b). Vyas and Argal (2013) assessed the antiurolithiatic activity of ethanolic root extract (ELC) and OA isolated from roots of using zinc disk implantation model in albino wistar male rats. Significant reduction in the calcium output in a dose-dependent manner along with X-ray imaging duly supported their promising antiurolithiatic potential. Further, OA found to possess significant reduction in weight of calculi at very low dose in comparison to reference drug Cystone (Vyas and Argal 2013).

3.11 Antioxidant Activity

Free radical, a multifaceted molecule, generally believed to play an important role in the pathogenesis of various human diseases such as ischemic heart disease, atherosclerosis, diabetes, human neurodegenerative disorders, inflammation, cancer, etc. And number of plants and their metabolites have been reported to possess protective activity against free radical-induced damages in various experimental models (Hou et al. 2003).

A valuable study was carried out by Asadu et al. (2015) to evaluate in vitro antioxidant potential of methanolic extract of *Lantana* leaves using 2,2-diphelyl-1-picryl-hydrazyl-hydrate (DPPH), superoxide (O_2^-), hydroxyl (OH), and nitric oxide (NO•) radicals. The extracts showed antioxidant activity by scavenging free radicals on account of flavonoids, tannins, alkaloids, saponin, reducing sugar, vitamin A, vitamin C, and vitamin E. Kumar et al. (2014) conducted another study using leaves of Chandigarh purple (CP) and yellow (CY) variety, Chandigarh yellow turning pink variety (YTPV), and Palampur red variety (PRV) and assessed their antioxidant potential. Highest total antioxidant capacity expressed by CYV and PRV extract was related to their phenolic content. Bhakta and Ganjewala (2009) compared the antioxidant potential of premature and older leaves using DPPH models, wherein studies indicated the less activity of older leaves on account of loss of secondary metabolites.

Plant organs of the *Lantana* and nature of the solvent found to have great impact on the antioxidant potential, as evidenced by the reports of Ismail and Ali. Their findings highlighted 60–70% higher activity of leaf extract in comparison to stems. Further, aqueous ethanol cleaved extract expressed a significant free radical scavenging activity up to 87% (Ismail and Ali 2016; Da Porto et al. 2000). In vitro

antioxidant studies of lantadene A highlighted the importance of hydroxyl groups, proximity of aromatic rings, and their high molecular weight in scavenging the superoxide anion radicals (Grace-Lynn et al. 2012).

3.12 Antihypertensive Activity

Hypertension, a life-threatening disorder, is a major contributor to cardiovascular and cerebrovascular events, and according to the World Health Organization (WHO), it is a major cause of premature death worldwide (Tapela et al. 2020). Patients with hypertension are more inclined for holistic herbal approaches to managing their health, due to associated long-term use of allopathic drugs (Xiong et al. 2013). Available reports suggest the extracts of the herbs being "natural" and consequently "safer" with better acceptability with the human body and may provide adjunctive therapeutic approach for the treatment of hypertension (Tabassum and Ahmad 2011; Yuan et al. 2016). Matta et al. explored antihypertensive activity of ethanolic extract of *Lantana* leaves (EELC) on high salt-loaded wistar albino rats. Encouraging results suggested that extract induced a dose-dependent decreased mean arterial blood pressure and protected rats against renal and vascular injuries, possibly by antioxidant activity, reducing work load of the heart and maintaining ionic levels by negative chronotropic effect (Matta et al. 2015). The presence of verbascoside in the *Lantana* as a potent cardiotonic and vasodilator agent has also been reported with potential to act as an antihypertensive agent (Ghisalberti 2000).

3.13 Antidiabetic Activity

Diabetes, a group of metabolic diseases, is being characterized by high blood glucose levels (Alberti and Zimmet 1998). Jawonisi and Adoga's research group evaluated the effect of ethanolic, aqueous, and butanolic extracts of *Lantana* leaf on alloxan-induced diabetes in rats. Investigations highlighted the hypoglycemic activity of aqueous and butanolic fraction on an account of the presence of triterpenes (Jawonisi and Adoga 2015). Isolated stearoyl glucoside of ursolic acid, that is, Urs-12-en-3β-ol-28-oic acid 3β-D-glucopyranosyl-4′-octadecanoate, is known to possess antidiabetic potential by lowering sugar in streptozotocin-induced diabetic rat model (Kazmi et al. 2012).

3.14 Nephroprotective Activity

Drug-induced nephrotoxicity has been reported with number of medications (Jafari et al. 2013), as exemplified by cisplatin, a chemotherapeutic agent, but its clinical use

is severely limited by serious side effects such as nephrotoxicity. Thus, it is obligated to look forward for alternative systems of medicine and number of reports indicating recommendation of NPs as nephroprotective agents (Cummings and Schnellmann 2002; Mohan et al. 2006). Significant improvement in renal parameters was observed with methanolic extract of *Lantana* in cisplatin-induced nephrotoxicity in rat, and positive correlation was observed with phytoconstituents (phenylethanoids, flavonoid, iridoids, and phenolic acids) as nephroprotective agent (Abdel-Hady et al. 2018). Another study suggested the therapeutic utility of OA (from *Lantana* root) in renal injury after its nephron protective activity in a dose-dependent manner (Vyas and Argal 2012).

3.15 *Toxicological Aspects of* Lantana

"Natural" is not synonymous with "safe"; consumers often equate "natural" with "safe," and it is well documented in various reports that constituents in natural products (NPs) can result in toxicity (Gaston et al. 2020). *Lantana* is a widely grown noxious weed in tropical and subtropical regions of the world (Sharma et al. 1988). Well-documented reports indicate its outspread as a weed and toxicity in grazing animals. The domination of *Lantana* over other species has been reported due to allelopathic action of triterpenoids and phenols; however, the toxicity was observed only after consumption of high amount of plant material. More susceptibility of cattle, sheep, and goats to lantadenes A, B, and D and icterogenic acid toxicity, in comparison to no susceptibility of rats, horses, neonatal calves, and lambs toward lantadene A, further heightened that poisoning susceptibility varies with different animals. The prominent clinical sign of poisoning includes constipation, photosensitization, and jaundice. Further, histological studies showed that lesions in the liver of *Lantana*-poisoned animals are consistent with intrahepatic cholestasis and hepatotoxicity (Sharma 1989).

3.16 *Other Uses of* Lantana

Apart from invasive and its medicinal importance, various reports also indicate the use of *Lantana* as vermicompost. Its vermicompost was seen to be a good organic fertilizer with the tendency to enhance the nitrogen content and hence improve the fertility of rocky, grave, or hard laterite soils (Fan et al. 2010). It is used as green manure in India, on account of high content of nitrogen (N) in *Lantana* leaves and twigs (Hussain et al. 2015). Fast decomposition of *Lantana* leaves and twigs leads to the release of N into the soil. Further *Lantana* ash is reported with high content of microelements including manganese and potassium effective in manuring commercial plants (Munir 1996; Suthar and Sharma 2013).

Growing population and rising demand of furniture triggered a challenge to the oversupply of canes and timber. *Lantana* provides an instructive example; different research groups reported its potential in pulp, paper, furniture, toy industries, and articles of household in Southern India (Neelagar et al. 2018). Reports by Chatterjee's research group indicated that furniture resembling to cane in design and performance with reduced cost is being made by tribal communities around the Mudumalai Tiger Reserve in India, and to further explore the business opportunities in furniture industry using termite-resistant *Lantana* weed, another initiative, that is, Women Empowerment through *Lantana* Furniture and Artifacts and Restoration of Environment (WELFARE) was launched in the fringe of the Corbett National Park, India (Chatterjee 2015).

4 Formulations of *Lantana*

Despite of tough competition with latest synthetic formulations derived from computational and combinatorial chemistry, NP-based formulations are the choice by people due to their safety, efficacy, and socioeconomic benefits. After innumerable studies for the extraction and isolation of phytoconstituents of *Lantana*, considerable efforts have been made toward its herbal formulations (Obeid et al. 2017).

4.1 Gold Nanoparticles

Development of noble metal nanoparticles using plant extract has emerged as an economical approach in drug development process because of its simple, cost-effective, and high yield returns (Mahl et al. 2010; Zhang et al. 2012). Gold nanoparticles are extensively being used in last few decades in biomedicines and nanomedicines and in drug delivery system, and their administration in human has been found to be safe (Tiwari et al. 2011; Connor et al. 2005; Kumar et al. 2013a).

For the first time, AuNP synthesis using the root aqueous extract of *Lantana*, followed by their evaluation as an antioxidant and cytotoxic potential in human breast cancer cell line (MDA-MB-231), has been reported by the researcher Ramkumar and his team (Ramkumar et al. 2017). Encouraging outcomes of preponderant activity by newly synthesized AuNPs against human breast cancer (MDA-MB-231) cells and normal Vero cells could be via apoptosis induction or DNA fragmentation.

Methylene blue (MB), a thiazine-based cationic dye, is commonly used as dyeing material for silk, cotton, wood, and paper. Acute exposure to MB resulted in harmful effects like vomiting, cyanosis, shock, jaundice, etc. in humans and aquatic animals and indicated the importance of remediation of MB from wastewater (Hameed et al. 2007). Kumar et al. reported first time the usage of *Lantana* flower in the fabrication of AuNPs against photocatalytic degradation activity of the MB. Significant

photocatalytic degradation of MB (>62%, 10 mg/L) proved to be a rapid, inexpensive, and eco-friendly approach for industrial-scale production of *Lantana* flower AuNPs (Kumar et al. 2016).

4.2 Silver Nanoparticles

Among all the noble metal nanoparticles, silver nanoparticles (AgNPs) have gained boundless interests, because of their unique properties such as chemical stability and catalytic and most important antibacterial, antifungal, antiviral, and anti-inflammatory activities (Ahmed et al. 2016).

After several attempts of green synthesis of AgNP approach, Patil et al. reported for the first time synthesis of AgNPs using terpene-rich petroleum ether extract (TRE) of *Lantana* leaves. Prepared nanoparticles were explored for antibacterial, antioxidant, and cytotoxic potential. Dose-dependent antioxidant potential was observed using dot blot rapid screening method in comparison to standard ascorbic acid. A significant antimicrobial activity was observed for gram-positive (*S. aureus*) than gram-negative (*P. aeruginosa* and *E. coli*) bacteria by AgNPs synthesized by using petroleum ether extract of *Lantana* leaves (Patil 2017). The presence of high peptidoglycan content in the cell wall of gram-positive bacteria and interactions of positively charged Ag^+ ions with negatively charged thick peptidoglycan layer could be the possible reasons for their increased antibacterial activity. The synthesized AgNPs showed dose-dependent cytotoxicity on brine shrimp with LD_{50} value 514.50 μg/mL. Researchers proposed that AgNPs enter the cell and exert their cytotoxic effect by deactivating the bacteria cell membrane via forming stable S–Ag bond with thiol group of enzymes or denaturing DNA by breaking hydrogen bonds between nitrogen bases of DNA. Efforts were made by Ajitha et al. wherein they prepared AgNPs of *Lantana* leaves through simple green route and evaluated their antibacterial activity based on Kirby-Bauer disk diffusion method. Significant antibacterial activity was rendered against pathogenic bacteria including *Bacillus* spp. and *Pseudomonas* species (Ajitha et al. 2015).

4.3 Cream Formulation

A number of mosquito repellent formulations have been developed, keeping in mind the pleasure and comfort of cream usage especially with plant-based essential oils (Oyedele et al. 2002). This further engaged the interest on *Lantana* by another group of researchers, where they formulated creams with methanol, hexane, and ethyl acetate fractions of *Lantana* leaves for the first time and tested for antimalarial efficacy against female *Aedes aegypti* using Odomos as a positive control. They claimed general protective nature of all the formulations against mosquito bites,

without any allergic reaction by the human volunteers, and maximum protection was observed with methanol extract formulated based cream (Keziah et al. 2015).

4.4 Pulsatile Drug Delivery System

Pulsatile drug delivery system aims to release drugs at substantially constant release rate per unit time, in order to manage the disease while reducing the treatment-associated side effects. Considering the physiological need of the disease, drug is being delivered at specific time and results in enhanced therapeutic efficacy and compliance (Kalantzi et al. 2009).

Verma et al. (1997) reported for the first time the synthesis of super hydrophilic biocompatible *Lantana* polyacrylonitrile composite from the leaf oil and claimed for its superior antibacterial property. Inhibition zones of 8–10 and 7–8 mm were observed in the composite membrane of *B. subtilis* (gram-positive bacteria) and *E. coli* (gram-negative bacteria), respectively. Further study indicated sustained diffusion of *E*-caryophyllene, an active antibacterial component from porous composite membrane of polyacrylonitrile matrix making it an excellent antibacterial system (Verma and Balasubramanian 2014).

5 Conclusion and Perspectives

Better understanding of underlying pathology has rendered us with modern culture of synthetic drugs and systematic approaches to treat and manage different diseases. But the associated side effects of existing approaches and development of resistance are continuously necessitating and knocking the door for natural therapies. Indigenous traditional herbals are representing an alternative and gaining huge interest day by day by human beings for the treatment and prevention of a variety of ailments. Over the past decades, the weed *Lantana* has attracted lot of interest due to its striking features, and the present review is an explicit account of its therapeutic potentials and chemical constituents till date. Despite its vast diversity, there is a significant lack of research to make its reach to the clinical trials, emphasizing the need for further detailed investigations so as to bridge the explanatory gap between its different constituents and their proposed mechanism. On similar grounds, a small effort has been made by the authors in their laboratory using computer-aided drug design-inspired designing and synthesis of modified derivatives of naturally driven lantadenes to explore their potential in skin cancer treatment. Encouraging outcomes of this research work have been provisionally protected in terms of Indian patent. Thus, it can be envisaged that in coming years, rigorous and robust methodology may warrant some promising lead molecules for *Lantana*.

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Chapter 8
Algal Phytochemicals from Different Algal Forms with an Emphasis on Genomic Insights into Their Nutraceutical and Pharmaceutical Applications

Avik Kumar Choudhury and Rohan Kr Biswas

1 Introduction

The use of plants as whole or a its part(s) for treating different diseases is not new, but very old, as written in "Vedas" and "Puranas and other old scriptures where the uses of traditional medicines were mentioned. The consumption of different forms of raw plants was the earliest mode of medicinal treatments (Narayanaswamy 1981). Traditionally, local rural populations have largely remained dependent on plant-based home remedies [e.g., crude extracts of Tulsi (*Ocimum tenuiflorum*), Haldi (*Curcuma longa*), Kalmegh (*Andrographis paniculata*), Ashwagandha (*Withania somnifera*), Peepal (*Ficus religiosa*), Nagkesar (*Mesua ferrea*), Dalchini (*Cinnamomum verum*), Adrak (*Zingiber officinale*), etc.]. As science progressed, research into traditional medicines clearly established that phytochemicals and secondary metabolites derived from these plants were actually being used in traditional medicines. The branch of medical science known as "Ayurveda" was developed on the basis of the science of phytochemicals. This utilization of plant-based medicines largely derived from phytochemicals has remained a common method for treatment for several diseases in African nations as well (Mišurcová et al. 2012). With the advancement of science as knowledge on indigenous plants increased, the demand for more plant-based resources with varied applications in different fields continues to increase as well. Moreover, the chemical nature of these phytochemicals has also been worked out, which established these phytochemicals with potential pharmaceutical and nutraceutical applications (Brielmann et al. 2006). Crude phytochemicals derived from angiosperm sources (e.g., *Ocimum* spp., *Rauwolfia*

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M. K. Swamy, A. Kumar (eds.), *Phytochemical Genomics*,
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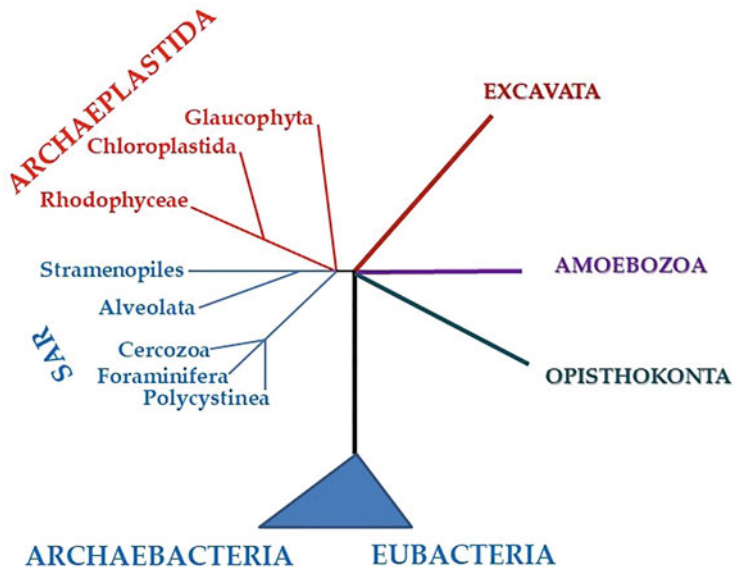

Fig. 8.1 Phylogenetic position of the Archaeplastida and SAR clades that are considered to be the closest relatives of Viridiplantae or modern-day land plants based on chloroplast membrane structure. (Adopted from Adl et al. 2005)

spp., *Santalum* spp., *Curcuma* spp., *Cucumis* spp., etc.) and other plant forms like the gymnosperm *Taxus baccata* have also been well documented to be important source for medicinally important phytochemicals (Nisar et al. 2008). However, lower plant groups like algae, bryophytes, or pteridophytes have been overlooked as potent source for important phytochemicals until recently possibly because of nonavailability of suitable biomass or the complications of extraction process.

Algae are one of the most abundant forms of plant on earth, and many of these forms inclusive of both microscopic and macroscopic entities have recently been separated into different groups like Apicomplexa, Alveolata, Chromalveolata, and Heterokontophyta, which deviate from the traditional concept of "Algae." This is because the chloroplast morphologies of these groups are dissimilar with angiosperm chloroplast, which are considered to be the true representative of "modern-day plants" as their chloroplast membrane structures are derived through primary endosymbiosis (Adl et al. 2005; Lee 2018). The nearest relative among the traditional eukaryotic algal groups (Fritsch 1935; Bold and Wynne 1985) has recently been confined to the class Archaeplastida (inclusive of Chlorophyta, Rhodophyta, and Glaucophyta) with closest similarity to the modern-day land plants, the Viridiplantae (Fig. 8.1) (Rockwell et al. 2014). The other traditional algal groups like diatoms, brown algae, and dinoflagellates are categorized under the "SAR clade" where the chloroplast evolution is an outcome of secondary endosymbiosis with presence of chloroplast endoplasmic reticulum (CER) along with the double membrane of chloroplast (Adl et al. 2005). However, all these algal groups have different classes

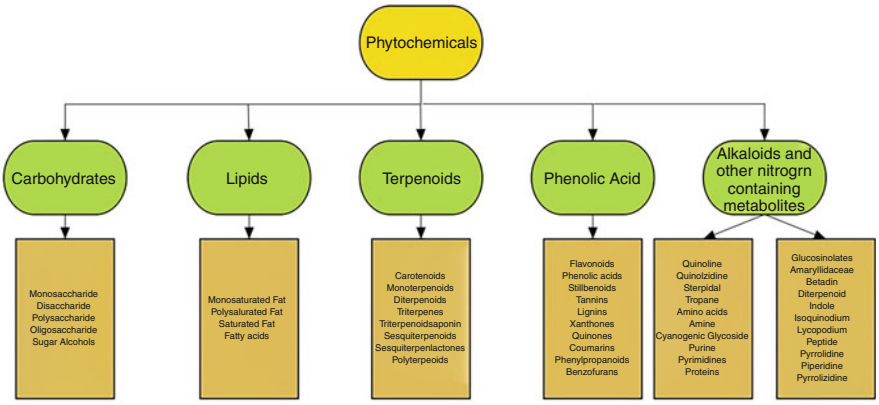

Fig. 8.2 The different categories of phytochemicals that are available from different plant sources. (Adapted and modified from Jareonsin and Pumas 2021)

of phytochemicals and secondary metabolites, which are often more diversified than those available from higher angiosperm and gymnosperm groups.

Plant-based phytochemicals have broadly been divided into five broad classes that include carbohydrates, lipids, terpenoids, phenolic acids, and nitrogenous and non-nitrogenous alkaloids, which can be further divided into several subclasses based upon their chemical nature (Jareonsin and Pumas 2021) (Fig. 8.2). Although phenolic compounds remain largely restricted to higher plant groups as secondary metabolites excluding algal sources, the other groups of phytochemicals are abundantly present in different micro- and macro-algal forms. Among them, carbohydrate derivatives have both sulfated and nonsulfated forms that are often commercially exploited as has been done with carotenoids and long-chain polyunsaturated fatty acids (LCPUFA) (Koyande et al. 2019). The majority of algae-derived nitrogenous alkaloids tend to show toxicity like purine derivatives and amide residues (Lee 2008), which are beyond discussion in the present work. The phytochemicals synthesized in different algal forms are mainly responsible for providing a defense system against abiotic stress pertaining to ROS scavenging activity by different classes of carotenoids. Other biomolecules like vitamins, terpenoids, and PUFA are also reported from several algal taxa, which have been documented to be effective against different classes of nonpathogenic diseases inclusive of tumor-forming cancerous cells (Abd El-Hack et al. 2019).

Even though algae inhabit diverse habitats, marine algae have remained as a source of food, fodder, and other common economically important products. Algae-derived products like agar agar, carrageenan, polysulfated galactans, and phycocolloids have been long been extracted from red algal (e.g., *Porphyra* spp., *Gelidium* spp., *Gracilaria* spp., *Kappaphycus* spp.) and brown algal resources (*Macrocystis* spp., *Laminaria* spp., *Sargassum* spp.) (Lee 2008). Studies have revealed that several other unicellular microalgae like *Dunaliella* spp. and *Haematococcus* spp. and diatoms are rich sources of different phytochemicals.

Thus, the present work discusses about algae-based phytochemicals and their sources with an emphasis on commercially important biomolecules. The molecular regulation of the biosynthesis of some of these important phytochemicals is taken into consideration. Furthermore, the aspect of therapeutic application of these phytochemicals for a wide array of diseases will be the main thematic area of the present chapter.

Among the algal phytochemicals, the extraction and exploitation of algal polysaccharide has remained the foremost process to be used commercially. These polysaccharides not only are building blocks of algal cells but also are important storage products as well (Fig. 8.3). Thus, the present chapter begins with algal polysaccharide and subsequently extends to other phytochemicals like pigments, vitamins, terpenoids, and polyunsaturated fatty acids (PUFA).

2 Algal Polysaccharides

Algal polysaccharides can be broadly classified into storage and structural polysaccharides, respectively (Lee 2008) (Fig. 8.3a, b). Structural polysaccharides are mainly responsible for protection and structural integrity, whereas storage polysaccharides act as the principal sources of energy. Algal polysaccharide compositions and their proportions alter on the basis of taxa, habitats (freshwater, marine, or terrestrial) (Becker 2007; Aquino et al. 2011; Cheng et al. 2011; Rodrigues and da

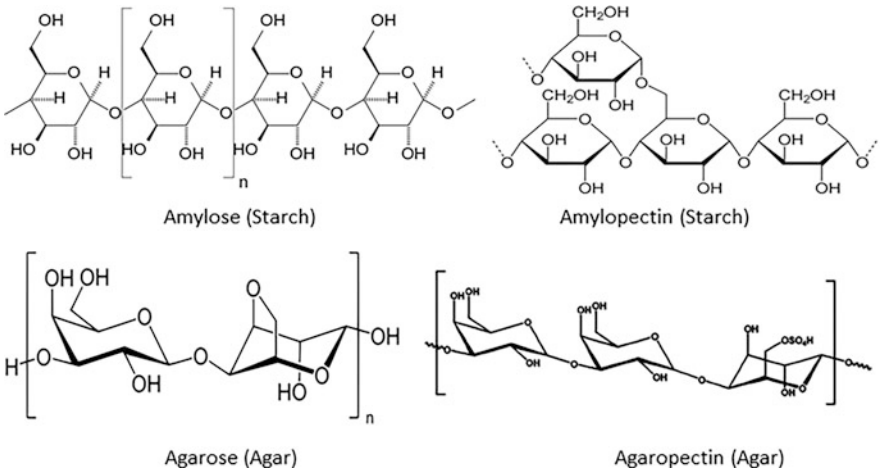

Fig. 8.3 (**a**, **b**) Chemical structures of different storage and structural polysaccharides in algae. (Sources: Lee (2008), https://commons.wikimedia.org/; https://www.vectorstock.com/; https://www.fao.org/; https://www.wikiwand.com/; https://www.carbosynth.com/; https://www.elicityl-oligotech.com/)

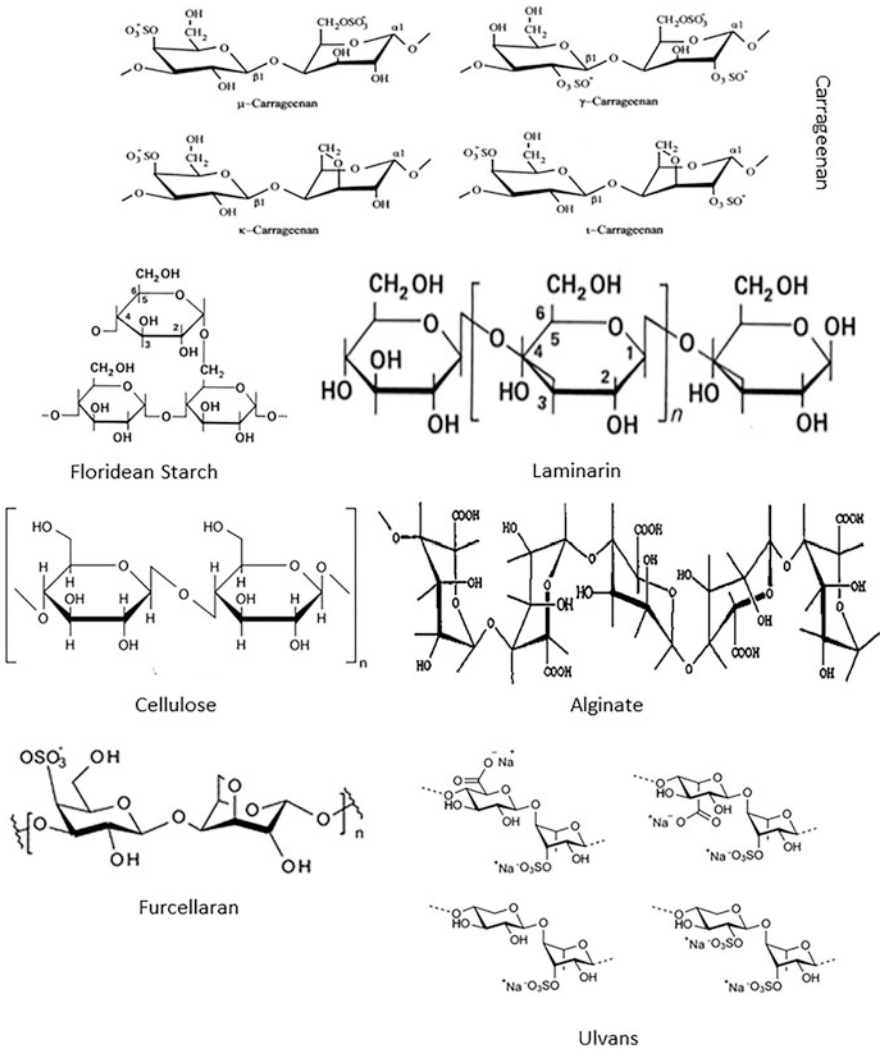

Fig. 8.3 (continued)

Silva Bon 2011; Rodrigues et al. 2012), morphological stage of life cycle, salinity tolerance, and location within the cell (Usov 1998).

2.1 Algal Polysaccharides as Storage Product

Storage polysaccharides can be broadly categorized into three major groups based upon their chemical structures and localization in the cell, namely, starch, floridean

starch, and laminarin. Even though glycogen is abundant in both green algae and cyanobacteria, starch remains as conventional starch in chlorophytes. Both in starch and glycogen, α-(1,4) glycosidic bonds link units of glucose and α-(1,6) linkages form branch points although the number and position of the monomeric units, and branches vary in these polysaccharides on the basis of taxon specificity (Chao and Bowen 1971). Floridean starch in red algae and laminarin in brown algae are mainly localized in the cytosol as compared to conventional starch that remains concentrated in plastid entities.

2.1.1 Starch

Starch is made up of variable proportions of amylose and amylopectin that ranges from 5% to 30% and about 70% or more, respectively, depending upon available environmental conditions and nutrient availability (Ball and Deschamps 2009). Starch synthesized by green algae has a well-organized tandem-cluster structure, whereas cyanobacterial starch tends to have a more randomly branched structure (Nakamura et al. 2005).

2.1.2 Laminarin

Laminaran or laminarin is the main storage polysaccharide in brown algae, which is formed by β-(1,3) D-glucan and β-(1,6) branch points with mannitol or glucose as terminal reducing ends. The higher number of branch points promotes better solubility in cold water as compared to lesser number of branch points that require warm water to solubilize (Rupérez et al. 2002; Jaulneau et al. 2010).

2.1.3 Floridean Starch

Floridean starch abundant in red algae is structurally similar to conventional starch of green algae and higher plants but without amylose. The other difference with plastidial starch is that this starch is synthesized in the cytosol. Interestingly, plastid-derived enzymes like isoamylases and starch synthases remain operative in the cytosol by utilizing UDP-glucose as the source of glucans for polymerization into starch, thereby restricting floridean starch synthesis in the cytosol (Mišurcová et al. 2014).

2.2 Structural Polysaccharides in Algae

The main function of this class of polysaccharide is to provide protection to the algal cells and maintain structural integrity of more complex algal forms. These

polysaccharides have a more heterogenous composition as compared to storage polysaccharides with variable proportions of branched and sulfated polysaccharides, proteins, and different ions.

2.2.1 Cellulose

Cellulose predominates the structural polysaccharides found in algae, which is made up of linear chain of D-glucose units joined together by β-(1,4) glycosidic bonds. The location of hydrogen bonds between and within the cellulose microfibrils determines the crystallization properties of cellulose molecules. Based upon this property, the natural form of cellulose I exists as I_α and I_β respectively, with I_α being abundant in algae and is I_β mainly found in angiosperms (Kroon-Batenburg and Kroon 1997; Nishiyama et al. 2003).

2.2.2 Alginates

Unsulfated alginates are abundantly present in the matrix and cells of brown algae along with fucans and other heteroglycans. These alginates are composed of heteropolysaccharides consisting of β-(1,4)-linked D-mannuronic (M) and α-(1,4)-linked L-guluronic (G) acids, with positional difference in monomeric composition, thereby constituting either homopolymeric (MM or GG) or heteropolymeric (MG or GM) blocks (Miller 1996; Rioux et al. 2007). The composition of G and M is critical in the gelling properties of alginates with higher M/G ratio providing more elasticity, whereas lower M/G ratio makes the alginates brittle (Fenoradosoa et al. 2010). Alginate composition also depends on algal taxa, habitat conditions, seasonal variations, and part of the brown algal thallus from where it is extracted (Larsen et al. 2003).

2.3 Sulfated Polysaccharides

Sulfated polysaccharides are recorded in all marine algae especially seaweeds with possible roles in halotolerance, mechanical flexibility, osmoregulation, and adaptation to marine habitat (Aquino et al. 2011; Rodrigues et al. 2012). Chemically, these are natural glycosaminoglycans with hemi ester sulfate groups in the sugar residues (Shanmugam and Mody 2000). Red algae synthesize sulfated galactans that are composed of β- or α-galactose units either in their D- or L-configurations (Usov 1998). Green seaweeds produce sulfated glucans, sulfated galactans, and sulfated arabinogalactans, whereas spirulan is the only sulfated polysaccharide reported in cyanobacteria (Shanmugam and Mody 2000; Costa et al. 2010; Aquino et al. 2011). The naming of these sulfated polysaccharides has been mainly based on the taxa from where it is extracted like spirulan (*Spirulina platensis*), Fucan (*Fucus* spp.),

Ulvan (*Ulva lactuca*), and Furcellaran (*Furcellaria lumbricalis*). The degree of sulfation and sugar compositions varies not only among different algal groups but also between different strains as well. Galactose residues dominate in red algae, whereas rhamnose, glucose residues, and uronic acid are more prominent in green algae (Shanmugam et al. 2002; Rupérez and Toledano 2003; Mao et al. 2006, 2009; Zhang et al. 2008). In cyanobacterial taxa like *Arthrospira platensis*, there is abundance of rhamnose and guluronic residues (Table 8.1) (Majdoub et al. 2009).

2.3.1 Agar Agar and Carrageenan

Agar and carrageenan have long been reported as the main sulfated polysaccharides available from red seaweeds mainly belonging to the order Gigartinales. Agar consists of a gelling fraction in agarose and a non-gelling fraction as agaropectin. Chemically, agar is made up of a linear chain of alternating 3-linked β-D-galactopyranosyl and 4-linked 3,6-anhydro-a-L-galactopyranosyl units with low sulfate levels, whereas carrageenan is composed of linear chains of repeating disaccharide units with alternating 3-linked β-D-galactopyranose (G-units) and 4-linked α-D-galactopyranose (D-units) or 3,6-anhydro-α-D-galactopyranose (DA-units) (Usov 1998; Shanmugam and Mody 2000; Lahaye 2001). Based upon the level of sulfate esterification, these polysaccharides can be classified into several groups like kappa, iota, lambda, gamma, theta, epsilon, and mu (κ, ι, λ, γ, θ, ε, and μ).

2.3.2 Furcellaran

Another recently found sulfated polysaccharide furcellaran has been isolated and commercially exploited from the red macroalga *Furcellaria* sp., commonly known as Danish agar. This polymer is mainly composed of 3-linked b-D-galactopyranose, 4-linked 3,6-anhydro-a-D-galactopyranose, and 3-linked b-D-galactopyranose 4-sulfate residues (Laos and Ring 2005; Laos et al. 2005). Porphyran extracted from *Porphyra umbilicalis*, another red algal polysaccharide, is composed of a variety of differently sulfated galactose residues in variable proportions.

2.3.3 Ulvans

Ulvans, a group of water-soluble, sulfated heteropolysaccharide abundantly available from members of Ulvales (*Ulva*, *Enteromorpha*), are composed of a variety of sugar residues like xylose, rhamnose, glucuronic acids, iduronic acids, and sulfate groups. Compositionally, these heteropolysaccharides are more complex than the extracted sulfated polysaccharides from red algae (Jiao et al. 2011).

Table 8.1 The different sources of sulphated polysaccharides isolated from diverse algal sources and their compositional differences as documented from available literatures (modified from Mišurcová et al. 2014)

Category	Algal division	Algal taxa	Most abundant neutral sugar residues (NSR)	Method of extraction	References
Sulfated polysaccharide	Rhodophyta (red algae)	*Schizymenia binderi*	Galactose (49.8%)	Water	Zúniga et al. (2006)
		Chondrus crispus	Galactose (120.7 g/kg)	Enzymatic	Rupérez and Toledano (2003)
		Porphyra tenera	Galactose (120.7 g/kg)	Enzymatic	Rupérez and Toledano (2003)
		Nothogenia fastigiata	Mannose (62.3 mol %)	Hot water	Kolender et al. (1997)
	Chlorophyta (green algae)	*Ulva conglobate*	Rhamnose (71.9 mol %)	Hot water	Mao et al. (2006)
		Monostroma lattisimum	Rhamnose (78.65 mol %) Rhamnose (86.77 mol %)	Water	Zhang et al. (2008) Mao et al. (2009)
		Monostroma nitidum	Rhamnose (79.4 mol %)	Water	Zhang et al. (2008)
		Codium dwarkense	Galactose (43.62 mol %)	Cold water	Shanmugam et al. (2002)
		Codium dwarkense	Mannose (32.4 mol %)	Hot water	Shanmugam et al. (2002)
		Avrainvillea erecta	Glucose (66.32 mol %)	Hot water	Shanmugam et al. (2002)
	Cyanobacteria	*Arthrospira platensis*	Rhamnose (49.7%)	Ultrafiltration	Majdoub et al. (2009)
		Spirulina platensis	Guluronic acid (18.3%)	Hot water	Abd El Baky et al. (2013)
		Spirulina platensis	Guluronic acid (2.46%)	Ethanol	Abd El Baky et al. (2013)
				Ion exchange	

(continued)

Table 8.1 (continued)

Category	Algal division	Algal taxa	Most abundant neutral sugar residues (NSR)	Method of extraction	References
	Phaeophyta (brown algae)	*Laminaria saccharina*	Fucose (36.7%)		Ushakova et al. (2009)
		Laminaria digitata	Fucose (31.1%)	Ion exchange	Ushakova et al. (2009)
		Fucus distichus	Fucose (40.8%)	Ion exchange	Ushakova et al. (2009)
		Fucus serratus	Fucose (24.8%)	Ion exchange	Ushakova et al. (2009)
		Fucus vesiculosus	Fucose (92.3%)	Ion exchange	Dürig et al. (1997)
		Fucus vesiculosus	Fucose (43.9 g/kg)	Ethanol	Rupérez and Toledano (2003)
		Ascophyllum nodosum	Fucose (26.6%)	Ion exchange	Ushakova et al. (2009)
		Chorda filum	Fucose (64%)	Ion exchange	Ushakova et al. (2009)
		Analipus japonicus	Fucose (44.1%)	Ion exchange	Ushakova et al. (2009)
		Punctaria plantaginea	Fucose (44.3%)	Aqueous calcium chloride	Bilan et al. (2014)
		Padina tetrastromatica	Fucose (54 mol%)	Water	Karmakar et al. (2009)
		Turbinaria ornata	Fucose (30.3 mol %)	Methanol: chloroform: water	Thuy et al. (2015)
		Sargassum polycystum	Fucose (20.3 mol %)	Methanol: chloroform: water	Thuy et al. (2015)
		Undaria pinnatifida	Fucose (7.1 g/kg)	Enzymatic	Rupérez and Toledano (2003)

2.3.4 Spirulans

Sodium Spirulan (Na-SP) and calcium spirulan (Ca-SP) are the main sulfated polysaccharide isolated from *Spirulina platensis* by hot water extraction. They are mainly rhamnose and xylose residues with sulfate substitution at position 4 (Lee et al. 2000; Yamamoto et al. 2003). Ca-SP show significant antiviral activity, whereas therapeutic activity of Na-SP has been documented as antithrombin agent.

2.4 Therapeutic Applications of Algal Polysaccharides

Fucoidans isolated from different species of brown algae have been reported to show anti-inflammatory, antiviral, antiangiogenic, immunomodulatory anticoagulant, and antiadhesive properties (Damonte et al. 2004; Cumashi et al. 2007) although species-specific compositional variations change the intensities of these therapeutic applications (Cumashi et al. 2007). Fucoidans isolated from *Fucus vesiculosus* have shown that over sulfation of these sulfated polysaccharide resulted in downregulation of both mitogenic and chemotactic activity of vascular endothelial growth factor 165 (VEGF165) that was responsible for anti-carcinogenic effects on carcinoma and melanoma cell proliferation (Koyanagi et al. 2003). Likewise, fucoidans isolated from *Cladosiphon novae-caledoniae* also inhibited VEGF expression in HeLa cells of human uterine carcinoma that reduced malignancy and vascular tubule formation (Ye et al. 2005). Different studies have further documented not only those therapeutic applications of algal polysaccharides that remain restricted to anticancer activities but also those obtained from red alga (*Porphyridium* sp.) and cyanobacteria (*Nostoc commune*, *Spirulina maxima*, *S. platensis*) that are beneficial for prevention of cardiovascular diseases also (Ku et al. 2015; Ngo-Matip et al. 2014) (Table 8.2). The sulfated polysaccharides extracted from a diverse group of green algae, rhodophytes, and dinoflagellates have shown antiviral properties (Chen et al. 2016; Winter et al. 2014).

Table 8.2 The therapeutic applications of some of the algal polysaccharides isolated from different cyanobacteria, rhodophytes, and dinoflagellates (modified from Abd El-Hack et al. 2019)

Activity	Microalgal taxa	Bioactive compound	References
Micro- and macronutrients	*Dunaliella salina*, *D. bardawil*, *Chlorella* spp.	Glycogen	Santos-Sanchez et al. (2016), Salmeán et al. (2015)
Prevention of cardiovascular disease	*Porphyridium* sp., *Nostoc commune*, *Spirulina maxima*, *Spirulina platensis*	Algal polysaccharide, non-lipid polysaccharide-rich fraction	Ku et al. (2015), Ngo-Matip et al. (2014)
Antiviral activities	Cyanobacteria, red algae, dinoflagellates	Sulfated polysaccharides	Chen et al. (2016), Winter et al. (2014)
Antitumor activities against carcinogenic cells	*Gymnodinium* spp., *Aphanizomenon flos-aquae*, *Chlorella pyrenoidosa*, *Spirulina* sp., *Porphyridium* sp.	D-galactan sulfate in association with L-(+)-lactic acid	Gardeva et al. (2014)

3 Pigments

Pigments are the foremost phytochemicals abundantly available in algal forms. Other than the reaction center, which is the chlorophyll a, a diverse array of tetrapyrrole ring (chlorophyll, phycobilisomes) and conjugate bond (carotenoids) containing pigments are reported from algal sources. Among tetrapyrrole-containing pigments, both closed and open tetrapyrrole ring configurations are abundantly present in algal forms and cyanobacteria. The occurrence of open tetrapyrrole forms is unique to algal forms without any representative taxa among higher plant groups including bryophytes, tracheophytes, and other vascular phanerogams. Carotenoids are essentially tetraterpenoids although their primary role as accessory pigment of light reaction of photosynthesis as protective molecule against photobleaching makes it more relevant to be categorized under this section.

3.1 Phycobilisomes

The open tetrapyrrole pigment molecules collectively regarded as phycobilisomes (PBS) are abundantly present in cyanobacteria, red algae, and cryptophytes although the phenomenon of incident light wavelength complementation in phycobilisomes is observed only in cyanobacteria (Kehoe and Grossman 1994; Lee 2008). They are composed of a light-sensing chromophore part known as phycobilin, which combines with the apoprotein component to form the functional phycobilisome holoprotein.

3.1.1 Structure and Composition of Phycobilisomes

In phycobilisomes, the chromophore component remains in an open tetrapyrrole configuration, allowing it in making H-bond in water, which makes it a water-soluble pigment (Glazer 1989). The phycobilisome is radiating accessory pigment on the surface of thylakoid membrane that funnels the incoming incident visible light to the reaction center chlorophyll a, thereby inhibiting photobleaching. The constitutive part of phycobilisomes is made up of allophycocyanin (APC) and phycocyanin (PC), whereas the inducible part is composed either of PC or phycoerythrin (PE). Each of the components of the radiating phycobilisome structure, namely, APC, PC, and PE, is made up of hexamers ($\alpha_3\beta_3$) in a heterodimer configuration ($\alpha\beta$). The different components of phycobilisomes (APC, PC, and PE) are joined together through linker proteins (Lee 2008). As reported from the freshwater cyanobacterial taxa *Fremyella diplosiphon* UTEX 481, the two major subunits of the *F. diplosiphon* PBS are the core and rods, with six of the rods emerging from the core. These PBS may contain various combinations of three major types of chromophores containing proteins, namely, phycoerythrin (PE) [absorption maximum

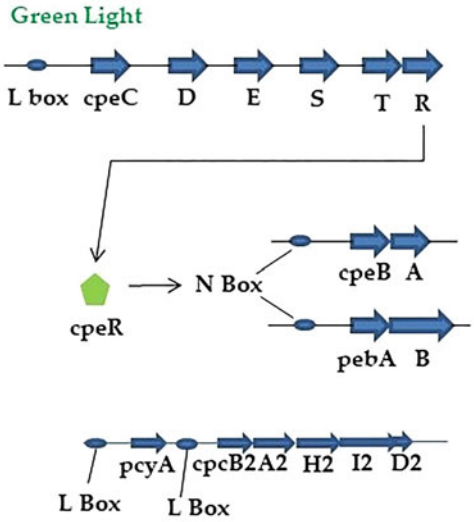

Fig. 8.4 Regulation of phycobilisome gene expression under green light (GL) exposure referred to as the Control of Green Light Induction (Cgi) method in *Fremeyella diplosiphon*

(A_{max}) = 565 nm], phycocyanin (PC) (A_{max} = 620 nm), and allophycocyanin (APC) (A_{max} = 650 nm) (Bogorad 1975; Gantt 1981; Glazer et al. 1982; Sidler 1994). Each of these is unique disc-shaped structures that are formed of six monomers stacked in two cylindrical trimers, with each monomer containing an α- and β-subunit. Discs are connected by non-chromophorylated linker proteins, which also attach the rods to the core and the core to the thylakoid membrane successively (Adir 2005). AP is found within the core whose α- and β-subunits are encoded by the *apcA1B1*genes. The genes responsible for encoding the core-linker protein (*apcC*) and the core-membrane linker (*apcE*) are located near the *apcA1B1* genes. The core-proximal disc of each rod consists of a PC called "constitutive PC" (PCc or PC1) encoded by the genes *cpcB1A1*, which remains next to APC and forms the core component of all phycobilisomes. Green light (GL) and red light (RL) exposures do not have any effect on these genes, and both transcripts and the corresponding protein abundance from this operon are equal in both exposures. The core-distal regions of the rods show differential responses to different light exposures during CCA in this species. During growth in GL exposure, as many as three separate discs of PE may be present. The β- and α-subunits of PE are encoded by the *cpeBA* operon. The PE discs are attached with each other and with the core proximal portion of the rod through the action of three PE linkers, encoded by the *cpeCDE* operon. During growth under RL exposure, the distant two discs of the PBS rods contain a second form of PC known as "inducible PC" (PC_i or PC2). The β- and α-subunits of this phycobiliprotein (PC_i or PC2) are encoded by the *cpcB2A2* genes. The genes *cpcB2A2* are cotranscribed with the genes *cpcH2I2D2*, which encode the corresponding linker proteins (Fig. 8.4). The expression of this large transcription unit *cpcB2A2H2I2D2*, referred together as "*cpc2*," is highly and continuously upregulated in RL. On the other hand, both *cpeCDE* and *cpeBA* operons are upregulated under GL induction in the presence of a small activator molecule

CpeR. The *cpeR* gene is located downstream of the *cpeCDE* genes along with two additional genes, namely, *cpeS* and *cpeT* in the *F. diplosiphon* genome. In GL induction, *cpeR*, cpeCDE, and *cpeST* genes are cotranscribed and form a regulatory circuit that operates through the serial expression of two operons, namely, *cpeCDESTR* and *cpeBA*, respectively, in succession. CpeR has a dual role in synchronizing transcript accumulation of the *cpeCDE* and *cpeBA* operons in GL and as a global activator that coordinates the expression of genes encoding proteins involved in PBS biosynthesis in GL (Fig. 8.4).

3.1.2 Pharmaceutical and Nutraceutical Applications of Phycobilisome and Other Metabolites from Cyanobacteria

Phycobilisome has been documented to be an excellent coloring agent due to the ease of extraction process from cyanobacteria. However, phycobilisomes have also been documented to show antioxidant, anticancerous, neuroprotective, anti-inflammatory, hepatoprotective, and hypocholesterolemic activities (Sonani et al. 2016). In vitro assessment of antioxidant activities of PC showed that it can scavenge alkoxy, hydroxyl, and peroxy radicals and reduce lipid peroxidation (Bhat and Madyastha 2000; Benedetti et al. 2004; Bermejo et al. 2008; Sonani et al. 2015). The ethanolic extract of cPC from *Aphanizomenon flos-aquae* inhibits the growth of AML cells by arresting them in G0-G1 phase (Li et al. 2006). Similar potential anticancer properties have been detected against cancer cell lines from different solvent-based extracts from *Aphanizomenon flos-aquae* and *Phormidium molle* (Bechilli et al. 2011). A host of other compounds isolated from cyanobacterial sources has also been documented to have pharmaceutical applications. A potent neurotoxin Cryptophycin I and a semisynthetic form of the same Cryptophycin 8 isolated from *Nostoc* sp. GSV 20 have shown significant antiproliferative effect against tumor cells under in vivo conditions (Carmicheal 1992; Moore 1996). A polyketide molecule borophycin extracted from the marine cyanobacteria *Nostoc spongiaeforme* var. *tenue* and *Nostoc linckia* showed significant cytotoxic effects against different types of carcinoma cells (Davidson 1995; Banker and Carmeli 1998). Another bioactive molecule Cucarin A isolated from the filamentous cyanobacterium *Lyngbya majuscula* showed inhibitory effect on a series of cancer cell line cells by inhibiting tubulin polymerization (Suzuki et al. 1999; Oftedal et al. 2010). Calothrixins A and B, alkaloids derivatives, and scytonemin differentially regulated cell cycle and mitosis that accounted for cytotoxicity in HeLa, human fibroblast, and endothelial cells (Pang et al. 2010; Rickards et al. 1999) (Table 8.3).

3.2 Carotenoids

Carotenoids are the most common natural pigments, which are excellent antioxidants, scavenging singlet molecular oxygen, and peroxyl radicals due to the presence

Table 8.3 Some of the significant therapeutic phytochemicals extracted and isolated from different cyanobacterial sources with their possible mode of action and clinical status as documented in different studies (modified from Abd El-Hack et al. 2019)

Name of the phytochemical	Source	Mode of action	Clinical status	References
Apratoxin A	*Lyngbya majuscula*	Cytotoxic	Inhibits growth of breast cancer tumor cells	Balaji et al. (2017)
Coibamide A	*Leptolyngbya* sp.	Cytotoxic	Cytotoxic to colon tumor cells	Medina et al. (2008)
Scytonemin	*Stigonema* sp.	Antiproliferative and anti-inflammatory	Inhibits proliferation of Jurkat T cell	Stevenson et al. (2002)
C-Phycocyanin	*Spirulina platensis*	Anticancer activity through MAPK signaling pathway	Inhibits cell proliferation and colony formation ability of MDA-MB-231 cells	Jiang et al. (2018)
Curacin A	*Lyngbya aestuarii*	Antitumor activity	Inhibit carcinoma and adenocarcinoma cell lines	Hassouani et al. (2017)
Calothrixins	*Calothrix* sp.	Cytotoxic	Human Jurkat cells growth reduced to 18% after 24 h of application	Chen et al. (2003)

of conjugate bonds and aromatic ring in the terminal ends. Carotenoids are also involved in cellular signaling as an influencer and trigger redox-sensitive regulatory pathways (Ambati et al. 2018). Several types of division and class specific carotenoids are found in algae (Christaki et al. 2013).

3.2.1 Biosynthesis of Carotenoids

In plant systems, the precursor for all carotenoids is a C5 compound isopentenyl pyrophosphate (IPP), and the carotenoids are synthesized via the plastidial 2-C-methyl-D-erythriol 4-phosphate (MEP) pathway or non-mevalonate (non-MVA) pathway. Chlorophyta exclusively use the 1-deoxyxylulose 5-phosphate/2-C-methylery-thritol 4-phosphate pathway for the biosynthesis of isoprenoids (Schwender et al. 1996) although the biosynthesis and accumulation of β-carotene in *Dunaliella salina* proceed via the glyceraldehyde 3-phosphate/pyruvate pathway (Capa-Robles et al. 2009). Geranylgeranyl diphosphate (GGPP) is derived from dimethylallyl pyrophosphate (DMAPP), where farnesyl pyrophosphate (FPP) is an intermediate compound. The synthesis of FPP from DMAPP is catalyzed by FPP synthase (FPPS), and synthesis of GGPP from FPP is catalyzed by GGPP synthase (GGPPS) (Hirschberg et al. 1997). The first committed step in the carotenoid biosynthesis is the condensation of two molecules of geranylgeranyl pyrophosphate (GGPP) that yields a colorless carotenoid and phytotene catalyzed by phytotene

synthase (PSY EC 2.5.1.32), which is considered to be the rate-limiting step of carotenoid biosynthesis (Sandmann et al. 2006; Varela et al. 2015). PSY is found to be upregulated in several unicellular chlorophyte microalgae such as *Haematococcus pluvialis* (Vidhyavathi et al. 2008), *Chlamydomonas reinhardtii* (Bohne and Linden 2002), and *Dunaliella salina* (Coesel et al. 2008). Generally, there are two classes of PSY family of genes that are found in Chlorophyceae (Ye et al. 2008), although a unique PSY encoding gene has been documented in taxa like *C. reinhardtii*, *Volvox carteri*, or *Chlorella vulgaris* (Ye et al. 2008). From phytotene, phytofluene is subsequently synthesized by phytotene desaturase [(PDS) EC 1.3.5.5]. ζ-carotene (zeta carotene) is then derived from phytofluene, and this step is also catalyzed by PDS. From ζ-carotene, neurosporene is developed with the help of ζ-carotene desaturases [(ZDS) EC 1.3.5.6], which further forms lycopene, which is the first colored carotenoid (Varela et al. 2015). Here, the biosynthesis process bifurcates, and in one branch, δ-carotene is produced from lycopene catalyzed by lycopene ε-cyclase (LCYe). Lycopene β-cyclase (LCYb) further converts δ-carotene to α-carotene, which is formed. This cyclation step of converting lycopene to α- or β-carotene form as a branch point is often considered as a control step in different algal forms (Harjes et al. 2008). Further downstream, α-cryptoxanthin is formed catalyzed by carotene ε-hydroxylase (CYP97C3), and zeinoxanthin is formed by carotene ε-hydroxylase (CYP97A5). From both α-cryptoxanthin and zeinoxanthin, lutein is formed, and the reactions are catalyzed by CYP97A5 and CYP97C3, respectively (Hirschberg et al. 1997; Varela et al. 2015). In another branch, γ-carotene is formed from lycopene, and β-carotene is formed from γ-carotene, and both reactions are catalyzed by LCYb. From β-carotene, two molecules are formed. In one reaction, canthaxanthin is formed with the help of β-carotene C-4-oxygenase or β-carotene ketolase (BKT). From canthaxanthin, astaxanthin is formed, and the reaction is catalyzed by β-carotene 3,3′-hydroxylase (CrtR-b) (Huang et al. 2006). In selected green algal taxa like *Haematococcus pluvialis* and *Chlorella zofingiensis*, astaxanthin is also synthesized from β-carotene by BKT and carotene β-hydroxylase (CHYb). In another reaction, β-cryptoxanthin is formed from β-carotene, and zeaxanthin is formed from β-cryptoxanthin. Both reactions are catalyzed by CHYb. Subsequent production of antheraxanthin and violaxanthin from zeaxanthin is catalyzed by with zeaxanthin epoxidase (ZEP). Antheraxanthin can be synthesized from violaxanthin de-epoxidase (VDE). Zeaxanthin and violaxanthin are interconverted by ZEP and VDE, respectively (Kim et al. 2009; Sandmann et al. 2006). From antheraxanthin, capsanthin is formed, and from violaxanthin, capsorubin is formed with the help of capsanthin-cabsorubin synthase (CCS). Neoxanthin production from violaxanthin is mediated by neoxanthin synthase (NSY) (Fig. 8.5) (Gupta et al. 2021; Hirschberg et al. 1997; Varela et al. 2015). The specific genes of carotenoid synthesis like *pds*, *chyB*, and *bkt* are upregulated under high light stress, whereas NaCl stress upregulates only the *bkt* gene. Under low irradiance levels, sugar additives like sucrose, glucose, and mannose promoted the expression of *pds*, *chyB*, and *bkt* genes for astaxanthin biosynthesis (Li et al. 2008, 2009; Huang et al. 2006, 2008; Sun et al. 2008). Studies have clearly revealed that the carotenoid-specific genes in eukaryotic

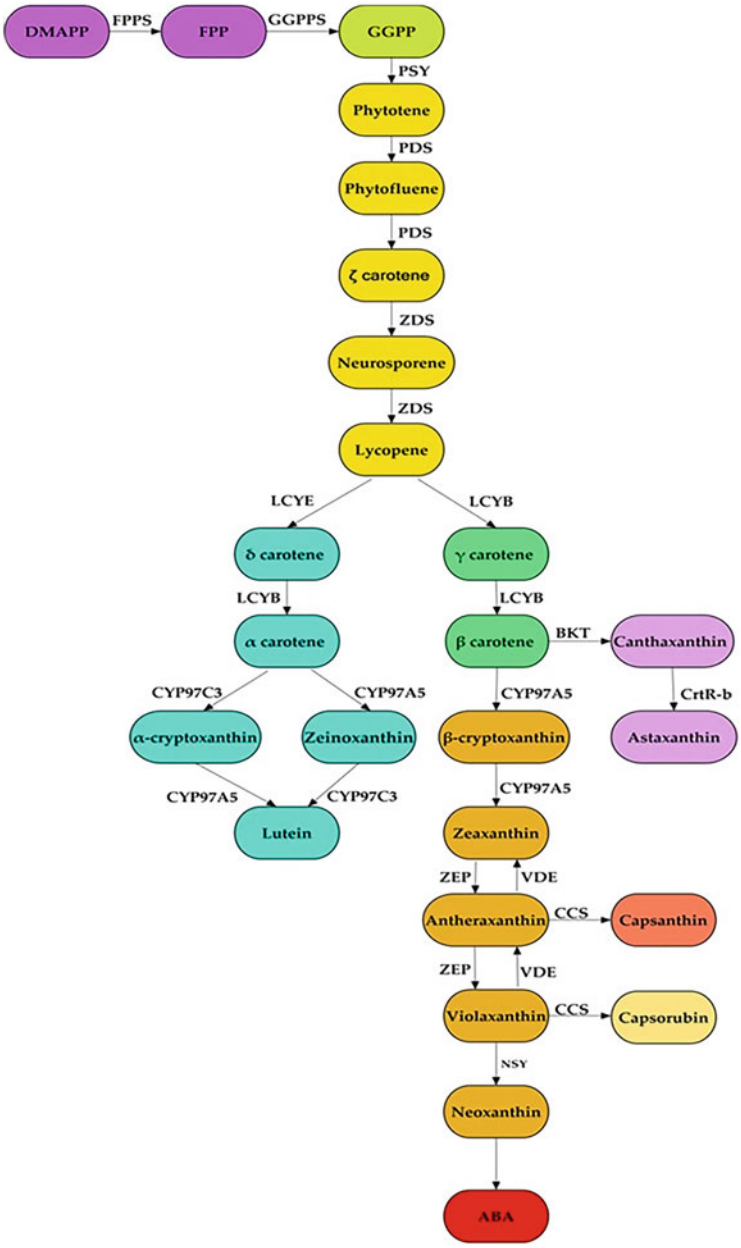

Fig. 8.5 Schematic representation of the carotenoid biosynthesis pathway as documented in different algal forms like *Dunaliella* spp. and *Haematococcus* spp.

algae are derived from cyanobacteria through endosymbiotic gene transfer (EGT) and gene duplication (Tran et al. 2009; Ni et al. 2012). However, the presence of only class I PSY genes in Chlorophyta (except Prasinophyceae) and Streptophyta and class II PSY genes in Rhodophyta is possibly an outcome of gene loss (Masamoto et al. 2001; Chen et al. 2010; Takaichi et al. 2016). In contrast, the absence of α-carotene and subsequent downstream products in members of Haptophyta, Ochrophyta, and Cryptophyta suggests that the loss of LCYe genes prevents the ε-ring formation and subsequent carotenoid derivatives (Wang et al. 2018). Thus, the processes of gene duplication and gene loss has accounted for unbalanced carotenogenesis gene distribution in different algal lineages that alter the pigment composition between different algal groups.

3.2.2 Application of Carotenoids as Therapeutic Agents

Carotenoids in combination with chemotherapeutic agents like 5-fluorouracil have been reported to yield "complete remission" of colorectal cancer as compared to only chemotherapeutic agent, which yielded "partial remission" (Fleischauer et al. 2003). Astaxanthin has been documented to arrest cell cycle at G0/G1 phase when applied in different doses in human gastric cancer cell lines. Astaxanthin has also been reported as efficient antioxidant, which enhances immunity against tumor formation by preventing genotoxicity and cytotoxicity mediated by ROS formation (Kowshik et al. 2014). Further clinical study showed that fucoxanthin isolated from *Undaria pinnatifida* promoted apoptosis and DNA fragmentation in human colon cancer and leukemic cells, thereby showing anti-malignant activities. However, the inhibitory effect against malignant cells was found to be more potent for siphonoxanthin isolated from *Codium fragile* as compared to fucoxanthin from *Undaria pinnatifida* (Fung et al. 2013). Other than anticancerous properties, carotenoids have other cosmeceutical and nutraceutical applications that have been represented in the under mentioned table (Table 8.4).

4 Vitamins

Algae are one of the good sources of vitamins like vitamins A, B_1, B_2, B_6, B_{12}, C, and E (Koyande et al. 2019). It is also found that algal species required different combinations of vitamin B, like thiamin (B_1), biotin (B_7), and cyancobalamin (B_{12}) (Croft et al. 2006). Though the vitamin biosynthesis pathways are less known in algae (Croft et al. 2006), there are some studies that explain the routes and the enzymes that are involved in vitamin biosynthesis.

Table 8.4 A comprehensive documentation of the different algal carotenoids that have been documented to show cosmeceutical, antimicrobial, pharmaceutical, and nutraceutical applications (modified from Meléndez-Martínez et al. 2019; Wang et al. 2015)

Properties	Algal carotenoids
Cosmeceuticals	• Colorless carotenoids phytotene and phytofluene have UV radiation (UVR) absorbing properties. Specifically, phytotene absorbs UV-B (280–320 nm) and phytofluene absorbs UV-A (320–400 nm) region. Cosmetics made using these carotenoids protect skin from UVR damage (Meléndez-Martínez et al. 2019) • β-carotene has been shown to protect against photodamage caused by infrared and visible radiations, and it may an effective antioxidant in sunscreen (Meléndez-Martínez et al. 2019) • Provitamin A carotenoids produce retinoic acid, which like other retinoids intervene in processes like keratinocyte proliferation, epidermal differentiation and keratinization, reduction of inflammation or oxidation, enhancement of the penetration of agents administered topically. Thus, retinoids are applied for different purposes, for example, improving wound healing, preventing skin aging, or the treatment of acne, psoriasis, or other skin conditions (Meléndez-Martínez et al. 2019) • Astaxanthin protects against erythema or reduced wrinkling (Meléndez-Martínez et al. 2019) • It has been reported that astaxanthin can suppress skin hyperpigmentation (Wang et al. 2015) • Canthaxanthin is useful to treat erythropoietic protoporphyria (Meléndez-Martínez et al. 2019) • Lutein can also protect from photodamage (Meléndez-Martínez et al. 2019) • Lycopene, along with phytotene and phytofluene, prevents the UV-induced erythema formation (Meléndez-Martínez et al. 2019) • Fucoxanthin isolated from *Laminaria japonica* has been reported to suppress tyrosinase (the key enzyme of melanin synthesis (Stoyneva-Gärtner et al. 2020) activity in UVB-irradiated guinea pigs and melanogenesis in UVB-irradiated mice (Wang et al. 2015) • Zeaxanthin is also considered to have antityrosinase properties (Stoyneva-Gärtner et al. 2020) • Fucoxanthin counteracts oxidative stress caused by UV radiation and thus is applicable for use in cosmeceuticals (Wang et al. 2015) • Cantaxanthin from the marine eustigmatophycean genus *Nannochloropsis* is used in tanning products (Stoyneva-Gärtner et al. 2020) • β-carotene is used in the formulation of hair conditioners, shampoos, and after shave lotions (Stoyneva-Gärtner et al. 2020)
Antimicrobial	• Zeaxanthin, particularly polar phenolic complexes, has high antimicrobial activity (Parsaeimehr and Chen 2013) • Fucoxanthin has antiviral properties (Ambati et al. 2018)
Antioxidant	• Astaxanthin is the main carotenoid found in *Haematococcus pluvialis*, which is an excellent antioxidant, better than vitamins C and E or other carotenoids (Wang et al. 2015) • B-Carotene has a strong antioxidant capacity, which helps to counteract the free radicals involved in gastrointestinal cancer, arthritis, or premature aging (Wang et al. 2015)

(continued)

Table 8.4 (continued)

Properties	Algal carotenoids
	• Canthaxanthin and lutein also has antioxidant activities (Sathasivam and Ki 2019)
Pharmaceutical and nutraceutical	• Lutein plays an active role in preventing acute and chronic coronary symptoms. It also helpful in maintaining normal visual functions. It hinders the development of cataracts, stimulates the immune response, delays progression of early atherosclerosis, avoids gastric infection, and inhibits macular degeneration linked to age (Wang et al. 2015) • Astaxanthin has effective role against benign prostatic hyperplasia and prostate and liver tumor. It can be actively used against liver neoplasms, which has affecting role in cardiovascular health issues (Sathasivam and Ki 2019). It can be used to protect organisms against various disorders like atherosclerosis, coronary disease, ischemic brain development, chronic inflammatory diseases, metabolic syndrome, diabetes, gastrointestinal and liver diseases as well as neurodegenerative diseases (Alzheimer's and Parkinson's) or to improve cognitive functions (Christaki et al. 2013) • B-Carotene also prevents night blindness and liver fibrosis (Sathasivam and Ki 2019) • Canthaxanthin and lutein, along with astaxanthin and violaxanthin, prevent acute and chronic coronary syndromes and chances of stroke, also help in the prevention of cataracts, and prevent macular degeneration associated with age. It also prevents retinitis and avoid gastric infection by *Helicobacter pylori* (Sathasivam and Ki 2019) • Astaxanthin and violaxanthin both have anti-inflammatory activity (Sathasivam and Ki 2019) • Fucoxanthin have anti-obesity properties (Sathasivam and Ki 2019) • Zeaxanthin, along with β-carotene, also prevents of acute and chronic coronary syndromes, helps to maintain a normal visual function, prevents of cataracts, and prevents macular degeneration associated with age (Sathasivam and Ki 2019) • B-Carotene prevents depression, asthma, infertility, psoriasis, high blood pressure, malnutrition, and macular degeneration (Ambati et al. 2018) • Fucoxanthin has antidiabetic, anti-inflammatory, anti-allergic, anti-osteoporotic properties (Ambati et al. 2018) • Fucoxanthin can protect the liver, blood vessels of the brain, bones, skin, and eyes (Christaki et al. 2013) • Reduced plasma cholesterol and atherogenesis, fat accumulation, and inflammation in liver (Udayan et al. 2017)

4.1 Biotin

Biotin (B_7) is a cofactor required for the transfer of carbon dioxide (CO_2) in many carboxylase enzymes that play a vital role in different metabolic reactions like fatty acid synthesis, branched chain amino acid catabolism, citric acid cycle, and gluconeogenesis. Though the function of biotin in the growth of algae has been well documented in published works, there is a dearth of information about the

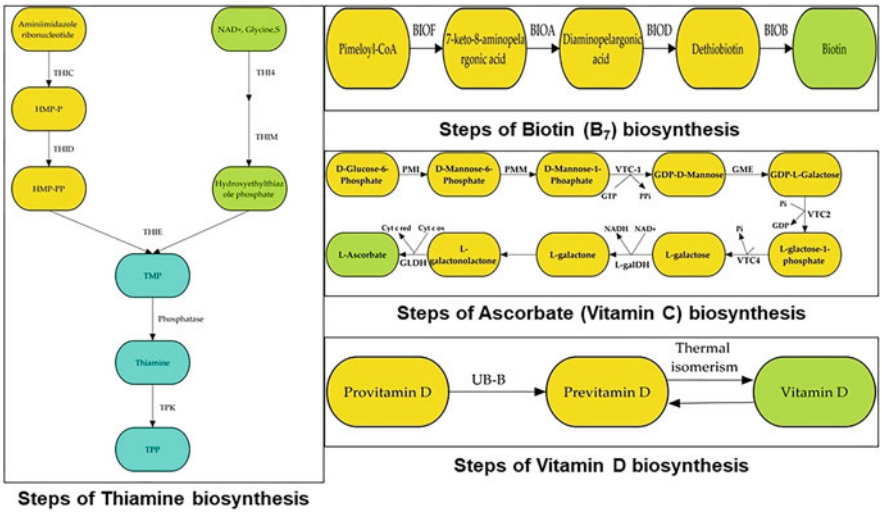

Fig. 8.6 The specific steps of enzymatic biosynthesis of different types of vitamins namely Biotin (B_7), Ascorbate, Vitamin D and Thiamine

biosynthetic routes of biotin in the algal kingdom (Chui et al. 2012). The precursor molecule for biotin synthesis is pimeloyl-CoA thatis an ω-carboxyacyl CoA, which is a S-pimeloyl derivative of coenzyme A (Schneider et al. 2012). From pimeloyl-CoA, 7-keto-8-aminopelargonic acid is formed, and the reaction is catalyzed by BIOF (7-keto-8-aminopelargonic acid synthase, EC 2.3.1.47). In the next step, diaminopelargonic acid is formed with the help of BIOA (diaminopelargonic acid synthase, EC 2.6.1.62). Dethiobiotin then formed with the help of BIOD (dethiobiotin synthase, EC 6.3.3.3) and from dethiobiotin. Biotin is synthesized, and this is catalyzed by BIOB (biotin synthase, EC 2.8.1.6). In algal species, BIOD is attached upstream of BIOA gene (Chui et al. 2012) (Fig. 8.6).

4.2 Thiamine

Thiamine also played an important role in intermediary carbon metabolism. The active form of thiamin is thiamin pyrophosphate (TPP), and it is essential in all organisms (Croft et al. 2006). In model green alga *Chlamydomonas reinhardtii*, TPP biosynthetic pathway is well studied, and it is also found that the pathway is regulated by riboswitches. Hydroxymethyl pyrimidine pyrophosphate and hydroxyethylthiazole phosphate are condensed together to form thiamine monophosphate (TMP). The reaction is catalyzed by thiamine monophosphate synthase (THIE, EC 2.5.1.3). Hydroxyethylthiazole phosphate is synthesized from glycine, NAD^+, and an indefinite sulfur donor, and THI4 (thiamine thiazole synthase, EC 2.8.1.10) and THIM (hydroxyethylthiazole kinase, EC 2.7.1.50) are

involved in it. While hydroxymethyl pyrimidine pyrophosphate (HMP-PP) is synthesized from aminoimidazole ribonucleotide, THIC (phosphomethylpyrimidine synthase, EC 4.1.99.17) and THID (hydroxymethylpyrimidine/phosphomethylpyrimidine kinase, EC 2.7.4.7) are involved (Croft et al. 2007). From aminoimidazole ribonucleotide, at first hydroxymethylpyrimidine phosphate (HMP-P) formed with the help of THIC and then hydroxymethylpyrimidinepyrophosphate (HMP-PP) with the help of THID (Fig. 8.6). TPP biosynthetic pathway is basically composed of two moieties, a thiazole and a pyrimidine moiety. Hydroxymethyl pyrimidine pyrophosphate represents the pyrimidine pathway, and hydroxyethylthiazole phosphate represents the thiazole pathway (Croft et al. 2006). From TMP, thiamine is formed, and the reaction is catalyzed by an unknown phosphatase. And from thiamine, TPP is formed into thiamine pyrophosphokinase (TPK, EC 2.7.6.2) (Croft et al. 2007). In *C. reinhardtii*, the splicing of the THI4 and THIC transcripts is altered that encode the first enzymes of the thiazole and pyrimidine branches of thiamine biosynthesis. This results in an increase in intracellular thiamine and TPP levels. In *C. reinhardtii*, the *pyr1* mutant is resistant to the thiamine analogue pyrithiamine because of a mutation in the THI4 riboswitch that prevents the repression of THI4 gene by TPP. Using these riboswitches, a more effective regulation of thiamine biosynthesis in *C. reinhardtii* can be achieved at physiological concentrations of the vitamin (Croft et al. 2007).

4.3 Cobalamin

Cobalamin is an important vitamin with importance in human health too. Plants do not produce cobalamin as they lack the enzymes (Croft et al. 2005). Algae also are not able to produce the vitamin (Croft et al. 2006). Though many algae are rich in cobalamin and in some species like *Porphyra yezoensis* (Nori), the amounts are as high as found in animal liver cells. Many algal groups are cobalamin auxotroph, and the source of this vitamin in algae is a symbiotic relationship with gram-negative soil bacteria like *Mesorhizobium* sp. and *Halomonas* sp. (Croft et al. 2005).

4.4 Ascorbic Acid

Ascorbate (vitamin C) is an enzyme cofactor in eukaryotes that plays an important role in protecting photosynthetic eukaryotes against damaging reactive oxygen species (ROS) derived from the chloroplast. Plants and algae use different enzymes than animals to biosynthesize ascorbate. In plants, mannose-6-phosphate isomerase (PMI, EC 5.3.1.8) converts D-glucose-6-phosphate to D-mannose-6-phosphate. In the next step, D-mannose-1-phosphate is derived from D-mannose-6-phosphate with the help of phosphomannomutase (PMM, EC 5.4.2.8). GDP-D-mannose then formed from D-mannose-1-phosphate with the help of mannose-1-phosphate guanylyltransferase (VTC1, EC 2.7.7.13), and the step is catalyzed by GTP → PPi

conversion. In the next step, GDP-L-galactose formed with the help of GDP-D-mannose epimerase (GME, EC 5.1.3.18). L-Glactose-1-phosphate then formed in the next step with the help of GDP-L-galactose phosphorylase (VTC2, EC 2.7.7.69). L-Galactose is formed and subsequently catalyzed by L-galactose-1-phosphate phosphatase (VTC4, EC 3.1.3.25). From L-galactose, L-galactose is formed by the enzyme L-galactose dehydrogenase (L-galDH, EC 1.1.1.316). L-Galactonolactone is formed from L-galactose, and from L-galactonolactone, L-ascorbate is formed by the help of L-GalL dehydrogenase (GLDH, EC 1.3.2.3) (Wheeler et al. 2015). Data of vitamin D biosynthesis in algae is limited. Some scientists reported vitamin D_2, vitamin D_3, and their provitamins in algae. Microalgae live on the surface of water, and vitamin D is probably synthesized by the exposure of UV-B that converts provitamins D in vitamin D. To synthesize vitamin D_3, algae should produce 7-dehydrocholesterol if using the same pathway as vertebrates. But the sterols found in algae are diverse. Rhodophyta contains cholesterol, and some of them contain desmosterol, while brown algae contain fucosterol. Because of this huge diversity, it is difficult to put a conclusion on the production of vitamin $D_{2/3}$ in algae (Jäpelt and Jakobsen 2013). High vitamin contents have been reported for seaweeds like laver (*Porphyra umbilicalis*), sea spaghetti (*Himanthalia elongata*), and *Gracilaria changii*, which is quantitatively equivalent to vegetables like lettuce and tomatoes (Norziah and Ching 2000; Ferraces-Casais et al. 2012). Other Kelp species like *Macrocystis pyrifera* is rich in vitamin E, whereas seaweeds like *Codium fragile* and *Gracilaria chilensis* have been documented to be excellent source of β-carotene (Ortiz et al. 2009; Skrovankova 2011). Details of different algal sources and the corresponding vitamin extracted or isolated from these seaweeds have been represented here (Table 8.5).

Table 8.5 The different algal/seaweed sources for different types of vitamins as reported from different studies (modified from Wells et al. 2017)

Division	Algal taxa	Vitamin obtained	References
Rhodophyta Rhodophyta Ochrophyta	*Porphyra umbilicalis* *Gracilaria changii* *Himanthalia elongata*	Vitamin C	Norziah and Ching (2000), Ferraces-Casais et al. (2012)
Ochrophyta	*Eisenia arborea*	Vitamin C	Hernández-Carmona et al. (2009)
Ochrophyta	*Macrocystis pyrifera*	α-tocopherol	Ortiz et al. (2009), Skrovankova (2011)
Chlorophyta Rhodophyta	*Codium fragile* *Gracilaria chilensis*	β-carotene (pro-vitamin A)	Ortiz et al. (2009)
Chlorophyta Haptophyta Chlorophyta Chlorophyta	*Tetraselmis suecica*, *Isochrysis galbana*, *Dunaliella tertiolecta*, *Chlorella stigmatophora*	Lipid-soluble (A and E) and B-group vitamins including vitamins B1, B2 (riboflavin), B6 (pyridoxal), and B12	Fabregas and Herrero (1990)
Rhodophyta	*Pyropia yezoensis*, *P. tenera*	Vitamin B12, vitamin B12 analog	Watanabe et al. (2014), Takenaka et al. (2001), Yamada et al. (1999)

5 Terpenoids

These are the broadest group of phytochemicals that are abundantly reported from different marine algae with significant antioxidant activities and reported therapeutic role in cancer treatment (Huang et al. 2012). Even though carotenoids constitute the largest fraction of algae-derived tetraterpenoids, there are other mono-, di-, tri-, tetra-, mero-, and sesquiterpenoids that contribute to the phytochemical pool of algal terpenoids (Sathasivam and Ki 2018). Since a separate section for carotenoids has already been mentioned previously, so here other terpenoids are only taken into consideration showing antioxidant and anti-carcinogenic effects that pertain to pharmaceutical applications.

5.1 Monoterpenes

The red macroalga *Plocamium cartilagineum* has been documented to produce different halogenated monoterpenes like furoplocamioid C, prefuroplocamioid, pirene, and cyclohexane with antioxidant and anticancer activity against human melanoma and cancerous colon cells (De Inés et al. 2004; Shapumba et al. 2017). Similar antioxidant especially H_2O_2 scavenging activity has also been reported from monoterpene lactone isolated from the brown seaweed *Sargassum ringgoldianum* (Yang et al. 2011) (Table 8.6).

5.2 Diterpenes

Different brown algal systems like *Bifurcaria bifurcata* and *Dictyota dichotoma* are known to produce diterpenes like eleganolone and eleganonal that have shown neuroprotective function against neuroblastoma and cytotoxic effects on liver and breast cancer cell lines, respectively (Silva et al. 2019; Ayyad et al. 2011). The red alga *Sphaerococcus coronopifolius* has been reported to produce the diterpene sphaerodactylomelol that inhibits the proliferation of human liver cancer cells (Rodrigues et al. 2015). Downregulation of Bcl2 and regulatory pathways like JAK2/STAT3, PI3K/Akt, and NF-κB by these diterpenes is the main mode to inhibit cancer cell proliferation (Table 8.6).

5.3 Triterpenes

The dichloromethane extract of triterpenoids from *Sargassum wightii* and methanolic extract of *Sargassum* sp. and *Eucheuma cottonii* displayed significant

Table 8.6 The different types of non-carotenoid terpenoids documented from different algal sources with their possible therapeutic application with emphasis on anticancer and antioxidant activities

Terpenoid type	Bioactive molecule	Algal source	Therapeutic role	Reference
Monoterpene	Furoplocamioid C, prefuroplocamioid	*Plocamium cartilagineum*	Anticancerous activity	De Inés et al. (2004), Shapumba et al. (2017)
	Lactone derivative	*Sargassum ringgoldianum*	H_2O_2 scavengers	Yang et al. (2011)
Diterpenes	Eleganolone, eleganonal	*Bifurcaria bifurcata*, *Dictyota dichotoma*	Neuroprotective against neuro-blastoma cells, cytotoxic against liver and breast cancer cell lines	Silva et al. (2019), Ayyad et al. (2011)
	Sphaerodactylomelol	*Sphaerococcus coronopifolius*	Inhibit cancer cell proliferation	Rodrigues et al. (2015)
Triterpenes	Dichloromethane extract	*Sargassum wightii*	Free radical scavengers	Syad et al. (2013), Nurjanah et al. (2017)
	Methanolic extract	*Sargassum* sp. *Eucheuma cottonii* *Gracilaria salicornia*	Antioxidant activity	Ghannadi et al. (2016), Arsianti et al. (2020), Rajamani et al. (2018), Wu et al. (2012), Li et al. (2013)
Sesquiterpenes		*Ulva fasciata*	Free radical scavenger	Chakraborty and Paulraj (2010)
	Compositacin D and G Cycloelatanene A and B	*Laurencia* spp.	p53-dependent cyclin inhibition	Rocha et al. (2018), Kim et al. (2008)
	Caulerpenyne	*Caulerpa taxifolia*	Inhibition of cell cycle of neuro-blastoma cells	Barbier et al. (2001)

free radical scavenging activity (Syad et al. 2013; Nurjanah et al. 2017). Likewise, methanol-based extraction from *Gracilaria salicornia* showed significant antioxidant activities on human colon cancer cells (Table 8.6). Specific solvent extracts from different seaweed like *Eucheuma cottonii*, *Padina boergesenii*, *Kjellmaniella crassifolia*, *Laurencia mariannensis*, *Lacerta viridis*, and *L. obtuse* have been documented to show anti-carcinogenic effects on a series of different carcinoma

cells, both for treatment and preventive measures (Ghannadi et al. 2016; Arsianti et al. 2020; Rajamani et al. 2018; Wu et al. 2012; Li et al. 2013).

5.4 Sesquiterpenoids

Different types of sesquiterpenoids are isolated from *Ulva fasciata*, an Indian green seaweed that has been reported to be potent-free radical scavengers with significant antioxidant properties (Chakraborty and Paulraj 2010). Members of the genus *Laurencia* spp. have been a major source of sesquiterpenoids like compositacin D and G, as well as cycloelatanene A and B that were reported to promote apoptosis in cancer cells through activity of caspase and p53-dependent cyclin inhibition (Rocha et al. 2018; Kim et al. 2008) (Table 8.6). *Caulerpa taxifolia*, a green macroalgae, has been documented to produce caulerpenyne that inhibits cell cycle progression of neuroblastoma cells from G2 to M phase (Barbier et al. 2001).

6 Polyunsaturated Fatty Acids (PUFA)

Polyunsaturated fatty acids (PUFA) are very important component of human as well as animal diet. PUFA can be divided into two classes, n-6 class and n-3 class, and the precursor molecules are α-linoleic acid (ALA) and linoleic acid (LA), respectively. Algae are the ultimate source of PUFA and very long-chain PUFA (VLCPUFA) and are transported to higher trophic states by the food chain (Harwood 2019). In fact, in recent times, the bio-production of PUFA by both freshwater and marine algae is a subject for intensive research, and it gets commercial attention too. Though fishes are a source of PUFA, the increasing demand cannot be met by this sole source, and algae flourished as an important alternative. Moreover, the quality of fish oil depends on many things like the species of fish, the climate, season, geographical location, and food consumed by fish, and the purification process of PUFA from low-grade fish oil is technically difficult as techniques like adsorption chromatography, fractional or molecular distillation, enzymatic splitting, low temperature crystallization, supercritical fluid extraction, and urea complexation are involved. On the other hand, some marine algae contain large amount of high-quality PUFA. These are used in aquaculture operations and can be cultivated with cheap organic substances, without proper sunlight, under controlled environment (Guschina and Harwood 2006). Some algal sources of PUFA are shown in the undermentioned table (Table 8.7).

It is found that PUFA and VLCPUFA have pharmaceutical properties also. These applications of PUFA and VLCPUFA have been enlisted below. It is found that the consumption of n-3 classes PUFA, that is, ALA, EPA, and DHA, is inversely correlated with coronary heart diseases, and they play vital role in the treatment of dyslipidemias (Zuliani et al. 2009) (Figs. 8.7 and 8.8). PUFA also have anti-inflammatory effects in the brain. It is found that arachidonic acid-derived bioactive mediators regulate the peripheral immune function and have been shown to regulate

Table 8.7 The different algal sources and the details of PUFA and VLCPUFA compositional differences as documented from available references (Li-Beisson et al. 2019; Lang et al. 2011)

Name of the species	Class	PUFA and VLCPUFA accumulated
Chlamydomonas reinhardtii	Chlorophyceae	PA, OA, GLA, SDA, ALA
Chlorococcum infusionum	Chlorophyceae	PA, OA, LA, SDA
Desmodesmus maximus	Chlorophyceae	PA, OA, ALA
Dunaliella salina	Chlorophyceae	PA, OA, LA, ALA
Haematococcus pluvialis	Chlorophyceae	PA, OA, LA, ALA, SDA, EPA
Monoraphidium minutum	Chlorophyceae	PA, SA, OA, LA, ALA, SDA
Scenedesmus obliquus	Chlorophyceae	PA, SA, OA, LA, ALA, SDA
Tetracystis intermedia	Chlorophyceae	PA, SA, OA, LA, ALA
Volvox tertius	Chlorophyceae	PA, SA, OA, ALA
Ankistrodesmus spp.	Chlorophyceae	PA, OA, LA, ALA, SDA, EPA
Chlorella vulgaris	Trebouxiophyceae	PA, OA, LA, ALA
Trebouxia simplex	Trebouxiophyceae	LA, ALA
Halochlorococcum marinum	Ulvophyceae	PA, OA, LA
Ulothrix mucosa	Ulvophyceae	PA, LA, ALA, SDA
Emiliania huxleyi	Haptophyceae	PA, SA, OA, SDA, DHA
Pavlova lutheri	Haptophyceae	PA, OA, LA, ALA, SDA, EPA, DHA
Phaeodactylum tricornutum	Bacillariophyceae	PA, OA, LA, SDA, ESIA, EPA, DHA
Nannochloropsis gaditana	Eustigmatophyceae	PA, OA, ALA, EISA, EPA
Nannochloropsis oculata	Eustigmatophyceae	PA, EPA
Monodus subterraneous	Eustigmatophyceae	EPA
Ectocarpus siliculosus	Phaeophyceae	PA, SA, LA, ALA, SDA, EISA, EPA
Heterococcus chodati	Xanthophyceae	PA, LA, EISA, EPA
Tribonema vulgare	Xanthophyceae	PA, ALA, EISA, EPA
Compsopogon hookeri	Rhodophyceae	PA, LA, EISA, EPA
Porphyridium purpureum	Rhodophyceae	PA, LA, EISA, EPA
Cosmarium cucumis	Conjugatophyceae	PA, OA, LA, ALA, SDA
Micrasterias radiate	Conjugatophyceae	PA, OA, LA, ALA, SDA
Klebsormidium elegans	Conjugatophyceae	PA, LA, ALA
Euglena gracilis	Euglenophyceae	PA, OA, LA, ALA, DHA
Isochrysis spp.	Prymnesiophyceae	PA, OA, LA, ALA, EPA
Lobosphaera incisa	Trebouxiophyceae	AA
Odontella aurita	Mediophyceae	EPA, DHA

microglia activation (Layé et al. 2018). PUFA also have positive effects on cardiovascular disease (CVD) outcomes (Bowen et al. 2016). PUFA significantly decrease the blood pressure, angiotensin II formation, angiotensin-converting enzyme (ACE) activity, and tumor growth factor-beta (TGF-β) expression and increase the endothelial nitric oxide (NO) formation and trigger the parasympathetic nervous system (Cicero et al. 2009). PUFA protect neuronal cells from oxidative damage, controlling inflammation, regulating neurogenesis, and preserving neuronal function (Hashimoto et al. 2014). They reduce depressive, psychotic, and suicidal symptoms,

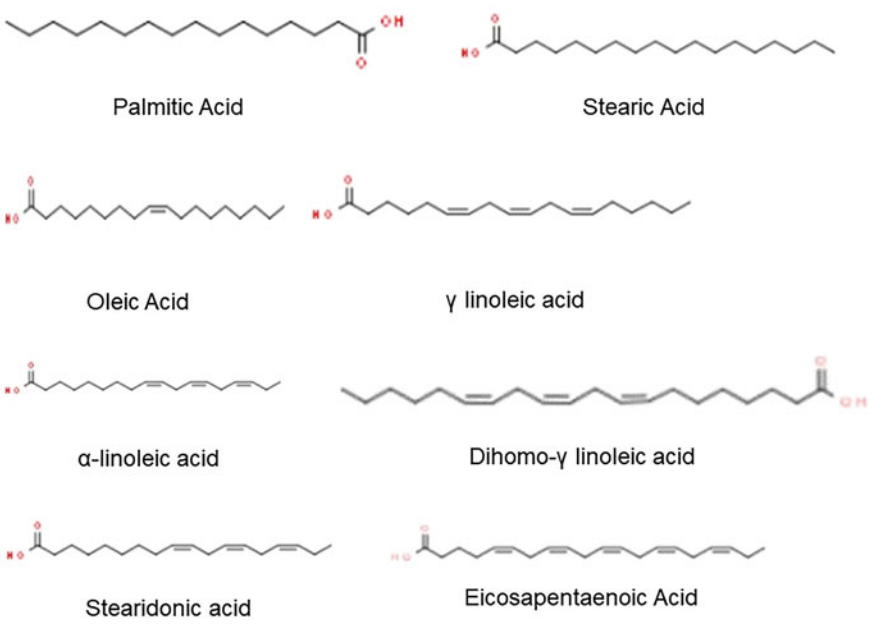

Fig. 8.7 The chemical structures of the different linear chain LCPUFA reported from algal sources

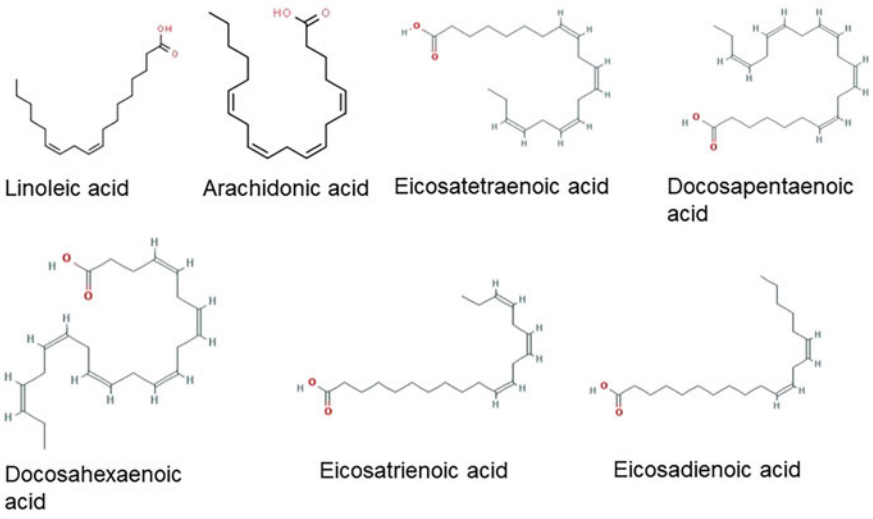

Fig. 8.8 The chemical structures of the different circular LCPUFA reported from algal sources

as well as aggression (Hashimoto et al. 2014). n-3 PUFA may be beneficial in certain neuropsychiatric illnesses such as dementia, mood disorder, and PTSD (Hashimoto et al. 2014).

7 Application of Biotechnological Tools for Better production of Algal Phytochemicals

Many of the bioactive phytochemicals are produced commercially by scaling up the production through recombinant technologies by using different host systems (transgenic animals, transgenic plants, yeast, and bacteria) via genetic engineering. However, each of these host systems has different benefits and drawbacks based on the targeted biomolecule that they are used for. If the targeted plant molecule is from a eukaryotic source, then bacteria and yeast cannot be used as a host system as they are incapable of posttranscriptional and posttranslational modifications (e.g., splicing, glycosylation, protein assembly) (Koo et al. 2013). Although bacterial host has largely remained as a preferred choice for recombinant proteins, the endotoxins and protease contaminations may not be suitable for generation of pharmaceutically important biomolecules. Likewise, even though yeast has remained a preferred low-cost eukaryotic host system, yet modifications like hypermannosylation lead to protein misfolding and concomitant malfunctions (Yusibov and Mamedov 2010). Plant-based host system can be a possible answer to these drawbacks due to their eukaryotic nature yet devoid of properties like endotoxin formation and hypermannosylation. However, these systems are also hindered as a host system due to factors like allergic reactions to plant-derived compounds, permissibility of medical applications, and low productivity rates (Koo et al. 2013).

Due to close similarity with higher plants, eukaryotic algae especially green algae have been considered as an excellent host for the selective expression of phytochemical genes (Novoveska et al. 2019; Saini et al. 2019). Due to the close similarity with higher plants, minimal genetic modifications of pathway would yield necessary phytochemicals. Moreover, the production of biomass can be achieved easily under varied environmental conditions due to their high adaptability to environmental conditions. Furthermore, different types of posttranslational modifications occur in green algal hosts that enable differential expression of targeted proteins more efficiently as compared to other hosts (Weiner et al. 2018; Scaife et al. 2015; Jareonsin and Pumas 2021). However, heterotrophic algal hosts are preferred over autotrophic hosts due to the following reasons:

1. They can easily grow under low or no light conditions that negate the need for the availability of light.
2. They can utilize a host of carbon sources other than CO_2 and even grow well in wastewater.
3. They can be cultivated with cheap nutrients that allow easier scaling up of production as compared to autotrophs.
4. They can be grown in photobioreactors also that reduce space requirement (Jareonsin and Pumas 2021).

Eukaryotic algae carry nuclear, mitochondrial, and chloroplast genomes where chloroplast gene transformation yields higher proteins as compared to nuclear gene transformation (Faè et al. 2017). The chloroplast gene transformations have a host of

Table 8.8 Some of the green microalgal strains that have been utilized as expression vectors with details of plasmids, selectable markers/reporter genes, and methods of gene expression (modified from Jareonsin and Pumas 2021)

Strains	Plasmids	Gene expression method	Markers/reporter genes	References
Scenedesmus acutus	pCXSN-GEP	*Agrobacterium*	Hygromycin B	Suttangkakul et al. (2019)
Chlamydomonas reinhardtii	pET-vp28 pER123 pSL18_HR	Glass bead Glass bead Electroporation	Spectinomycin Paromomycin Paromomycin	Kiataramgul et al. (2020) Mooi et al. (2018) Perozeni et al. (2018)
Chlorella pyrenoidosa	pGreeII 0029	Electroporation	NptII, eGFP	Run et al. (2016)
Chlorella vulgaris *Chlorella ellipsoidea*	pCAMBIA1304 pPt-ApCAT pSoup	Electroporation Electroporation Electroporation	Hygromycin Chloramphenicol NptII	Koo et al. (2013) Niu et al. (2011) Bai et al. (2013)
Dunaliella salina	pUCG-Bar	Electroporation	Herbicide PPT	Jia et al. (2012)

ribosomes and protein translation machinery like chaperones, protein sulfide isomerases, and peptidylprolyl isomerases that allow better production of phytochemicals (Rasala and Mayfield 2015; Jareonsin and Pumas 2021). Among the potential green algal hosts, *Scenedesmus acutus* (Suttangkakul et al. 2019), *Chlamydomonas reinhardtii* (Kiataramgul et al. 2020), *Chlorella* spp. [*Chlorella sorokiniana* (Sorokin 1967), *Chlorella vulgaris* (Mathieu-Rivet et al. 2014; Koo et al. 2013), and *Chlorella ellipsoidea* (Bai et al. 2013)] have been preferred as an excellent host for heterologous protein expression due to the low cost of production, high growth rate, ease of culturing under inexpensive resources, and adaptation to different conditions (Klamczynska and Mooney 2017; Yang et al. 2016). Different methods for genetic transformation in algae are available that include electroporation, particle bombardment, *Agrobacterium*-dependent transformation and PEG (polyethylene glycol)-mediated transformation (Kim et al. 2014). Although particle bombardment and electroporation have remained the main methods for introduction of foreign DNA in the algal host, reports on *Agrobacterium*-dependent transformations have remained less (Barrera and Mayfield 2013). Thus, the method of transformation employed in algal cells is an important parameter to be considered while designing the transformation process (Jareonsin and Pumas 2021). Selection of vector is the other foremost factor to be considered for transformation giving importance to the aspects of promoter selection, transcription regulation, marker/reporter gene selection, and enhanced gene expression. Some of the more common gene expression tools for algal transformation have been included in Table 8.8. Even though many of the plant-based phytochemicals are produced on a commercial level for their pharmaceutical importance, wastage of biomass and the quality of the crude product have remained a cause of concern. Thus, in recent times, algae have also been utilized as

Table 8.9 The different phytochemicals that have been manufactured through algal hosts with their importance as high value compounds (modified from Jareonsin and Pumas 2021)

Microalgal system	Phytochemicals produced	Role	Reference
Synechococcus elongatus	Cannabidiol (Cannabinoids)	Treatment for medical conditions like AIDS, neuropathy	Laban (2019)
Chlamydomonas reinhardtii	Terpenoids	Dietary supplement, pigment	Lauersen (2018)
Dunaliella sp.	β-carotene, astaxanthin	Antioxidant, anti-allergic, anti-inflammatory	Saha et al. (2018), Barkia et al. (2019)
Haematococcus sp.	Pigments: β-carotene, astaxanthin	Antioxidant, anti-inflammatory	Barkia et al. (2019)
Scenedesmus sp.	Pigments: β-carotene, Lutein	Dietary supplement	Chen et al. (2017)
Botryococcus braunii	Carotenoids	Antioxidant, medically important	Niehaus et al. (2011)
Porphyridium sp.	PUFAs: Arachidonic acid protein-pigment complex: B-phycoerythrin	Food supplement, medically important	Li et al. (2020)

important hosts for terpenoid phytochemicals like pigments that are not only produced by higher plants but by algal plastids as well (Bock and Warzecha 2010). Table 8.9 in the present work puts forward the different high value phytochemicals that have been expressed through algal hosts in recent times.

8 Conclusion and Future Perspective

The present work puts together comprehensive information on the aspect of phytochemicals obtained from algae including both microscopic and macroscopic forms. As evident, algae are sources for high value phytochemicals that can be used for a broad spectrum of areas that encompass dietary supplements to anticancer compounds. Even though the use and applications of phytochemicals have long been studied, algal resources have not been looked thoroughly as compared to higher plants. However, as evident from this work, the algal resources have applications mainly in the fields of pharmaceutical and nutraceuticals. In the present era as climate continues to change, there is an impending fear of losing habitats for proliferation of both natural and cultivable plants. Thus, we need to look into alternative plant forms for important phytochemicals that would require less space, yet the quality of phytochemicals will not be compromised. So, it becomes obvious that algal forms are the way not only to look forward into not only for their ability to produce high-quality phytochemicals but also to enhance the yield of such phytochemicals by in vitro manipulation of culturing conditions. Moreover, as health

incidences continue to be more complicated due to changing environmental conditions, alternate modes of treatment for specialized diseases will be the need of the hour. Thus, newer pharmaceutical and nutraceutical treatment processes will emerge in the coming days where algal phytochemicals can be an important component.

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Part II
The Emergence of Phytochemical Omics and Transgenics

Chapter 9
Emergence of Phytochemical Genomics: Integration of Multi-Omics Approaches for Understanding Genomic Basis of Phytochemicals

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1 Introduction

Since time immemorial, humans and plants have been in harmonious association, and this association has shaped the cultures and societies of the world (Seymour 2016). Humans use plants for a variety of purposes such as food, fiber, shelter, and medicine (Plotkin and Balick 1984). Plants provide a wide variety of chemical constituents, known as phytochemicals, essential for the plant themselves and also for the sustenance of human life (Grusak 2002). These naturally occurring phytochemicals are of various types and have diverse functions and roles within the plant system. Primary metabolites such as carbohydrates, amino acids, nucleic acids,

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M. K. Swamy, A. Kumar (eds.), *Phytochemical Genomics*,
https://doi.org/10.1007/978-981-19-5779-6_9

lipids, and proteins are common to all plants and play diverse roles in plants (Briskin 2000). On the other hand, certain plants possess unique medicinal properties due to the presence of the combinations of secondary metabolites, nowadays also known as specialized metabolites (SMs). There are nearly 391,000 species of plants, and it is believed that 17,810 species are highly medicinal (Yamazaki et al. 2018). Several classes of specialized metabolites such as terpenes, terpenoids, nitrogen-containing compounds, carotenoids, phenolics, alkaloids, and organosulfur compounds, based on their biosynthetic origins, have been identified using various approaches (Bellik et al. 2013; Nielsen et al. 2019). The diversification of plant metabolites has evolved with respect to the conditions in which they survive (Rai et al. 2017). The plant metabolites are synthesized by plants in various cells and organs (Karuppusamy 2009). Trichomes, the epidermal outgrowths in plants, also produce an array of important specialized metabolites (Hachez 2017; Stanojković et al. 2020). The diversified functions of the metabolites are due to their complex chemical structures (Rai et al. 2017). Apart from playing diverse roles in plants such as defense responses, deterrence to herbivory, and attractants toward pollinators or symbionts, specialized metabolites are also important for humans and possess antioxidant, antibactericidal, and anti-inflammatory activities (He and Giusti 2010). Due to their unique properties, many of them have been used in treating diseases such as haemorrhoids, liver disorders, cardiovascular disease, inflammatory digestive disorders, blood vessel disease, asthma, diabetes, cancer, urological disorders, neurological generative disease, skin diseases, and many more ailments (Leicach and Chludil 2014; Hussein and El-Anssary 2018; Seca and Pinto 2019). For example, berberine derived from *Berberis vulgaris* is used for anticancer ailment (Sun et al. 2009). Opium poppy (*Papaver somniferum*) produces benzylisoquinoline alkaloids like sanguinarine, papaverine, and noscapine that have many medicinal properties including cough suppression and anticancer properties (Beaudoin and Facchini 2014). Berberine can also be obtained from *Thalictrum minus* L. (Kobayashi et al. 1991). Steroidal alkaloid solasodine could be obtained from *Solanum laciniatum*. Solasodine is the precursor from which corticosteroids and anti-fertility drugs were synthesized (Chandler and Dodds 1983). Glucosinolates synthesized by the Brassicaceae family have anti-defense properties (Sanchez-Pujante et al. 2017), and they also exhibit antifungal activities (Wittstock and Burow 2010; Ishida et al. 2014). Cardenolides in *Digitalis thalpi* are synthesized in the leaves (Corchete et al. 1990). Plant fibers composed of cellulose, hemicellulose, and lignin are known to reduce cholesterol levels in the human body (Agarwal and Chauhan 1988; Soliman 2019). Cholesterol may lead to coronary heart diseases in humans (Grundy 1990; Després et al. 2000). Many plants synthesize phytosterols and phytoestrogens that play an important role in decreasing cholesterol synthesis (Kerckhoffs et al. 2002). Red yeast rice, a fermented rice variety found in China and Japan, is known to have monacolin-related compounds, sterols, and isoflavones that assist in reducing cholesterol levels (Heber et al. 1999; Segura 2003). These phytosterols and phytoestrogens help in reducing cholesterol-related health issues in humans (Chen et al. 2008). Soy and legume proteins are proven to reduce hyperlipidemia (Chen et al. 2014). Medicinal products are substances that contain a compound with

defined pharmacological and beneficial therapeutic effects (Aronson 2017). The γ-oryzanol from rice bran is an antioxidant and can be used as a cholesterol-reducing agent. β-glucans from barley flour enhance lipid metabolism (Baiano 2014; Bhat et al. 2020). Some plants such as *Taxus brevifolia*, *Catharanthus roseus*, and *Camptotheca acuminata* are few of the most important sources of anticancer drugs (Cragg and Newman 2005; Kaur et al. 2011). *C. roseus* produces important specialized metabolites such as ajmalicine, vincristine, vinblastine, and catharanthine (Tikhomiroff and Jolicoeur 2002). Camptothecin is obtained from *C. acuminata.* The source of the potent anticancer drug taxol is *T. brevifolia* (Cragg and Newman 2005). *Crocus sativus* produces terpenes and terpenols including safranal and other compounds such as crocin (Alavizadeh and Hosseinzadeh 2014). Polyphenols act as antioxidants and possess many health benefits (Segovia et al. 2014). Artemisinin from *Artemisia annua* L. is used as antimalarial drug, and now, research has shown that it has the potential to kill cancer cells (Nakase et al. 2008). According to Saito (2013), the main metabolites found in *Arabidopsis* were flavonoids, glucosinolates, camalexin, coumarins, sinapoyl esters, amides, and caryophyllene. A huge diversity of plants containing several thousands of metabolites exists in nature. Many plants have been exploited for various important primary and secondary metabolites. Meanwhile, many plants are yet to be explored for their important metabolites that are essential for drug discovery. The discovery of recent technologies enables us to screen more plants for their phytochemicals. Their role in treating human diseases has led to increased interest in investigating the mechanisms of their biosynthesis and the identification of genes involved in their biosynthetic pathways (Boudet 2007; Tohge et al. 2007; Bhambhani et al. 2017). There is also a rising interest in identifying a large number of yet unidentified phytochemicals present in the plants (Saxena et al. 2013).

Omics approaches, such as transcriptomics, proteomics, and metabolomics, have been increasingly used for the investigation of the genomic basis of phytochemicals, their production, and their functions in many plant species (Saito 2013). Omics tools can help us elucidate the phytochemical biosynthetic pathways and their regulatory aspects (Rai et al. 2017; Pathak et al. 2019). The advancements in genome sequencing technologies have led to the sequencing of a large number of medicinal plants that have contributed towards the understanding of genes responsible for producing a particular metabolite (Yang et al. 2016). The gene-to-metabolite connections have been explicated, specifically by employing the combined studies of transcriptomics and metabolomics (Sheth and Thaker 2014; Liu et al. 2020). Several metabolomic studies have also been performed on the crop plants such as rice (Kusano et al. 2011), maize (Amiour et al. 2012; Casati et al. 2011), and wheat (Bowne et al. 2012). Further, high-throughput sequencing technologies also help us study the responses in plants following various stress conditions (Zhuang et al. 2014; Soda et al. 2015; Liu et al. 2017; Meena et al. 2017; Li et al. 2019). Genes responsible for various cellular metabolisms, stress, and systemic responses are identified using a combination of phytochemical genomics techniques (Harborne 1973). Many recent approaches also help us identify microregulators of metabolite biosynthesis such as noncoding RNAs (ncRNAs) including long non-coding RNAs, microRNAs, and

Table 9.1 List of some important drugs obtained from plants (Rates 2001)

Plant species	Drug/s	References
Artemisia annua	Artemisinin	Balint (2001)
Atropa belladonna	Atropine	Catch and Evans (1960)
Camptotheca acuminata	Camptothecin	Carte et al. (1990)
Capsicum annuum	Capsaicin	Veeresham (2012)
Catharanthus roseus	Vincristine and vinblastine	Chu et al. (1997), Volkov and Grodnitskaya (1994)
Cinchona ledgeriana	Quinine and quinidine	Staba and Chung (1981)
Coffea arabica	Caffeine, theobromine, theophylline	Mazzafera et al. (1991)
Coleus forskohlii	Forskolin	Veeresham (2012)
Cryptolepis sanguinolenta	Neocryptolepine, biscryptolepine	Cimanga et al. (1996)
Curcuma longa	Curcumin	Verghese (1993)
Digitalis purpurea	Digoxin	Wade (1986) and Bucca (2018)
Erythroxylon coca	Cocaine	Leete (1980)
Galanthus nivalis	Galantamine	Veeresham (2012)
Gossypium spp.	Gossypol	Hansen and Jaroszewski (1996)
Huperzia serrata	Huperzine A	Kennedy et al. (2010)
Murraya koenigii	Mahanine, mahanimbine	Pandit et al. (2010)
Papaver somniferum	Morphine and codeine	Wold (1978)
Silybum marianum	Silymarin	Veeresham (2012)
Tanacetum parthenium	Parthenolide	Awang et al. (1991), Zhou et al. (1999)
Taxus brevifolia	Taxol	Fett-Neto et al. (1994)

circular RNAs. Noncoding RNAs act as key regulators of gene expression in plants (Zhu and Wang 2012; Chekanova 2015; Shafiq et al. 2016; Wang et al. 2017). Many ncRNAs are also proven to have roles in the regulation of the synthesis of specialized metabolites in plants (Ou et al. 2017; Narnoliya et al. 2019). Integration of all these omics technologies can help us understand gene-metabolite networks in medicinal plants, storage, and evolution of specialized metabolites within the diverse groups of plants or plant families (Tohge and Fernie 2010; Nakabayashi and Saito 2015). Additionally, the integration of genetic engineering technologies, including gene editing tools, enables us to improve the medicinal plants for the metabolites that are of commercial and economic importance (Bourgaud et al. 2001; Debnath et al. 2006; Yadav et al. 2017). This chapter is aimed to introduce the concepts of phytochemical genomics, various techniques that are used in phytochemical genomics, and some of the examples of medicinal plants, where these technologies have been successfully utilized. Table 9.1 summarizes a list of some important medicinal plants and the important drugs/compounds that are obtained from them.

2 Emergence of Phytochemical Genomics

Many plants possess medicinal properties because of specialized metabolites present in them. Numerous metabolites have been identified from plants (Keurentjes et al. 2006; Shitan 2016; Hussein and El-Anssary 2018). However, a large number of plants are yet to be analyzed for the existence of metabolic diversity (Hall 2006). Increased profiling of plants and deciphering of metabolic diversity has led to a new and yet emerging field known as phytochemical genomics (Saito 2013; Muranaka and Saito 2013). Phytochemical genomics aims to investigate the genomic basis of metabolic diversity in plants (Saito 2013). The high-throughput technologies such as genomics, transcriptomics, proteomics, and metabolomics are important for identification and characterization of the metabolites and investigation of their genomic basis (Tohge et al. 2007; Saito 2013). The deployment of integrative omics approaches can help us analyze multiple plants at a time within a relatively shorter time (Choi 2018). Metabolomics identifies all the related compounds in the biosynthesis of a phytochemical (McGhie and Rowan 2011; Tzin et al. 2019). Transcriptomic and metabolomic data provide hints about the expression patterns and functions of genes and metabolite accumulation (Sawada et al. 2009; Ge et al. 2015). Therefore, this emerging discipline offers great opportunities for understanding the genomic basis of the metabolite synthesis, accumulation, and storage. Further, omics data obtained can be integrated with the gene editing and gene engineering technologies for the improvement of the bioactive compounds in the plants. Figure 9.1 explains the integration of multi-omics approaches to plants for understanding the genomic basis of phytochemicals.

2.1 Genomics

A large population globally rely on traditional sources of plant-based drugs for their primary health care (Farnsworth and Soejarto 1988; Elujoba et al. 2005; Payyappallimana 2010). Many traditionally-used plants are reservoirs of important medicinal compounds (Scartezzini and Speroni 2000; Namukobe et al. 2011; Yadav et al. 2014). Globally, medicinal plants are exploited for the extraction of bioactive compounds from them (Balunas and Kinghorn 2005; Jamshidi-Kia et al. 2018). The current practices may not be sustainable to meet the global demands for medicinal drugs (Chen et al. 2016). Therefore, to avoid excessive pressure on naturally occurring medicinal plants, efforts must be taken to look for alternative sources of drugs or more sustainable practices should be devised (Phillipson 1994; Farombi 2003). One alternative approach is to metabolically engineer plants for important metabolites and/or use genetic modification tools to improve the metabolites (Verpoorte et al. 2002; Verpoorte and Memelink 2002). However, medicinal compounds are most often synthesized by complex pathways involving multiple genes and gene networks (Ncube and Van Staden 2015). For a better understanding of the

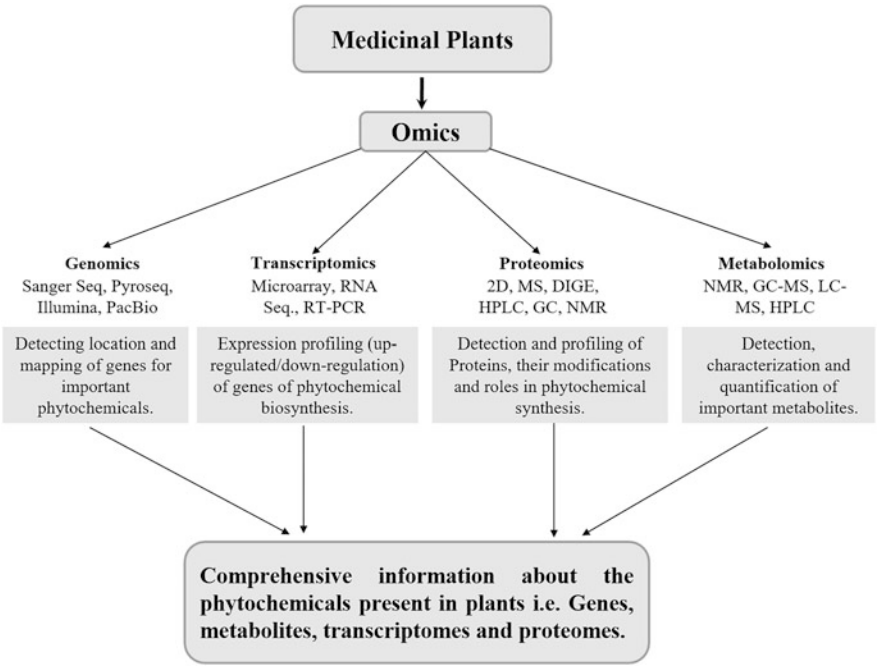

Fig. 9.1 Integrative omics tools for understanding genomic basis of phytochemicals. The diagram explains the application of various omics tools to medicinal plants for elucidation of mechanisms of synthesis of metabolites existing in them. Various high-throughput techniques that can be applied to each omics tools such as Sanger seq, Pyroseq, Illumina, and PacBio (genomics); microarray, RNA Seq, and RT-PCR (transcriptomics); 2D, MS, DIGE, HPLS, GC, and NMR (proteomics; and NMR, GC-MS, LC-MS, and HPLC (metabolomics). The information obtained from the omics tools can be further integrated with gene editing tools such as ZFN, CRISPR/Cas9, MNs, and TALENs for metabolite engineering or improvement of the metabolites in plants

genes and gene regulatory networks, we must understand the genome structure and function of the plants (Hao and Xiao 2015). Identifying genes that govern the synthesis of a particular plant metabolite is essential for further gene editing and genetic engineering ventures (Oksman-Caldentey and Inzé 2004). We must also understand the gene metabolite links and the evolutionary patterns of the metabolites (Hao and Xiao 2015). The genomic DNA is present in the organisms' chromosomes, which contains the genes that code mRNAs and its translation results in the production of proteins (Clancy and Brown 2008). The genome sequence of an organism contain essential information about various traits of the plants including the existence of phytochemical diversity (Yang et al. 2019a). Genomics aims to identify the organism's complete hereditary material (Quiroz 2002). It involves recombinant DNA technology, sequencing methods, and bioinformatics pipelines to sequence, analyze, and annotate gene functions of organisms (The European Bioinformatics Institute EMBL-EBI 2020). Conventionally, Sanger sequencing is used for the sequencing of genomes (Zhang et al. 2014b). However, recent high-throughput

next generation sequencing (NGS) technologies have become more affordable and cheap (Beedanagari and John 2014). Since many metabolites are produced by plants, their genetic dissection is important to further improve medicinal plants (Chakraborty 2018). The application of phytochemical genomics approaches to medicinal plants can help us study and understand the genes involved in the synthesis of the phytochemicals (Saito 2013). These advancements also help researchers to understand the metabolic pathways leading to the production of secondary metabolites. The development of bioinformatics tools and online genomic databases has increased the accessibility of genomic information. Table 9.2 details some of the important medicinal plants whose genomes have been sequenced. Table 9.2 also explains the significant findings of the genome sequencing studies in the medicinally important plants. Functional studies of the identified genes can contribute to molecular modification, genetic transformation, and metabolic engineering of important genes to enhance target drug production in medicinal plants (Montecillo et al. 2020).

2.2 Transcriptomics

The DNA is transcribed into RNA, and the expression of RNA differs under a set of given conditions (Clancy 2008; Finotello and Di Camillo 2015). The total RNA of a cell or an organism is known as a transcriptome (Srivastava et al. 2019). It includes coding and noncoding RNAs present in an organism (Thompson et al. 2016). The transcription of the genes is highly dynamic (Hager et al. 2009). To study the functions of genes, it is important to profile their expression under a given set of conditions (Alberts et al. 2002). Next-generation sequencing technologies allow to profile the whole transcriptomes of organisms (Ekblom and Galindo 2011). Total transcriptomes of organisms can be studied using either DNA microarrays or RNA sequencing technologies (Wang et al. 2009a; Lowe et al. 2017). The analysis of transcriptomes of whole organisms provides important insights into their functions and their regulations (Manzoni et al. 2018). The availability of reference genomes is important for DNA microarray-based transcriptome studies, whereas sequencing-based transcriptome studies can be applied to organisms even if reference genomes are unavailable (Wang et al. 2009a). The latter allows de novo sequencing of organisms. The next-generation sequencing technologies are strengthened by the bioinformatics pipelines (Tan et al. 2019). Whole genome sequencing data of only a few medicinal plants are available because of the challenges associated with it due to the complex structure of genomes, the cost of the sequencing technologies, and the bioinformatics resources (Kim et al. 2019). Therefore, transcriptome sequencing technologies can be applied to medicinal plants for understanding the expression of genes, their functions, and genetic regulation of medicinally important compounds (Wang et al. 2009a). Table 9.3 represents some of the medicinal plants whose transcriptomes are sequenced and the major findings of the sequencing studies vis-a-vis biosynthesis of important metabolites, identification of genes, transcription

Table 9.2 Genomic information of few important medicinal plants (Medicinal Plant Genomics 2017; Chakraborty 2018)

Sl. no.	Name of the medicinal plant	Genomic information	References
1.	*Calotropis gigantea*	A total of 18,197 high-confidence genes were annotated	Hoopes et al. (2017)
2.	*Camptotheca acuminata*	Candidate orthologs for genes involved in camptothecin biosynthesis were identified	Zhao et al. (2017)
3.	*Catharanthus roseus*	Revealed details about monoterpene-derived indole alkaloid (MIA) pathway	Kellner et al. (2015)
4.	*Glycine soja*	SNPs and indels present in domesticated *Glycine max* was absent in *G. soja*, which may be the reason for non-domestication of the latter	Kim et al. (2010b)
5.	*Salvia miltiorrhiza*	32,483 protein-coding genes with a repetitive DNA content of approximately 64.84% were observed	Song et al. (2020)
6.	*Ziziphus jujuba*	Final assembly of 437.65 Mb contains 32,808 genes. Study revealed frequent inter-chromosome fusions and segmental duplications and no whole genome duplications in the jujube genome	Liu et al. (2014)
7.	*Pogostemon cablin*	Study predicted 110,850 protein-coding genes. It revealed clear evidence of whole-genome octuplication (WGO). Expansion of type a of TPS gene family suggest its role in the synthesis of sesquiterpenes	He et al. (2018)
8.	*Ocimum tenuiflorum*	Assembled genome of 374 Mb, with a genome coverage of 61%. Revealed the genes that are responsible for specialized metabolism	Upadhyay et al. (2015)
9.	*Salvia miltiorrhiza*	Transcription factors involved in the tanshinone and phenolic acid biosynthetic pathways are identified. 82 terpene synthase genes and 437 CYPs were also identified	Xu et al. (2016)
10.	*Lonicera japonica*	Whole-genome duplication. Gene expression analysis not only revealed biosynthetic genes of carotenoid accumulation and also the role of carotenoid degradation in its flower coloration	Pu et al. (2020)
11.	*Dianthus caryophyllus*	A total of 43,266 complete and partial protein-encoding genes were deduced. Intensive characterization of the carnation genes was revealed	Yagi et al. (2014)
12.	*Gelsemium elegans*	43.16% of the genome had repetitive elements. Among the predicted protein-coding genes, 84.56% were functionally annotated	Liu et al. (2019)
13.	*Artemisia annua*	The whole genome sequencing revealed the expansion and functional diversification of genes encoding enzymes required for terpene biosynthesis and involved in artemisinin biosynthetic pathway	Shen et al. (2018)
14.	*Capsicum annuum*	Revealed biosynthesis of capsaicinoids. 34,476 protein-coding genes identified	Qin et al. (2014), Kim et al. (2014)
15.	*Lycium chinense* Mill.	The cp genome length was 155,756 bp, with a large single-copy region of length 86,595 bp, small single-	Yang et al. (2019b)

(continued)

Table 9.2 (continued)

Sl. no.	Name of the medicinal plant	Genomic information	References
		copy region of length 18,209 bp, and a 37.8% GC content. 114 genes were encoded, out of which 16 are duplicated. Most genes had start codons as ATG and some had ACT/ACG. *L. chinense* was found as a sister taxon to *L. barbarum*	
16.	*Rhodiola crenulata*	A total of 31,517 protein-coding genes were identified. The genomic sequence will be useful for interpretation of the evolutionary mechanism of the stress resistance gene and the biosynthesis pathways of the medicinal ingredients like salidroside	Fu et al. (2017)
17.	*Isatis indigotica*	Several candidate genes for the biosynthesis of active compounds such as terpenoids, phenylpropanoids, and indole were characterized	Kang et al. (2020)
18.	*Atalantia buxifolia*	Genomic basis of apomixis. 30,123 protein-coding genes was identified	Wang et al. (2017)
19.	*Glycyrrhiza uralensis*	A total of 34,445 protein-coding genes were predicted. Some of the genes involved in triterpenoid saponin biosynthesis	Mochida et al. (2016)
20.	*Andrographis paniculata*	Study predicted 25,428 protein-coding genes and provided insights into the diterpenoid biosynthesis	Sun et al. (2019)

factors, and other regulators such as noncoding RNAs. The studies depicting transcriptome sequencing of more such plants have provided a deeper understanding of the biosynthetic pathways of important metabolites. Similar studies in the future must also be aimed at unravelling the secrets of bioactive components of medicinal plants by integrating with other omics approaches such as genomics, proteomics, metabolomics, and transcriptomics.

2.3 Proteomics

The total protein of a cell is known as a proteome (Ponomarenko et al. 2016). The proteome of an organism is a highly dynamic complement of the genome and performs a wide array of functions (Graves and Haystead 2002). It is possible nowadays to investigate the total proteome of a cell, tissue, or organism with the help of advanced proteomics technologies (Aslam et al. 2017). Proteomics involves the characterization of proteins, analysis of structure, functions, interactions, modifications, and investigation of their expression (Graves and Haystead 2002; Agrawal et al. 2013). The changes in the expression levels of genes are manifested in the proteome profiles, and to gain insights into the links between genes, their expression, and proteins, proteomics must also be integrated into genomics and transcriptomics (Kumar et al. 2016). Since the comprehensive understanding of the genes and their

Table 9.3 List of few medicinal plants in which transcriptome sequencing was done (Xin et al. 2017)

Sl. no.	Name of the medicinal plant	Result of the transcriptome profiling	References
1.	*Zanthoxylum planispinum*	76 CYP genes and their isoforms were identified	Kim et al. (2019)
2.	*Artemisia annua*	The contigs corresponding to enzymes for terpenoids and flavonoids biosynthesis were identified	Wang et al. (2009b)
3.	*Epimedium sagittatum*	Information about the secondary metabolite pathways such as the flavonoid pathway were analyzed	Zeng et al. (2010)
4.	*Glycyrrhiza uralensis*	Genes related to the secondary metabolite pathway of glycyrrhizin, including cytochrome P450s and glycosyltransferase, were found	Li et al. (2010)
5.	*Panax quinquefolius*	Genetic mechanisms in the triterpenoid saponin biosynthesis were found	Wu et al. (2010)
6.	*Panax ginseng*	Nine genes involved in the biosynthesis of ginsenoside skeletons and its modification were identified	Chen et al. (2011)
7.	*Siraitia grosvenorii*	Seven CYP450s and five UDPGs genes involved in mogrosides biosynthesis were identified. Gives insights on the formation of major bioactive constituents in the fruit extract from *S. grosvenorii*	Tang et al. (2011)
8.	*Lonicera japonica*	Gene expression profiles in flowers of *Lonicera japonica* Thunb. and *L. japonica* Thunb. var. chinensis (*Watts*) were studied	Yuan et al. (2012)
9.	*Carthamus tinctorius* L.	Expression of chalcone synthase, chalcone isomerase, and anthocyanidin synthase in different flowering stages	Huang et al. (2012)
10.	*Picrorhiza kurroa*	Few genes involved in picroside biosynthesis were identified	Gahlan et al. (2012)
11.	*Lilium regale*	Two transcriptome sets, which can be used for marker development, comparative genomic studies and candidate gene approaches were developed	Shahin et al. (2012)
12.	*Polygonum cuspidatum*	Eighteen potential UDP-glycosyltransferase unigenes involved in the biosynthesis of glycosides were identified	Hao et al. (2012)
13.	*Nelumbo nucifera.*	A total of 231 genes displayed rhizome-specific expression	Kim et al. (2013a)
14.	*Aquilaria sinensis*	Discovering and identification of the genes involved in sesquiterpenoid production	Ye et al. (2015)
15.	*Fallopia multiflora*	Provided insights about the biosynthesis of 2,3,5,4′-tetrahydroxy stilbene-2-*O*-ß-D-glucoside (THSG)	Zhao et al. (2014a, b)
16.	*Erigeron breviscapus*	The molecular mechanism of scutellarin biosynthesis was studied, which plays a major role in the pharmaceutical activities of *E. breviscapus*	Chen et al. (2015)
17.	*Polygala tenuifolia*	Many transcripts involved in the biosynthesis of triterpene saponins and phenylpropanoids were identified	Tian et al. (2015)
18.	*Xanthium strumarium*		Fan et al. (2015)

(continued)

Table 9.3 (continued)

Sl. no.	Name of the medicinal plant	Result of the transcriptome profiling	References
		miRNAs that play a crucial role in the terpenoid biosynthesis in the glandular trichomes of *X. strumarium* was studied	
19.	*Andrographis paniculata*	A total number of 124 CYP450 transcripts were identified, and nearly 146 different transcripts coding for enzymes involved in the biosynthesis of terpenoids were found	Cherukupalli et al. (2016)
20.	*Forsythia koreana*	Gene ontology found the presence of lignan-biosynthetic enzyme genes in the callus transcriptome. It also predicted candidates for matairesinol-glycosylation enzymes	Shiraishi et al. (2016)
21.	*Picrorhiza kurroa*	Key transcription factors regulating picrosides biosynthesis were identified	Vashisht et al. (2016)
22.	*Ephedra sinica*	Biosynthetic genes of ephedrine alkaloids in aerial stems of *Ephedra* plants were revealed	Okada et al. (2016)
23.	*Pinellia ternata*	Transcripts encoding enzymes involved in benzoic acid and ephedrine biosynthesis were identified	Zhang et al. (2016b)
24.	*Swertia japonica*	Identified 37 unigenes as potential candidates of glycosylation of bioactive metabolites	Rai et al. (2016)
25.	*Plantago ovata*	Several genes involved in various biological processes were identified	Kotwal et al. (2016)
26.	*Dendrobium nobile*	Unigenes related to biosynthesis of dendrobine sesquiterpene backbone	Li et al. (2017a)
27.	*Dendrobium officinale*	Several unigenes were identified	Shen et al. (2017)
28.	*Elettaria cardamomum*	First work on cardamom transcriptome sequencing for wild and cultivar genotypes	Nadiya et al. (2017)
29.	*Cassia angustifolia*	Several CDS encoding signaling factors, protein-modifying or degrading enzymes, biosynthesis of phytohormone, phytohormone signaling, osmotically active compounds, free radical scavengers, chlorophyll metabolism, leaf cuticular wax, polyamines, and protective proteins were identified	Mehta et al. (2017)
30.	*Camellia sinensis*	First-time transcriptomic profiling of the defense against tea geometrid	Wang et al. (2018)

functions comes from proteomics studies, it is compulsory to perform proteome analysis of the samples of medicinal plants taken for transcriptome studies (Wang et al. 2019). Various techniques are used to study the protein components of the organisms (Lodish et al. 2000), such as conventionally chromatography-based techniques (ion exchange chromatography, size exclusion chromatography, affinity chromatography) and western blotting for purification or selective analysis of proteins (Lodish et al. 2000; Coskun 2016; Najafov and Hoxhaj 2017). Protein microarrays of different types are also used to analyze proteins. Gel-based approaches such as sodium dodecyl sulfate polyacrylamide gel electrophoresis

(SDS-PAGE), two-dimensional gel electrophoresis (2D-PAGE), and two-dimensional differential gel electrophoresis (2D-DIGE) are used to purify complex protein samples (Saraswathy and Ramalingam 2011; Kurien and Scofield 2012; Pasquali et al. 2017). Mass spectrometry (MS) techniques such as liquid chromatography–mass spectrometry (LC–MS) can be used to analyze complex protein mixtures, and MS-based techniques are highly sensitive (Kolker et al. 2006; Karpievitch et al. 2010). Various tools such as isotope-coded affinity tag (ICAT) labeling, stable isotope labeling with amino acids in cell culture (SILAC), and isobaric tag for relative and absolute quantitation (iTRAQ) are developed to study quantitative proteomics (Froment et al. 2005; Yeh et al. 2015). Some high-throughput techniques such as X-ray crystallography and nuclear magnetic resonance (NMR) spectroscopy are nowadays increasingly used for the elucidation of 3D protein structures (Berg et al. 2002). The proteomics research goes beyond just the identification of the proteins, it also studies the posttranslational modifications (PTMs) and protein-protein interactions (van Wijk 2001). Proteomic profiling provides the differential expression of proteins in different samples, and hence, the result could be used to compare the protein expressions of various tissues of plants under varying conditions (Graves and Haystead 2002; Tuli and Ressom 2009). Functional proteomics studies provide information about the biological functions of proteins (Monti et al. 2005, 2007). It involves affinity-based procedures and uses suitable tabs as baits for the interacting partners in a cell (Monti et al. 2005). Proteome profiles of medicinal plants can differ across cultivars or under different environmental conditions (Kim et al. 2016; Aghaei and Komatsu 2013). The synthesis of metabolites also changes in response to environmental conditions. Combining metabolomics and transcriptomics with proteomics can help us identify the enzymes that play important roles in mediating the pathways of medicinal compounds in plants (Saito and Matsuda 2010; Zhan et al. 2016). Various proteomic techniques are used to analyze the synthesis of bioactive compounds that give medicinal properties to plants (Sharma and Sarkar 2012; Hashiguchi et al. 2017). Therefore, proteomics combined with other omics technologies can help us better understand the regulation of the mechanisms governing biosynthesis of the bioactive phytochemicals in medicinal plants. Table 9.4 presents a non-exhaustive list of some examples of medicinal plants whose proteome information is obtained using different approaches.

2.4 Metabolomics

Metabolites are a key component of plant metabolism and play important roles in growth, development, and response to environments (Turner et al. 2016). Metabolomics is the comprehensive, unbiased, high-throughput analysis of complex metabolites present in plants (Hall et al. 2002). Metabolomics involves several steps including sample preparation, measurement, and data analysis (Khoomrung et al. 2017). Metabolite profiling plays a huge role in drug discovery and development

Table 9.4 Proteomics of some important medicinal plants (Hashiguchi et al. 2017)

Sl. no.	Name of the medicinal plant	Result of the proteomic sequencing	References
1.	*Cannabis sativa*	Identified a polyketide synthase enzyme involved in cannabinoid biosynthesis	Raharjo et al. (2004)
2.	*Panax ginseng*	A total of 192 differentially expressed protein spots were observed	Ma et al. (2016b)
3.	*Anemone flaccida*	Using bioinformatics, several enzymes involved in the triterpenoid saponin biosynthetic pathway were identified	Zhan et al. (2016)
4.	*Artemisia annua*	More proteins were identified from the plant with trichomes in comparison to the ones without trichomes	Bryant et al. (2016)
5.	*Salvia miltiorrhiza*	Proteins involved in tanshinone biosynthesis are studied. In the proteins identified from the root extracts, some are upregulated, while others are downregulated. The upregulated genes function in metabolism, stress defense, and redox homeostasis. Cytochromes involved in tanshinone biosynthesis were also found	Contreras et al. (2019)
6.	*Pinellia ternata*	A total of 24 proteins were identified such as small heat shock proteins, proteins involved in RNA processing, photosynthesis, protein degradation, and defense. The study provided details in the response of the plants to heat stress at proteome levels	Zhu et al. (2013)
7.	*Lithospermum erythrorhizon*	Study identified the candidate genes involved in the biosynthesis of shikonin. Polyphenol oxidase, cannabidiolic acid synthase-like, and neomenthol dehydrogenase-like proteins were specifically noted	Takanashi et al. (2018)
8.	*Curcuma comosa*	Superoxide dismutase (SOD) and ascorbate peroxidase (APX) that are associated with antioxidant activity and cysteine protease were identified. Some proteins were identified as lectins	Boonmee et al. (2011)
9.	*Calotropis gigantea*	Anticancerous peptides were identified	Rehman et al. (2020)
10.	*Hylocereus polyrhizus*	Proteomic analysis by iTRAQ revealed the molecular mechanism of betalain biosynthesis	Hua et al. (2016)
11.	*Catharanthus roseus*	Identified the novel proteins involved in the biosynthesis of alkaloids of the plant. Some unique sequences were also found	Jacobs et al. (2005)
12.	*Chelidonium majus*	A total of 1240 proteins were identified. The most abundant protein categories were energy, metabolism, photosynthesis, stress, and defense response	Nawrot et al. (2014)
13.	*Corydalis cava*	A total of 228 proteins were identified. The most abundant protein categories were energy, stress and defense response, nucleic acid binding, overall metabolism, and cell organization and structure	Nawrot et al. (2014)
14.	*Nigella sativa*	A total of 277 proteins were identified. The majority of proteins identified were involved in enzyme catalytic activity, nucleotide-binding, and protein binding	Alanazi et al. (2016)
15.	*Dipsacus asperoides*	iTRAQ technique was used and revealed 2149 proteins. UTP-glucose-1-phosphate uridylyltransferase, allene	Jin et al. (2020)

(continued)

Table 9.4 (continued)

Sl. no.	Name of the medicinal plant	Result of the proteomic sequencing	References
		oxide cyclase, and isopentyl diphosphate isomerase 2 were found to be the key proteins involved in Dipsacus saponin VI synthesis	

(Tian et al. 2016). Nowadays, many approaches are available that can be used for metabolite identification such as HPLC, NMR, GC-MS, UPLC, capillary electrophoresis-mass spectrometry (CE coupled to MS), and ^{1}H nuclear magnetic resonance (^{1}H NMR) (Sangwan et al. 2017). Recent developments in high-throughput techniques and their integration with data analysis allow easy separation, detection, and characterization of many metabolites and their related pathways (Piasecka et al. 2019). Integration of metabolomics with transcriptomics and genomics studies helps to study the links between metabolites and genes (Wen et al. 2016). Several databases have been recently developed, which help to understand the functions of genes in metabolite biosynthesis. For example, the plant metabolomics initiative, PlantMetabolomics.org, was started to study the functions of genes in *Arabidopsis* (Okazaki and Saito 2012; Bais et al. 2010). Similarly, tomato metabolites are curated in the Metabolome Tomato Database (Grennan 2009). Several other databases such as TERPMED have information about plant terpenoid and their importance for human health. MetaCyc has information on over 1800 pathways that integrate with metabolite data from more than 2000 plant organisms. The KNApSAcK database is an important repository of a large number of metabolites from thousands of plants (Shinbo et al. 2006; Takahashi et al. 2011; Afendi et al. 2011). KEGG PLANT of the KEGG Pathway database has data on secondary metabolites (Kyoto Encyclopedia of Genes and Genomes 2006). We have given a brief list of plants in Table 9.5 whose metabolomes are studied using different metabolomics studies.

3 Employing Genetic Engineering and Gene Editing Tools on the Information Obtained Using Omics Approaches

Genetic engineering and editing technologies can be employed on the information obtained using the omics tools. The availability of information about the genes, their functions, and the pathways in which they exert their regulatory roles allows us to pinpoint the gene engineering and editing interventions required for tweaking a particular metabolite in plants. Knowing about the undesirable traits and the necessity to eliminate their impacts requires the application of genetic engineering and genome editing tools (Patra and Andrew 2015). Genome editing refers to the modifications done to a specific genome sequence (Gaj et al. 2016; Doudna and Charpentier 2014)). It is a way of creating a particular genome with the addition of

Table 9.5 Metabolomics of some important medicinal plants

Sl. no.	Name of the medicinal plant	Result of the metabolic profiling	References
1.	*Salvia miltiorrhiza*	35 metabolites showing significant changes in rates of JA-mediated accumulation of secondary metabolites were observed	Ge et al. (2015)
2.	*Cannabis sativa*	Delta9-tetrahydrocannabinolic acid (THCA) and cannabidiolic acid (CBDA) can be used to differentiate the different cultivars	Choi et al. (2004)
3.	*Angelica acutiloba*	Twenty-two metabolites consisting of sugars, amino, and organic acids were identified	Tianniam et al. (2008)
4.	*Echinacea purpurea*, *E. pallida* and *E. angustifolia*	Alkamides were seen. The extracts were utilized for analyzing its medicinal uses in animals	Hou et al. (2010)
5.	*Medicago truncatula*	Investigation of the isoflavonoid metabolism in response to elicitation was studied	Farag et al. (2008)
6.	*Camellia sinensis*	First-time metabolomic profiling of the defense against tea geometrid. Higher accumulation of fructose and theanine in plants was found in response to tea geometrid attack	Wang et al. (2018)
7.	*Scutellaria baicalensis*	Over 2000 compounds including 781 were found to be of medicinal importance	Murch et al. (2004)
8.	*Lycium barbarum*, *Lycium chinense* and *Lycium ruthenicum*	Flavonoids, rutin, raffinose, galactinol, trehalose, citrulline, and DL-arginine were identified	Yao et al. (2018), Zhao et al. (2020)
9.	*Camptotheca acuminata*	A subset of alkaloids and alkaloid glycosides were more abundant in young bark	Wurtele et al. (2012)
10.	*Prunella vulgaris*	450 metabolites were detected	Wurtele et al. (2012)
11.	*Panax ginseng*	21 ginsenosides were characterized, in which six were malonyl-ginsenosides	Lee et al. (2017)
12.	*Taxus* spp.	2246 metabolites used in different primary and secondary metabolic pathways were identified	Zhou et al. (2019)
13.	*Weinmannia trichosperma* Cav.	25 metabolites including phenols and flavonols such as isoastilbin, neoisoastilbin, and neoastilbin were isolated	Barrientos et al. (2020)
14.	*Capsicum annuum*	Several metabolites from *Capsicum* fruits including capsaicinoids were studied	Aizat et al. (2014)
15.	*Cyrtopodium glutiniferum*	Phenolic compounds and phenanthrene were found in the plant	Araújo-Lima et al. (2020)

desirable traits or removal of undesirable traits to study functional genomics (Abdallah et al. 2015; Zaman et al. 2019). The expression of certain genes could be controlled using gene editing techniques, and it has revolutionized the way we think about gene modifications (Schaeffer and Nakata 2015). Gene editing tools are

so powerful and have huge potential to eradicate many gene-related issues in the world (Hu 2017; Shew et al. 2018). Since gene editing tools have huge potential in various areas of research such as agriculture, medicinal plant chemistry, genetics, and hereditary diseases, the Nobel Prize in Chemistry in 2020 is awarded to two scientists, namely, Emmanuelle Charpentier and Jennifer A. Doudna who developed CRISPR-Cas9 gene editing protocol (The Nobel Prize in Chemistry 2020; Cohen 2020). Unlike genetic engineering, no foreign DNA is incorporated into the plant in gene-edited crops (Metje-Sprink et al. 2019; Labant 2020). The process involves the introduction of DNA double-strand breaks (DSB) using various tools such as meganucleases, zinc finger nucleases, transcription activator-like effector nucleases, and CRISPR/Cas9 (Gaj et al. 2016; Khan 2019; Li et al. 2020). The double-strand breaks activate the DNA repair mechanism such as nonhomologous end joining (NHEJ) and homologous recombination (HR). NHEJ can cause insertions or deletions called "indels" and may result in frameshift mutation if they occur in the coding region of the gene (Su et al. 2016; da Silva et al. 2019). This results in gene knockout. The HR can be used for gene modification or gene insertions (Bortesi and Fischer 2015). Therefore, gene editing techniques can lead to gene knockout, insertions, and modifications, which can upregulate or downregulate the production of various metabolites (Adrio and Demain 2006, 2010). Several tools are employed for genome editing such as meganucleases, clustered regularly interspaced short palindromic repeats/Cas9 (CRISPR/Cas9), zinc finger nucleases (ZFN), and transcription activator-like effector nucleases (TALENS) (Li et al. 2020). The genome editing technologies can be easily combined with the information obtained using omics tools for the improvement of medicinal plants as depicted in Fig. 9.2.

3.1 TALENs (Transcription Activator-Like Effector Nucleases)

It is one of the most important targeted gene modification technologies, which consists of a nonspecific DNA-cleaving nuclease fused to a specific DNA binding domain of transcription activator-like effector (TALE) (Joung and Sander 2012). These nucleases cause double-strand breaks in the specific DNA sites, and the breaks are repaired by DNA repair machinery (Zhang et al. 2014a).

3.2 ZFN (Zinc Finger Nucleases)

Like TALEN, ZFN consists of a zinc finger binding domain (that recognizes the DNA sequence) and a nuclease domain of the FokI restriction endonuclease enzyme (Urnov et al. 2010). It increases the efficiency of the genome editing technique (Gaj et al. 2016) and enables the directed mutagenesis of specific genes for silencing and transgenic targeting for increasing the expression of genes (Wilson and Roberts 2014).

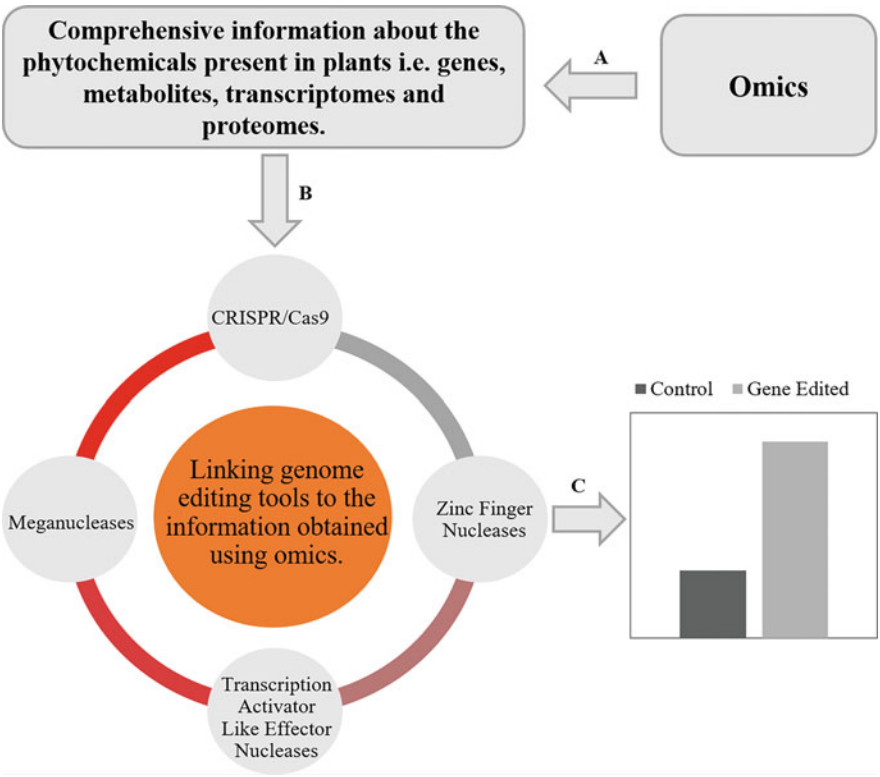

Fig. 9.2 Linking gene editing tools to the information obtained using omics tools for the improvement of medicinal plants. The information (A) obtained using omics tools can be integrated, and then gene editing tools (B) can be employed for rapid improvement of the medicinally important crops (C)

3.3 Meganucleases (MN)

Meganucleases are precise endonucleases and have a recognition site of nearly 12–40 bp (Ahmar et al. 2020; Stoddard 2006; Seligman 2002; Sussman et al. 2004). They also cause double-strand breaks in the DNA and rely on DNA repair mechanisms. Several different families of MNs have been identified based on the sequence and structure motifs (Sussman et al. 2004). Due to issues with the naturally occurring MNs, scientists have recently tried to engineer meganucleases to increase the efficacy and efficiency of gene editing in various organisms based on their requirements (Maeder and Gersbach 2016; Takeuchi et al. 2014).

3.4 CRISPR/Cas9

CRISPR stands for clustered regularly interspaced short palindromic sequence, and it modifies the DNA at specific sites (Jiao and Gao 2016). The tool is appropriate for the genome editing of plants in general. But monocotyledons steal the crown, especially those that have high genomic GC content (Schaeffer and Nakata 2015; Miao et al. 2013). It is a rapid and easy method used for targeted gene editing in different plant species. CRISPR, along with Cas protein, forms the CRISPR/Cas9 system (Zhou et al. 2014; Bortesi and Fischer 2015). CRISPR and Cas genes are essential for immunity in bacteria and archaea, and they use it against foreign genetic elements as a defense system and rely on guide RNA (gRNA) instead of synthetic DNA binding domain (Loureiro and da Silva 2019). The procedure of CRISPR/Cas9 involves various steps such as identification of the desired target gene and protospacer adjacent motif sequences, designing the gRNA, cloning of designed gRNAs, construction of the binary vector, transfer of the vector into the plant through transformation, development of transgenic plants, and genotyping of transgenic plants (for detailed review, refer to Ahmar et al. 2020). The efficiency of CRISPR depends on the sequence and location of the target (Belhaj et al. 2015). Gene knockout strategies were performed by Alagoz et al. (2016), where they manipulated the metabolic pathway of benzylisoquinoline alkaloid (BIA) in *Papaver somniferum*. The 4′OMT (3′-hydroxy-*N*-methylcoclaurine-4′-*O*-methyltransferase) was knocked out by targeting it and observing InDel mutations by NHEJ using CRISPR/Cas9 tool, and hence, the synthesis and the expression of BIA were reduced in opium poppy (Belhaj et al. 2015; Alagoz et al. 2016). Using this tool, we can analyze and incorporate plants with desired gene modifications, including both quantitative and qualitative metabolite composition (Alagoz et al. 2016). Transgenic *Cannabis* plants can be produced using the CRISPR tool (Schachtsiek et al. 2018).

Often, plants with glandular trichomes are excellent sources of secondary metabolites (Schuurink and Tissier 2019), and by employing CRISPR/Cas9, one can derive various compounds selectively (Fu et al. 2018; Glas et al. 2012). In the case of *Cannabis sativa,* the secondary metabolites such as cannabinoids are produced in glandular trichomes (Livingston et al. 2019). Unfortunately, not all plants are ideal for deriving secondary metabolites from glandular trichomes. For example, tobacco has a glandular trichome, but the secretion also contains nicotine and other stress-related protein products (Amme et al. 2005). Hence, plants such as tomato, cotton, and mint could be used as alternatives to obtain secondary metabolites (Wang et al. 2016; Kortbeek et al. 2016; Ma et al. 2016a).

3.5 Successful Examples of Gene Editing in Medicinal Plants

Recently, gene editing technologies have been applied to medicinal plants for the improvement of certain metabolites. All these genome editing tools can be used to

modify the genome of a plant (Rehman et al. 2020). The editing tools could be efficiently utilized for the deletion of unwanted genes or transcripts so that the resulting plant can be completely used without any fear of the intermixing of unwanted compounds already present in the plant (Agapito-Tenfen et al. 2018). Genome editing can have a huge impact on functional genomics, crop improvement, and commercial product development and it will play a crucial role in the study of complex traits and their benefits (Petolino 2015). Table 9.6 displays application of genome editing to certain genes for the improvement of the medicinal plants.

3.6 GMO and Gene-Edited Crops

Mutagenesis is a process by which a change or mutation occurs in DNA (Durland and Ahmadian-Moghadam 2020). It can be either advantageous or deleterious to the plant. If the mutation is beneficial, the mutant offspring will be better than the parent plant (Loewe and Hill 2010). This type of mutagenesis can be called random mutagenesis, and farmers have been selecting those plants with beneficial mutations for years. It can be confirmed that all of us consume a genetically modified crop (Phillips 2008). Non-random and highly planned mutagenesis are also done in plants by various techniques to improve them for human welfare (Sikora et al. 2011). Gene editing techniques, as mentioned above, use tools such as CRISPR/Cas9, ZFN, TALEN, and MNs, without the introduction of an external DNA (Rehman et al. 2020). It involves altering the base pair arrangements within the genome of the organism. On the other hand, GMOs are the plants where a donor DNA has been introduced into the host DNA (Shew et al. 2018). Crops are genetically modified for various purposes like increased production and resistance to pathogens (Phillips 2008; Key et al. 2008; Maghari and Ardekani 2011). Golden rice, Bt Brinjal, GM maize, and GM tomato are well-known GM crops (Zhang et al. 2016a; Raman 2017; Abbas 2018; Mishra 2019). Edible vaccines are also produced in this manner (Kurup and Thomas 2020; Gunasekaran and Gothandam 2020). It is expected that gene-edited crops might be preferred by consumers over GMOs since there is no foreign DNA in the former ones. However, policies must be designed to make it possible for the commercial production and sale of gene-edited crops (GEd). Since current GMO regulatory frameworks do not cover the GEd crops, national and international agencies and governments must take the required steps for easy introduction of the GEd crops into the market (Menz et al. 2020).

Table 9.6 Some important genome-edited medicinal plants

Sl. no.	Name of the plant	Gene edited	Improvement	Reference
1.	*Papaver somniferum*	3′-hydroxy-*N*-methylcoclaurine 4′-*O*-methyltransferase (4′OMT2) gene regulates benzylisoquinoline alkaloids (BIAs) metabolism and biosynthesis.	Reduction of BIAs (e.g., morphine, thebaine) in edited crops	Alagoz et al. (2016)
2.	*Salvia miltiorrhiza*	Rosmarinic acid synthase (RAS) gene	Decrease in rosmarinic acid (RA) and lithospermic acid B in the edited plant	Zhou et al. (2018)
		Diterpene synthase gene (SmCPS1)	Knockout for tanshinone biosynthesis	Li et al. (2017b)
3.	*Solanum lycopersicum*	Anthocyanin mutant 1 (*ANT1*)	Overexpression of ANT1 enhances anthocyanin synthesis	Čermák et al. (2015)
		Phytoene desaturase (*SlPDS*, Solyc03g123760.2.1) and Phytochrome interacting factor (PIF4)	Silencing of SlPDS resulted in photobleaching	Pan et al. (2016)
		Phytoene synthase (PSY1)	Carotenoid biosynthesis	Hayut et al. (2017)
4.	*Citrullus lanatus*	Phytoene desaturase (ClPDS)	Carotenoid biosynthesis	Tian et al. (2017)
5.	*Dioscorea zingiberensis*	Farnesyl pyrophosphate synthase gene (Dzfps)	CRISPR/Cas9-mediated mutagenesis of Dzfps gene reduces farnesyl pyrophosphate synthase (FPS) activity squalene content in edited plants	Feng et al. (2018)
6.	*Vitis vinifera*	Phytoene desaturase (VvPDS) gene	Carotenoid biosynthesis	Nakajima et al. (2017)
7.	*Citrus*	Phytoene desaturase (CsPDS)	Carotenoid biosynthesis	Jia and Wang (2014)
8.	*Taraxacum kok-saghyz*	Fructan:fructan 1-fructosyltransferase (1-FFT) is involved in inulin biosynthesis	Targeting of the 1-FFT gene increased the rubber production	Iaffaldano et al. (2016)

4 Production of Specialized Metabolites Using Tissue Culture and Hairy Root Culture

Tissue culture is the process of production of insect-free and pest-free cells or tissues separately from an organism under specific laboratory conditions (Hussain et al. 2012). The cells in the plant tissue culture are biosynthetically totipotent (Rao and Ravishankar 2002). The main advantage of tissue culture is that even rare and endangered plants can be maintained to produce specialized metabolites (Efferth 2019). The addition of precursors to the medium enhances the formation of secondary metabolites (Efferth 2019). A few examples of specialized metabolites produced using tissue culture include taxol, a diterpene alkaloid from Taxus tree that shows an anticancerous property. Phenylalanine is proven to increase taxol production in the cell culture. Callus culture of *Coscinium fenestratum*, which synthesized berberine, was performed since this important medicinal plant is on the verge of extinction (Nair et al. 1992). In 1986, Philip and Nainar successfully cultivated vanilla plantlets in vitro to obtain vanillin using in vitro culture by adding ferulic acid, which is a precursor of vanillin (Romagnoli and Knorr 1988). Some examples of tissue culture techniques for secondary metabolite production are shown in Table 9.7. Anthraquinone production was stimulated in *Cinchona ledgeriana by* using polymeric adsorbents like macro-reticular Amberlite XAD-7 (Robins and Rhodes 1986). Organ culture of *Fritillaria unibracteata* has also been done for the production of secondary metabolites (Gao et al. 1999; Hussain et al. 2012). Elicitors (a compound in small concentrations that are added to a living system to promote the synthesis of target metabolite) such as fungal carbohydrates and yeast extract has been used for enhancing secondary metabolite production (Hussain et al. 2012; Ramirez-Estrada et al. 2016). Another set of elicitors was used to improve isoflavonoid synthesis in *Lupinus mutabilis* (Tian 2015). Low concentration of indole-3-acetic acid was used to enhance the production of glucosinolates in *Brassica oleracea* var. italica (Sanchez-Pujante et al. 2017). Since roots show slow growth when cultured in vitro, hairy root culture is promoted (Nielsen et al. 2019). Hairy root cultures can be done by genetic transformation of plant cells with a pathogenic strain of *Agrobacterium rhizogenes* (Hidalgo et al. 2018). The T-DNA in plasmid of *A. rhizogenes* is responsible for the hairy root formation (Tian 2015). Some examples of hairy root culture techniques for specialized metabolite production are shown in Table 9.7.

Table 9.7 Application of metabolic engineering, tissue culture and hairy root culture to some of the medicinal plants

Sl. no.	Plant species	Metabolic approach	Target compound	Reference
1.	*Gentiana macrophylla Gentiana punctata Gentiana scabra*	Overexpression of transcription factors	Terpenoid	Tian et al. (2015)
2.	*Lobelia inflata*	Overexpression of transcription factors	Lobeline alkaloid	Yonemitsu et al. (1990)
3.	*Arabis caucasica Barbarea verna Nasturtium officinale Tropaeolum majus*	Overexpression of transcription factors	Gluconasturtiin Glucotropaeolin Glucoiberverin (All glucosinolates)	Wielanek et al. (2009), Wielanek and Urbanek (1999)
4.	*Lupinus mutabilis*	Overexpression of transcription factors	Isoflavonoids	Babaoglu et al. (2004)
5.	*Echium acanthocarpum*	Overexpression of transcription factors	Linolenic acid	Cequier-Sanchez et al. (2011)
6.	*Panax ginseng*	Overexpression of transcription factors	Ginsenosides	Kim et al. (2004), Palazón et al. (2003)
7.	*Centella asiatica*	Overexpression of rate-limiting step	Phytosterol and triterpene	Kim et al. (2010a)
8.	*Catharanthus roseus*	Overexpression of rate-limiting step	Hörhammericine	Magnotta et al. (2007)
9.	*Salvia miltiorrhiza*	Overexpression of enzymes in biosynthesis	Tanshinone	Kai et al. (2011)
10.	*Artemisia annua*	Overexpression of transcription factor	Artemisinin	Yu et al. (2012)
11.	*Hyoscyamus reticulatus*	Overexpression of abiotic elicitor	Tropane alkaloid	Khezerluo et al. (2018)
12.	*Papaver bracteatum*	Overexpression of a gene in biosynthesis pathway	Thebaine, codeine, and morphine	Sharafi et al. (2013)
13.	*Scutellaria bornmuelleri*	Elicitors	Flavonoids	Gharari et al. (2020)
14.	*Platycodon grandiflorum*	Overexpression of rate-limiting step	Phytosterols and triterpenoids	Kim et al. (2013b)
15.	*Glycyrrhiza uralensis*	Overexpression of genes	Flavonoids	Zhang et al. (2009)

5 Conclusions

Plants are an immense source of phytochemicals that are beneficial to humans. The presence of an array of such phytochemicals makes the plants, natural chemical factories. The whole plant or its various parts have been an important part of healthcare for a larger population of the world since ancient times. However, excessive reliance and overexploitation of precious natural resources may lead to the depletion of important phytodiversity. Therefore, alternative sustainable tools are needed to reverse the negative impacts on phytodiversity or to prevent further negative impacts in the future. Biotechnological interventions are needed to devise mechanisms to produce important phytochemicals, especially specialized metabolites that are important for human health and are scarce. Towards the end, we need a deeper understanding of the basic mechanisms governing the biosynthesis of important metabolites. Combined integrative omics approaches can help us elucidate such mechanisms. The combination of such omics approaches and their application to unravel the phytochemical secrets of plants has given birth to a new and emerging discipline known as phytochemical genomics. Further beyond phytochemical genomics, we can link the gene editing and gene modification technologies to the information obtained using omics to improve the phytochemical yields. Gene editing has particularly been increasingly used recently for a large number of plants for making changes in the genomes of many medicinal plants.

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Chapter 10
Application of Transcriptomics in Exploring Important Genes in Medicinal Plants

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1 Introduction

Medicinal plants have been a source of many active constituents that are lifesaving. They have a large number of active principles that are widely used in medicine and pharmaceutical companies. The majority of medicinal herbs serve as natural chemical factories. Several major modern medications have been developed from medicinal plants that have been used for centuries around the world to treat human illnesses and diseases. Many of these traditionally utilized herbs have been studied scientifically in order to develop some important lifesaving medicines. As per World Health Organization (WHO), nearly 80% of the civilization around the world is dependent on herbal medicines for basic healthcare (Vines 2004; Mohanty et al. 2017). According to Kew, out of total 30,000 plants, 17,810 plants are known to have therapeutic use (State of the World's Plants Report—2016). Since the introduction of lifesaving medications, a wide range of secondary metabolites extracted from plants has a positive impact on human healthcare. Secondary metabolites of medicinal plants are the most common natural products with therapeutic properties. Several unique and complicated routes interact via metabolic networks to produce such specialized metabolites. Secondary metabolites are intended to involve in a range of biological and operational roles in plants.

Transcriptomics is a powerful approach to find genes that are expressed differently under different conditions and involved in metabolic pathways. In earlier generations, studies are mostly relied on microarray-based methods where thousands of genes are present of array and utilized for expression of these genes in different plants/tissues/treatments. Microarray is a low-cost tool used in transcriptomics that

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M. K. Swamy, A. Kumar (eds.), *Phytochemical Genomics*,
https://doi.org/10.1007/978-981-19-5779-6_10

can measure the expression of thousands of genes; however, application of DNA microarrays is limited to study of only those transcripts, which are available in chip, and novel transcripts cannot be identified using microarrays. With advancement in technologies, ESTs and EST-based markers came into existence followed by whole transcriptome sequencing methods. EST-based approaches are proving to be a useful and low-cost tool for gene discovery. ESTs are derived from cDNA libraries and can investigate the expression profile number of genes at the same time. ESTs, on the other hand, are time-consuming, labor-intensive, and costly procedures. Next Generation Sequencing (NGS) technologies are widely used in transcriptomics currently because they allow for a multidimensional view of transcriptomes with high-throughput transcript data with ultra-high resolution with single base (Morozova et al. 2009). NGS technologies, including 454 sequencing, SOLiD sequencing, Solexa Sequencing, HeliScope Single-molecule Sequencer, PacBio RS, and Nanopore technologies are gaining attraction in medicinal plant transcriptome research, with the goal of discovering genes, analyzing gene expression profiles, annotating protein-coding genes, and identifying genetic markers.

Transcriptome has come out to be very important tool for harvesting knowledge from medicinal plants. It aids us with discovery of important genes associated with the biosynthetic pathways of medicinally important bioactive compounds. More than 100 studies are available in different plant species of medicinal importance to excavate the gene networks and pathways related to important economic traits using transcriptome analysis (Table 10.1).

2 Microarray-Based Identification of Genes Associated Therapeutic Traits

In case of medicinal plants, DNA-based microarrays have been found useful into two ways. First, global microarrays (where thousands of genes are present) are utilized to identify novel genes associated with synthesis of bioactive compounds. For example, in the case of *Salvia miltiorrhiza*, cDNA microarray analysis examines thousands of genes simultaneously out of which seven genes are in particular with tanshinone biosynthesis (Cui et al. 2011). Second is the customized microarrays where chip is customized with few genes and used for quality control of herbal formulations. For instance, 55 gene-based microarrays were developed to examine the quality control of herbal formulation for asthma (Zhao et al. 2013a). Similarly, 92 gene-based microarrays have been developed for herbal formulation of circulation problem (Kawamura et al. 2007). In another chip, nine genes are present to authenticate the herbal formulation of post stroke disorder (Rong et al. 2007). The DNA-based microarrays have wide application and exhaustively used in case of medicinal plants (for details see (Kiyama 2017)).

Table 10.1 Summary of transcriptome studies conducted in medicinally important plants

Plant species	Tissues used for transcriptome	Medicinally important compound (or compound category)	References
Armoracia rusticana	Leaf/root/sprout/stem	Asparagine, flavonoids	Näätsaari et al. (2014)
Artemisia annua	Glandular trichome, leaf, flower bud, cotyledon	Artemisinin (sesquiterpene)	Wang et al. (2009)
Artemisia tridentata	Leaf	Monoterpenes (including camphor, camphene, cineole, and thujone), sesquiterpenes, mumarins, and flavonoids	Bajgain et al. (2011)
Asparagus racemosus	Leaf and root	Alkaloids, amino acid, ascorbic acid, saponins	Upadhyay et al. (2014)
Azadirachta indica	Root, leaf, stem, flower, and fruit	Azadirachtin, nimbin, salannin	Krishnan et al. (2012), Narnoliya et al. (2014)
Boehmeria nivea	Leaf, root, stem,	Alpha amyrin, caffeic acid, rutin, lignin, chlorogenic acid	Liu et al. (2013)
Brassica juncea	Inflorescence/pod and seedling	Polyphenols, phenolic acids, flavonoids, carotenoids (zeaxanthin, lutein, β-carotene), alkaloids, phytosterols chlorophyll, glucosinolates, terpenoids, and glycosides	Paritosh et al. (2014)
Brassica oleracea	Sprout/seed	Glucosinolate (glycoside) glucoraphanin, sulforaphane	Gao et al. (2014)
Bupleurum chinense	Root	Saikosaponin (glycoside)	Sui et al. (2011)
Camellia sinensis	Leaf	Catechins (flavonoids), theanine	Wu et al. (2014)
Camptotheca acuminata	Leaf	Camptothecin, hyperoside, quinoline, strictosidine	Sun et al. (2011)
Capsicum annuum	Fruit	Capsaicin (phenols)	Lu et al. (2012)
Catharanthus roseus	Seedling, shoot, leaf, flower, root, cell suspension	Catharicine, vindoline/vinblastine/vincristine (alkaloids)	Kumar et al. (2014), Verma et al. (2014)
Centella asiatica	Leaf	Saponins (triterpenoid), asiaticoside, madecassoside	Sangwan et al. (2013)
Chlorophytum borivilianum	Leaf	Testosterone, flavones, steroidal saponins, catechin (flavonoid). (*S*)-tetrahydrocolumbamine (alkaloid)	Kalra et al. (2013), Kumar et al. (2016)
Cicer arietinum/ *C. reticulatum*	Root, shoot, seedling/flower/pod	Betaine, ascorbic acid	Deokar et al. (2014)
Cistus creticus	Trichomes	Resinoid, 3-hydroxy-3-methylglutaryl-coenzyme A	Falara et al. (2008)

(continued)

Table 10.1 (continued)

Plant species	Tissues used for transcriptome	Medicinally important compound (or compound category)	References
		reductase (terpenoid), tropinone reductase (alkaloid), chalcone synthase (flavonoid), cinnamate 4-hydroxylase (phenylpropanoid)	
Costus pictus	Leaf	Diosgenin (steroidal sapogenin), bixin, abscisic acid, and geranial and geraniol biosynthesis	Annadurai et al. (2012)
Citrullus lanatus	Fruit flesh	Cucurbitacin, triterpenes, sterols, and alkaloids	Guo et al. (2013b)
Crocus sativus	Flower bud, stigma	Crocin (carotenoid), picrocrocin (monoterpenoid glycoside), safranal (monoterpenoid aldehyde)	Hu et al. (2020), Gao et al. (2021b)
Digitalis purpurea	Leaf, stem, flower, root	Digitoxin, digoxin, cardiac glycosides	Wu et al. (2012)
Dioscorea zingiberensis	Leaves, rhizome	Diosgenin (steroids), dioscin	Li et al. (2018)
Eleutherococcus senticosus	Leaves	Eleutherosides, saponin, triterpenoid saponins, lignans, and phenolic compounds	Hwang et al. (2015)
Diospyros kaki	Flower	Chrysontemin (flavonoid), ursolic acid (triterpenoid compounds)	Luo et al. (2014)
Daucus carota	Root	Beta carotene, ascorbic acid, tocopherol	Rong et al. (2014)
Epimedium sagittatum	Leaf	Icariin, phytoestrogen	Zeng et al. (2010)
Erigeron breviscapus	Leaf/flower	Scutellarin and chlorogenic acids	Jiang et al. (2014)
Entada phaseoloides	Root, stem, leaves	Saponins (triterpenoid), phaseoloidin, glucoside	Liao et al. (2020)
Euryale ferox	Seeds	Ascorbic acid, beta carotene, beta sitosterol	Liu et al. (2018)
Fraxinus	Phloem plug	Catechin, rutin, fraxetin	Bai et al. (2011)
Fritillaria cirrhosa	Bulbs	Chinpeimine, sipermine, fritimine, minpeimine	Zhao et al. (2018)
Fritillaria hupehensis	Bulb, leaf, root, and stem	Hupehenine (alkaloid)	Guo et al. (2021)
Ginkgo biloba	Leaf	Amentoflavone, apigenin	He et al. (2015a)
Glycyrrhiza uralensis	Roots, stems, and leaves	Betulinic acid, flavonoids, glucosides, glycyrrhizic acid	Ramilowski et al. (2013)
Gymnema sylvestre	Leaves	Gymnemic acid, tannins, alkaloids	Kalariya et al. (2018)
Gynostemma pentaphyllum	Roots, leaves	Gypenoside, ginsenoside, beta sitosterol, flavones	Subramaniyam et al. (2011)
Hedera helix	Leaf, root	Glycoside, saponin	Sun et al. (2017)

(continued)

Table 10.1 (continued)

Plant species	Tissues used for transcriptome	Medicinally important compound (or compound category)	References
Hevea brasiliensis	Leaf, latex		Xia et al. (2011)
Hippophae rhamnoides	Leaf, root	Alpha carotene, beta amyrin	Jain et al. (2014)
Humulus lupulus	Lupulin/gland/ cone/leaf	Alpha pinene, alpha terpineol	Clark et al. (2013)
Ipomoea batata	Root	Anthraxanthin, flavoxanthin, auroxanthin (carotenoids)	Wang et al. (2010)
Isatis indigotica	Leaf/root	Beta sitosterol, isatin, palmitic acid	Tang et al. (2014)
Lilium "Sorbonne"	Flower	Carotenoid, flavonoid, anthocyanins	Zhang et al. (2015)
Lycium chinense	Whole plant		Zhao et al. (2013b)
Macleaya cordata, *M. macrocarpa*	Roots, leaves, and fruit shells		Zeng et al. (2013)
Magnolia sprengeri	Petal		Shi et al. (2014)
Mirabilis himalaica	Roots, stems, and leaves	Balanoinvolin, β-sitosterol, daucosterol, boeravinone D, chlorobenzene, boeravinone B	Gu et al. (2018)
Momordica charantia	Seeds	Ascorbigen, momordicin, charartin	Yi et al. (2021)
Narcissus pseudonarcissus	Leaf/bulb/ inflorescence	Galanthamine (alkaloid), harmanthamine (alkaloid), tazettine (alkaloid)	Singh and Desgagné-penix (2017)
Ocimum sanctum and O. basilicum	Leaf	Apigenin, ascorbic, alkaloids	Rastogi et al. (2014)
Opium poppy	Cell cultures	Opiods (alkaloids)	Desgagné-Penix et al. (2010)
Paeonia lactiflora	Red outer petal/ yellow inner petal	Astragalin, benzoic acid, palbinone (l) (triterpene)	Hao et al. (2016)
Paeonia suffruticosa	Flower buds	Paeoniflorin (monoterpene glycosides), gallic acid	Gai et al. (2012)
Panax japonicas	Leaf, flower, root, rhizome,	Saponins	Rai et al. (2016)
Panax notoginseng	Root	Ntoginsenoside, triacylglycerol (trilinolein)	Luo et al. (2011)
Panax quinquefolius	Roots, flowers, and leaves	Ginsenosides	Qi et al. (2015), Sun et al. (2010), Luo et al. (2011)
Paris polyphylla	Root	Dioscin, diosgenin	Liu et al. (2016)
Perilla frutescens	Leaves	Polyunsaturated oil, alpha linolenic acid	Fukushima et al. (2015)

(continued)

Table 10.1 (continued)

Plant species	Tissues used for transcriptome	Medicinally important compound (or compound category)	References
Picrorhiza kurroa	Root shoot, stolons	Iridoid glycosides, androsin, apocyanin	Kharb and Chauhan (2021)
Podophyllum hexandrum	Rhizome	Podophyllaotoxin, peltatin	Bhattacharyya et al. (2016)
Pueraria lobata	Roots and leaves	Allantoin, daidzein, isoflavonoids, formononetin, puerarin, daidzein-4′, 7-diglucoside, beta-sitosterol, 3′-methoxydaidzein, daidzein, daidzin, ononin	Wang et al. (2015)
Punica granatum	Fruit peel	Punicalagins, ellagic acid, punicic acid	Xue et al. (2017)
Ribes nigrum	Leaf bud	Anthocyanins, gamma linolenic acid, polyphenols	Russell et al. (2011)
Ricinus communis	Seed, leaf, flower	Cadimene, carotenoids, chelerythrine, ricinine (alkaloid)	Brown et al. (2012)
Salvia fruticosa	Glandular trichomes		Chatzopoulou et al. (2010)
Salvia guaranitica	Leaves	Cirsiliol	Ali et al. (2017)
Salvia sclarea	Calyx	Phellandrene, beta sitosterol, camphor, inalyl acetate and linalool	Legrand et al. (2010)
Scabiosa columbaria	Root, leaf, flower bud	Flavonoids, scabrioside (saponins)	Angeloni et al. (2011)
Sesamum indicum	Root, leaf, flower, seed, shoot tip	Sasamin, achilleine, gentic acid	Wei et al. (2011)
Sinopodophyllum hexandrum	Rhizome		Kumari et al. (2014)
Siraitia grosvenorii	Fruit	Mogrosides	Tang et al. (2011)
Solanum lycopersicum	Trichome	Pantothenic acid, biotin, lycopene	Spyropoulou et al. (2014)
Sonneratia alba	Root	Polyphenol, flavonoid, polysaccharides	Chen et al. (2011)
Stevia rebaudiana	Leaf	Ascorbic acid, beta carotene, beta sitosterol	Chen et al. (2014)
Swertia mussotii	Root, leaf, stem, and flower tissues	Amarogentin, swertiamarin, mangiferin, swerchirin, sweroside, amaroswerin, and gentiopicrin	Liu et al. (2017)
Taxus chinensis	Cell cultures	Taxol	Qiu et al. (2009)
Taxus maire	Roots, stems, and leaves	Taxol	Hao et al. (2011)
Taxus yunnanensis	Needles, branches, roots	Taxinine E, taxol, cephalomannine, α-conidendrin (phenolics)	He et al. (2018)

(continued)

Table 10.1 (continued)

Plant species	Tissues used for transcriptome	Medicinally important compound (or compound category)	References
Trachyspermum ammi	Inflorescence tissues	Carotenes, thymol	Soltani Howyzeh et al. (2018)
Trigonella foenum-graecum	Leaves, stem	4-Hydroxyisoleucine	Ciura et al. (2017)
Trillium govanianum	Rhizome, stem, leaf, and fruit	Saponins, sapogenins, and flavonoids	Singh et al. (2017)
Uncaria rhynchophylla	Capsule	Catechin, corynoxeine, trifolin, hirsutine, hyperin	Guo et al. (2014)
Vernicia fordii	Seed	Isodiverniciasin A, diverniciasin B, diverniciasin C, isoprincepin	Cui et al. (2018)
Withania somnifera	Leaf and root	Wuthanolides, withanoferins (steroids), somniferin (alkaloid)	Senthil et al. (2015)
Zanthoxylum planispinum	Tissues of leaf, early fruit and maturing fruit stage	Cineole, caryophyllene	Kim et al. (2019)

3 ESTs and EST-Based Markers in Medicinal Plants

In majority of medicinal plants, reference genome is not available. In such situation, ESTs and EST-based markers become key tools to identify and annotate important genes. High-throughput sequencing technologies have been noticeably utilized in generation of ESTs and further to develop EST-based markers in medicinal plants (Li et al. 2010; Zeng et al. 2010; Zhao et al. 2013c, 2015) For instance, in the case of *Glycyrrhiza uralensis*, 59,219 ESTs (average length 409 bases) were developed using 454 GS FLX platform and titanium reagents (Li et al. 2010). A total of 27,229 unique genes were identified, and out of them, 20,437 were annotated also. Further, genes associated with glycyrrhizin skeleton synthesis particularly with cytochrome P450 (125 homologues) and glycosyltransferases (172 homologues) were identified. These genes found to encode 16 enzymes of glycyrrhizin skeleton synthesis (Li et al. 2010). Similarly, in case of *Epimedium sagittatum*, EST dataset with 76,459 consensus sequences (including 17,231 contigs and 59,228 singletons) were developed, and of which 22,295 ESTs were successfully annotated (Zeng et al. 2010). A set of 2810 EST-SSRs were generated using 76,459 ESTs. Interestingly, majority (85.7%) of them showed good transferability across the different species of *Epimedium* and also showed high genetic diversity, which emphasized the potential of these EST-SSRs for genetic and genomics studies. In case of *Dendrobium officinale*, to identify the putative genes involved in establishing symbiotic association with fungus, 1437 ESTs were developed using suppression subtractive hybridization cDNA library (Zhao et al. 2013c). Out of 1437 ESTs, 579 were differentially expressed in *Dendrobium officinale* with symbiotically germinated seed as

compared to nonsymbiotically germinated seed. Two calcium-dependent protein kinase genes were characterized for germination under symbiosis in *Dendrobium officinale*. Further, using global transcriptomes of two species of *Gynostemma*, that is, *G. pentaphyllum* and *G. cardiospermum*, a total of 3891 EST-SSRs were developed (Zhao et al. 2015). Fifty percent of tested polymorphic EST-SSRs showed good transferability across 12 different species of *Gynostemma*.

4 Transcriptome Landscape in Medicinal Plants

Like food plants, medicinal plants have also been much explored for their transcriptome profiling, and number of genes and biosynthetic pathways have been identified to add the economic value of plants. For instance, in case of *Cannabis sativa*, transcriptome unveils the mystery of therapeutic value of marijuana strain of *Cannabis sativa* (Van Bakel et al. 2011); however, other strain of *Cannabis sativa* (hemp strain) has no medicinal value and is only used for fiber. The study identified that genes involved in cannabinoid pathway were significantly upregulated in Purple Kush genotype (marijuana strain) than the finola genotype (hemp strain). Most importantly, *tetrahydrocannabinolic acid synthase* (*THCAS*) gene exclusively expressed in marijuana strain and led to the synthesis of tetrahydrocannabinol (THC) (biochemical with high medicinal value). Likewise, in case of *Ferula asa-foetida*, de novo transcriptome analysis identified putative genes involved into terpenoid and coumarin biosynthesis pathways (Amini et al. 2019). Twenty-seven candidate genes were found to be involved in 2-C-methyl-derythritol-4-phosphate (MEP) and mevalonate (MEV) pathways. A total of 32,245 and 142 transcripts were matched with terpene synthase (TPS)/triterpene synthase (TTS), P450s, and phenylpropanoid pathway genes, respectively. Substantial number of differential gene expression in root tissue suggested their possible role in synthesis of oleo-gum-resin in *Ferula*. Full-length transcriptome of *Carthamus tinctorius* uncovered the biosynthetic genes of most bioactive compound, that is, flavonoid (Chen et al. 2018a). These flavonoids are very important to improve cerebral blood flow. Forty-four genes including *CtC4H2*, *CtCHS3*, *CtCHI3*, *CtF3H3*, *CtF3H1*, and their isoforms are reported to be upregulated under MeJA treatment and promote flavonoid biosynthesis. In another medicinal plant, namely, *Asarum sieboldii*, full-length transcriptome identified 63,023 transcripts, 555 alternative splicing sites, 10,869 long noncoding RNAs (lncRNAs) with 11,291 target sites, and 17,909 gene-based SSR markers (Chen et al. 2021). Ninety-six transcripts were identified to encode enzymes involved in asarinin (bioactive compound) metabolism, and 56 transcripts were found to be involved in aristolochic acid (toxic compound) biosynthesis.

Further, in case of *Cinnamomum camphora*, 23.76 Gb clean data were generated in two different chemotypes (unalool and borneol) including 156,184 unigenes (Chen et al. 2018b), 2863 genes were differentially expressed in two chemotypes and of which 67 genes were annotated to involve in terpenoid biosynthesis. In case

Table 10.2 List of SSRs developed in various medicinal plants using transcriptome sequencing

Plant species	Sequencing method	Total SSR	Reference
Panax notoginseng	454 pyrosequencing	2772	Luo et al. (2011)
Paeonia suffruticosa	Massive parallel pyrosequencing	2253	Gai et al. (2012)
Dendrobium officinale	454 pyrosequencing	1061	Guo et al. (2013a)
Glycyrrhiza uralensis Fisch	Illumina HiSeq 2500 sequencing	7032	Liu et al. (2015)
Morinda officinalis	Next generation sequencing	8064	Liao et al. (2019)
Salvadora oleoides	Illumina paired-end sequencing	7101	Bhandari et al. (2020)
Populus alba	Roche 454 GS-FLX platform	9559	He et al. (2015b)
Gastrodia elata	Illumina HiSeq 4000 sequencing	2298	Wang et al. (2020)

of *Dysphania schraderiana*, an important Tibetan medicinal plant, 52 million clean reads generated 40,142 unigenes and identified 2579 DEGs (flower versus leaf) including 2156 transcription factors (Fu et al. 2019). Similarly, Gao et al. (2021a) identified early bolting related genes in *Angelica sinensis* using transcriptome. Roots of *Angelica sinensis* is important for improvement of blood circulation; however, early bolting compromises the quality of roots. Transcriptome of early bolting and normal bolting genotypes identified 43,438 nonredundant transcripts and out of which 475 differentially expressed genes in early bolting and normal bolting genotypes. These DEGs were involved in flowering, pollen formation, and very long-chain fatty acid synthesis, which emphasized their role in early bolting (Gao et al. 2021b).

In another very important medicinal plant *Fritillaria* sp., transcriptome analyses have been conducted recently (Sharma et al. 2021; Kumar et al. 2021; Guo et al. 2021). The studies developed more than 340 million reads along with other molecular resources like 38,607 lncRNAs, 7914 SSRs, etc. Hundreds of DEGs in bulb tissue and their annotation to different biosynthetic pathways like phenyl propanoid, terpenoid, sesquiterpenoid, and triterpenoids suggested their role in synthesis of bioactive compounds primarily in bulb (Sharma et al. 2021). Triterpenoid biosynthesis in genes has also been excavated through transcriptome analysis in different medicinal plants including *Entada phaseoloides* (Liao et al. 2020), *Euphorbia jolkini*, etc. There is long list of studies where SSRs have been developed from transcriptome data, and few of them are listed in Table 10.2.

Apart from phytochemical biosynthesis, transcriptome facilitated the identification of important genes and pathways involved in seed germination in *Cinnamomum migao* (Huang et al. 2021), early microtuber formation and proliferation of axillary bud in *Dioscorea opposita* (Li et al. 2020), adaptation in *Rheum austral* (Mala et al. 2021), and polysaccharide metabolism in *Dendrobium houshanense* (Zhou et al. 2020).

5 Role of miRNA in Phytochemical Biosynthesis

MicroRNAs (miRNAs), short noncoding RNAs of 19–24 nucleotides (nt) in length, act as transcriptional and posttranscriptional regulators of gene expression. In case of medicinal plants, aside from the different genes, miRNAs found to play vital role in metabolite biosynthesis (Table 10.3). Several studies have suggested that miRNAs play a regulatory role in the formation of plant secondary metabolites (Bulgakov and Avramenko 2015; Singh et al. 2016a, b; Biswas et al. 2016), For instance, in *Ferula gummosa* (a well-known resource of galbanum, an aromatic gum resin),

Table 10.3 List of miRNAs and their targets involved in biosynthesis of metabolites

Sl. No.	Plant species	miRNA	Targets	Biosynthetic pathways	Reference
1	*Ferula gummosa*	miR2919, miR5251, miR838, miR5021, miR5658	–	Terpene biosynthesis	Najafabadi and Naghavi (2018)
2	*Xanthium strumarium*	miR7539, miR5021, miR1134	–	Terpenoid biosynthesis	Fan et al. (2015)
3	*Zingiber officinale*	miR1873	Phenylalanine ammonia lyase (PAL) enzyme	Gingerol and flavonoid biosynthesis	Singh et al. (2016a)
		miR838	Methofuran synthase (CYP71)	Terpenoid metabolism	
4	*Arabidopsis*	miR858		Flavonoid biosynthesis	Sharma et al. (2016)
5	*Osmanthus fragrans*	miR858	MYB genes	Flavonoid biosynthesis	Shi et al. (2021)
6	*Rauwolfia serpentina*	rse-miR828a	C1 protein	Flavonoid biosynthesis	Prakash et al. (2016)
		miR396b	Kaempferol 3-*O*-beta-D-galactosyltransferase enzyme		
7	*Podophyllum hexandrum*	miR172i and miR829.1	4-Coumarate–CoA ligase and chalcone synthase	Flavonoid biosynthesis	Biswas et al. (2016)
		miR1438	Caffeoyl-CoA *O*-methyl transferase	Lignin biosynthesis	
		miR5532	2-Hydroxyisoflavanone dehydratase	Isoflavonoid synthesis	
8	*Mentha*	miR414	*Terpene synthase 21* (TPS21)	Sesquiterpenoid and triterpenoid biosynthesis	Singh et al. (2016b)

transcriptome analysis facilitated identification of miRNAs and their targets. miR2919, miR5251, miR838, miR5021, and miR5658 found to be involved in terpene biosynthesis pathway (Najafabadi and Naghavi 2018). Similarly, miR7539, miR5021, and miR1134 target the upstream genes of terpenoid biosynthetic pathway and regulate the terpenoid biosynthesis in case of *Xanthium strumarium* (Fan et al. 2015). Transcriptomics studies in *Zingiber officinale* find out that miR1873 targets the gene encoding phenylalanine ammonia lyase (PAL) enzyme and controls the catalysis synthesis of ammonia and *trans*-cinnamic acid from L-phenylalanine step and ultimately regulates the biosynthesis of gingerol and flavonoids (Singh et al. 2016a). Further, miRNA858 also plays important role in flavonoid biosynthesis in *Arabidopsis* (Sharma et al. 2016) and *Osmanthus fragrans* (Shi et al. 2021) by targeting MYB genes (key gene of flavonoid biosynthesis).

In *Rauwolfia serpentina* (important endangered, pharmaceutical plant), 15 conserved potential miRNAs were identified, and out of which, rse-miR828a found to regulate the expression of anthocyanin biosynthesis-related genes by targeting C1 protein and miR396b targeted Kaempferol 3-*O*-beta-D-galactosyltransferase enzyme, which is involved in flavonol glycoside biosynthesis (Prakash et al. 2016). Similarly, in case of *Podophyllum hexandrum*, micro RNAs play multifarious role and regulate different biosynthetic pathways (Biswas et al. 2016). For instance, miR172i and miR829.1 regulate flavonoid biosynthesis by targeting 4-coumarate–CoA ligase and chalcone synthase, respectively; however, miR1438 is reported to regulate lignin biosynthesis by targeting Caffeoyl-CoA *O*-methyl transferase, and miR5532 regulates isoflavonoid synthesis by targeting 2-hydroxyisoflavanone dehydratase (Biswas et al. 2016). miR414 has role in regulation of sesquiterpenoid and triterpenoid biosynthesis by targeting *terpene synthase 21* (TPS21) that catalyzes reaction for terpene synthesis in *Mentha* (Singh et al. 2016b). Similarly, miR838 found to be involved in terpenoid metabolism in ginger by targeting CYP71 (which involved in methofuran synthase).

Apart from direct involvement, miRNA also found to play important role in combating abiotic stresses (Wang et al. 2021), which ultimately affect the phytochemical biosynthesis in terms either quality or yield or both. miRNA156, miR157d, and miRNA160 found to target key drought stress response genes like auxin response factor, cytokinin receptor, two-component response regulator, and DELLA in *Dendrobium houshanense* (Wang et al. 2021).

6 Important Genes Involved in the Biosynthesis of Bioactive Compounds

The availability of NGS technologies allows for large-scale investigation of transcriptomes of medicinal plants with the aim of identifying essential genes involved in the production of bioactive chemicals. With the use of NGS transcriptome analysis, a number of putative *CYP450* and glycosyltransferase

genes involved in the production of ginsenosides were discovered in three different *Panax* species: American ginseng (Jo et al. 2015; Qi et al. 2015; Sun et al. 2010), *Panax ginseng* (Gao et al. 2016), and *Panax notoginseng* (Luo et al. 2011). EST analysis identified putative glycyrrhizin biosynthetic genes in *Glycyrrhiza uralensis*. Three and six unigenes encoding *cytochrome P450s* and *glycosyltransferases*, respectively, were found to be potential candidates for glycyrrhizin production based on EST analysis and additional organ specific expression pattern analyses (Li et al. 2010). The components of alkaloid metabolism (benzyl isoquinoline alkaloids) in opium poppy cell cultures, as well as the genes involved in their biosynthesis, are revealed by deep transcriptome and proteome analyses (Desgagné-Penix et al. 2010). The main constituent of the essential oil of the medicinal plant *Trachyspermum ammi* is thymol. The gene family members *CYP450* (cytochrome P450s), TPs (terpene synthases), DHs (dehydrogenases), and TF (transcription factors) are involved in the thymol synthesis pathway and were retrieved through comparative transcriptomics investigations in *T. ammi* (Howyzeh et al. 2018). *Tripterygium wilfordii* is a plant that is used to diagnose a variety of inflammatory and autoimmune disorders. Eight potential (di)terpene synthases were identified and described based on transcriptome data generated from suspension cell cultures for their likely function in triptolide synthesis (Su et al. 2018). In case of *Amomum villosum*, transcriptome identified terpene synthase (TPS) genes, *AvTPS1* (AvPS: pinene synthase) is involved in the synthesis of α-pinene and β-pinene from GPP, and *AvTPS3* (AvBPPS: bornyl diphosphate synthase) is involved in the synthesis of bornyl diphosphate (Wang et al. 2018). Similarly, more than 20 genes were found to be involved in flavonoid biosynthesis and its regulation in *Ziziphus jujuba* leaf transcriptome data (Li et al. 2021). In the leaves of *Perilla frutescens*, 77 unigenes were found to encode 15 enzymes and involved in flavonoid biosynthesis, with high expression of the CHS gene enhancing flavonoid accumulation (Jiang et al. 2020). *Scutellaria viscidula* has roughly 177 genes involved in flavonoid production, including 23 enzyme producing genes (Bai et al. 2018). In case of *Gingko biloba*, SMRT sequencing identified 12 gene modules for flavonoid metabolism (Ye et al. 2019). In *Hedera helix*, a de novo leaf and root transcriptome analysis was performed to discover potential genes involved in the manufacture of triterpenoid saponins. In this investigation, 269 and 197 unigenes from the *CYP450* and GT families, respectively, were discovered (Sun et al. 2017). *Platycodon grandiflorum* is a perennial plant in the *Campanulaceae* family that is utilized as a medicinal herb for its ability to clear heat from the lungs, as well as its antitussive and expectorant effects. The transcriptome sequencing of *Platycodon grandiflorum* revealed around 21 candidate cytochrome *P450* genes and 17 candidate UDP-glycosyltransferase genes involved in triterpenoid saponin production, which aids our understanding of the biosynthesis of triterpenoid saponins at the molecular level (Ma et al. 2016). Transcriptome studies of rhizome of *Paris polyphylla* var. *yunnanensis* were used to identify putative genes for *Paris saponin* production (Gao et al. 2020). *Coptis deltoidea* contains a high concentration of benzylisoquinoline alkaloids (BIAs), which are very effective medicinally. A full-length transcriptome analysis was used to find putative genes involved in the biosynthesis of benzylisoquinoline

alkaloids and identified four STOX (*S*)-tetrahydroprotoberberine oxidase genes including *CdSTOX1*, *CdSTOX2*, *CdSTOX3*, *and CdSTOX4*, and three bHLH1 transcription factors involved in biosynthesis of benzylisoquinoline alkaloids (Zhong et al. 2020).

7 Metabolomics and Transcriptomics

Medicinal plants have a wide range of specialized metabolites with important pharmacological characteristics. Around 1,000,000 metabolites could be expected in plant kingdom, according to report (Afendi et al. 2012). Majority of the metabolites obtained from medicinal plants are extensively exploited in pharmaceutical companies. Pharmaceutical chemicals such as taxol, vincristine, and morphine are produced by medicinal plants. Because the world's population is growing, it's more crucial than ever to understand the whole biosynthesis of specialized metabolites in order to find or develop reliable sources in the future. The expression of transcriptomes from various traditional medicinal herbs was used to get species-specific understanding of plant metabolism. Chinese herbalists have employed *Artemisia annua* (sweet wormwood or Qing Hao) as a cure for over 2000 years (Maude et al. 2010). *Astragalus membranaceus* Bge. var. mongolicus improves people's health and energy levels. Metabolomics and transcriptomics approaches discovered 5435 metabolites, of which 2190 were annotated. This approach has considerable promise for uncovering novel metabolite structure and associated production pathways (Wu et al. 2020). Rutin (flavonoid) is one of the most important bioactive compounds in *Syringa oblata* that obstructs *Streptococcus suis* biofilm formation, an effective anti-biofilm medicinal plant. Transcriptomics investigated and identified important genes involved in the rutin formation in *S. oblata* in response to various light intensities (Liu et al. 2019). *Ziziphus jujuba* leaf are widely known for its therapeutic significance, of which around 778 metabolites are well investigated and characterized (Li et al. 2021). Similarly, *Perilla frutescens* (L.) is an important plant with nutritional and medical value. Application of transcriptomics enabled the identification of 9277 differentially expressed genes, and global metabolite profiling identified 223 flavonoid metabolites; these analyses added the valuable information on the flavonoid metabolism in *Perilla frutescens* (Jiang et al. 2020).

8 Conclusion and Future Perspectives

The use of high-throughput sequencing (454, Illumina, SOLiD, Helicos and Pacific BioSciences) in medicinal plant transcriptome investigations is only getting started. Many more important medicinal plants, particularly those in short supply or on verge of extinction, must be sequenced at the transcriptome level for biodiversity

conservation and long-term use. Currently, most medicinal plant research does not rely solely on RNA-Seq technology. In the advancement of transcriptomic technology, multi-omics that incorporate developing metabolomics and proteomics technologies will be critical. Transcriptomics and multi-omics will help to modernize medicinal plant research in the future. Hopefully, many more medicinal plants will be utilized to assess the transcriptome, and the results will be useful for future medication research and development.

Acknowledgments This study was supported by the Council of Scientific and Industrial Research (CSIR) for providing funds (MLP-201), V.J. also thanks to the Science and Engineering Research Board (SERB) for the Early Career Research Award and Department of Science and Technology (DST) for the INSPIRE faculty award. V.C. and H.G. thank to CSIR for Junior Research Fellowship. CSIR-IHBT communication number is 5024.

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Chapter 11
Metabolomics of Important Medicinal Plants

Jyotsna Baby, Toji Thomas, and T. Dennis Thomas

1 Introduction

According to Food and Agricultural Organization, more than 50,000 plant species have medicinal values across the globe (Schippmann et al. 2002). The biological diversity of medicinal plants and their vivid chemical capabilities have been used by man for the treatment of various diseases. This brought forth to traditional medicinal practices such as Ayurveda, Unani, Chinese, Middle Eastern, and African systems of medicine (Mamedov 2012). The conventional detection methods of plant metabolites mainly relied on phytochemical screening tests followed by simple techniques of separation such as thin layer chromatography (TLC). Isolation of metabolites by these methods is often tedious and not much efficient. Also the poor and low selectivity of detection makes hard the detection of trace amount of metabolites in the sample (Srivastava et al. 2014). A thorough understanding of the entire metabolites of plants became inevitable for discovering novel metabolites, along with their concerned biosynthetic genes. Thus, metabolomics, an ongoing extension of "omics", has emerged as a powerful tool (Sumner 2010).

Currently, there is an increased attraction toward herbal remedies, as the green medicine is assumed to be safe and eco-friendly. Rising incidences of harmful side effects from using synthetic drugs also persuade human beings to utilize natural products for various ailments (Chanda 2014). Along with this, there is an increased attention toward natural products; novel approaches are also developed to make a clear understanding of the mode of action of these green remedies, as demonstrated by the integrated "omics" approaches, which provides a holistic approach in drug research (Gonulalan et al. 2020).

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M. K. Swamy, A. Kumar (eds.), *Phytochemical Genomics*,
https://doi.org/10.1007/978-981-19-5779-6_11

A large number of phytochemicals with pharmacological value have been isolated and play predictable therapeutic roles in the clinical world (Gonulalan et al. 2020). The therapeutic effects exhibited by many of the medicinal plants are not always due to a single major compound, but in many cases due to synergistic or antagonistic activity of various compounds. If active compounds are present in very minute quantities in plants, it can affect its chemical characterization, and the process is very challenging also (Williamson 2001). In order to address this task, metabolomics can contribute an important role; it provides insight into the metabolome of plants. Metabolomics is observed as extension of the ongoing high-throughput technologies to the comprehensive analysis of small molecule metabolites of biological system (Sumner 2010). Besides, metabolomics confers several benefits as compared to other "omics;" this is because the biochemical phenotypes are not always represented by transcriptomic or proteomic approaches (Fernie and Stitt 2012). Metabolomics aims to measure the metabolites in the sample and provides information about the interconnection of metabolic pathways and the phenotype of the biological system (Johnson et al. 2016).

Metabolomics is also employed for obtaining valuable data to aid in discovering novel genes and the concerned pathways. The advancements in the sequencing technologies and metabolome-based genome-wide association study (mGWAS) are effectively utilized to unveil the genetic mechanisms behind diverse metabolome and how they are associated to the complex traits seen in plants (Hong et al. 2016). Wen et al. (2014) conducted mGWAS study in maize kernels, through which they identified 1459 locus-trait associations among three environments. Resequencing and analysis of association among candidate genes led to the identification of causal variants of five genes concerned with metabolic traits. Likewise, by combining metabolomics and transcriptomics, all genes of seco-iridoid pathway in *Catharanthus roseus* have been identified (Miettinen et al. 2014; Salim et al. 2014).

As per the estimates of the World Health Organization (WHO), about 80% of world's population use traditional medicines at a certain period of their lifetime (Chintamunnee and Mahomoodally 2012). Traditional herbal medicines are widely distributed across the globe and have been used for centuries without any rigorous rules (Efferth and Greten 2012). Together with this, there are chances for adulteration as products of medicinal plants are marketed and distributed (Perini et al. 2018). The bioactive compounds responsible for the therapeutic properties also need to be identified in order to do semi-synthesis and further development of novel new drug candidates from these lead compounds (Plazas et al. 2019).

In this context, metabolomics is a promising tool that can be effectively used to mine various bioactive metabolites from different medicinal plants (RaoGajula and Nanjappan 2021). In this chapter, we present how metabolomics approach can effectively be employed for medicinal plants. The chapter also discusses various strategies, technical advancements, data processing methods, and databases. In addition to this, various applications of metabolomics in connection with medicinal plants are also addressed.

2 Strategies Employed in Plant Metabolomics

Before the advent of "omics" era, investigation and extraction of bioactive metabolites from plants mainly relied on conventional extraction techniques like Soxhlet extraction, hydrodistillation, and maceration. The efficiency of these methods mainly depends on the solvents selected, polarity of selected compound, etc. (Azmir et al. 2013). Also, traditional methods of separation and bioassay-guided fractionation are too laborious as well as expensive. Hence, they are not cost-effective in an industrial approach for drug development (Yuliana et al. 2013). Revolutionizing changes have been made by the metabolomics technologies, which aim for targeted and global profiling of metabolites of the sample. The metabolomic profiling of medicinal plants is becoming crucial and aids in the development of novel phytotherapeutics (Shyur and Yang 2008). As herbal formulations are multi-compound medicines, metabolomic approach is vital for screening of multi-compounds. Hence, metabolomic approaches are gaining an upper hand in medicinal plant research (Lee et al. 2017). In addition to this, the technical advancements in mass spectrometry (MS) and nuclear magnetic resonance (NMR) make possible the estimation of a wide array of compounds and comparing the support data of novel compounds with natural products library (RaoGajula and Nanjappan 2021). The entire process of metabolomic analysis to derive meaningful data from the metabolome of medicinal plants is outlined in Fig. 11.1. Some of the major strategies employed in metabolomics are briefly discussed below.

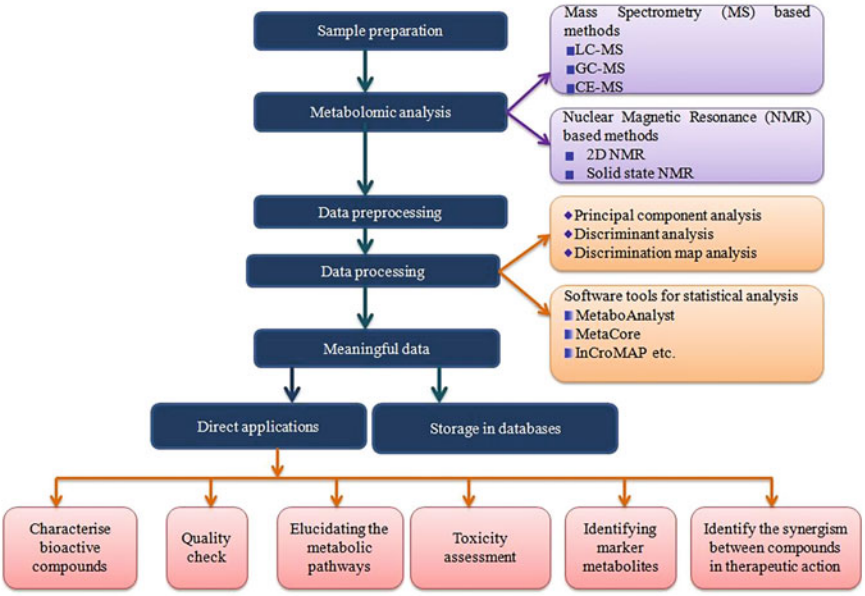


Fig. 11.1 The overall process of metabolomic analysis to mine useful data from medicinal plants

2.1 Metabolite Profiling

This involves the identification and quantification of a large group of compounds, sharing similar chemistry or related by metabolic pathways (Shafi and Zahoor 2021). Metabolic profiling includes nontargeted and targeted approaches.

2.1.1 Nontargeted Approach

The nontargeted/untargeted approach involves simultaneous measurement of a possible large number of metabolites in the sample (Shah et al. 2012; Schrimpe-Rutledge et al. 2016). This approach is appropriate for biomarker discovery and is employed for generating hypothesis. It is also called undirected or unbiased metabolomics (Wang et al. 2010). However, it cannot be regarded as truly unbiased because the researcher has to choose a combination of ionization mode and stationary phase, which in turn facilitates the detection of certain compounds and lowers the detection of some others (Ribbenstedt et al. 2018). Together with, it faces certain other challenges such as complicated protocols, longtime consumption to handle huge amount of raw data, problems of identification and characterization of unknown metabolites, reliability of the platform chosen, and greater chances of detecting more abundant metabolites (Roberts et al. 2012).

2.1.2 Targeted Approach

Metabolic profiling of known metabolites with distinct identities (already defined compounds) is done for the targeted approach (Shah et al. 2012). Also called directed or biased metabolomics, it mainly focuses on metabolites of a particular chemical class or any other predetermined group of compounds. This approach is hypothesis-driven and aims to verify biological pathways or authenticate an untargeted metabolomic study (Wang et al. 2010). Here, analysis can be carried out either in a quantitative or semiquantitative manner. Some advantages of targeted analyses like the downstream analysis are almost set free from analytical artifacts, and novel metabolic associations can be deciphered at particular physiological states (Roberts et al. 2012). Major metabolomic studies in medicinal plants, which employed targeted/untargeted approaches, are given in Table 11.1.

2.2 Metabolite Fingerprinting

This involves an untargeted approach, in which extensive identification and quantification of individual metabolites are not done. The data obtained were analyzed to recognize patterns specific to the fingerprint of metabolites at a given biological

Table 11.1 List of metabolomic studies of medicinal plants via targeted/untargeted approaches (from 2010 onward)

Sl. No.	Plant name	Metabolomic approach (targeted/ untargeted)	Major metabolites (identified/ discovered)/major findings	Reference
1	*Acanthopanax senticosus* (Rupr. et Maxim.) Harms	Targeted metabolomics	Compounds of C_6C_1 type greater in 9-year-old, $C_6C_3C_6$ type greater in 5-year-old and C_6C_3 type greater in 3-year-old plants	Xu et al. (2020)
2	*Aconitum pendulum* N. Busch	Targeted metabolomics	80 metabolites identified and 19 compounds were selected as biomarkers for the collected samples	Wang et al. (2022)
3	*Bryophyllum* spp.	Untargeted metabolomics	Phenolic compounds with therapeutic activity	García-Pérez et al. (2021)
4	*Centella asiatica* (L.) Urban	Targeted metabolomics	Confirmed the effect of methyl jasmonate in inducing the production of madecassic acid, madecassoside, asiaticoside and asiatic acid	James et al. (2013)
5	*Chrysanthemum morifolium* Ramat.	Targeted metabolomics	661 metabolites were identified among which 46 different metabolites are found simultaneously during different growth stages with flooding stress	Wang et al. (2019a)
6	*Cistanche salsa* (C. A. Mey.) G. Beck, *C. sinensis* G. Beck, *C. tubulosa* (Schenk) R. Wight, and *C. deserticola* Y.C. Ma	^{1}H NMR-based nontargeted to LC–MS-based targeted metabolomics	8-*epi*-loganic acid, acteoside, echinacoside, betaine, sucrose, mannitol, and 6-deoxycatalpol are identified as markers for discrimination among these four species	Liu et al. (2019)
7	*Citrus sinensis* L.	Untargeted metabolomics	Leaves have more flavonoid, condensed tannin, and phenol content, whereas flavedo has more carbohydrates	Lamine and Mliki (2021)
8	*Clausena lansium* (Lour.)	Untargeted metabolomics	364 metabolites identified, and 64 potential biomarkers were selected	Fan et al. (2020)
9	*Cuminum cyminum* L.	Untargeted metabolomics	45 metabolites with differential expression including quercetin, luteolin, kaempferol, and salvianolic acid	Pandey et al. (2015)
10	*Curcuma elata* Roxb., *C. aromatica* Salisb., *C. longa* L., *C. phaeocaulis* Val. and *C. caesia* Roxb.	Targeted metabolomics	*C. longa* has the highest quantity of curcuminoids, but some of the bioactive compounds like 6-gingerol was found the lowest in *C. longa*	Ye et al. (2022)

(continued)

Table 11.1 (continued)

Sl. No.	Plant name	Metabolomic approach (targeted/ untargeted)	Major metabolites (identified/ discovered)/major findings	Reference
11	*Ephedra sinica* Stapf.	Untargeted metabolomics	Identified that 22 chemical markers differ between stem and root	Lv et al. (2015)
12	*Eucommia ulmoides* Oliver	Untargeted metabolomics	2373 metabolites were identified, and 116 metabolites discovered	Chen et al. (2022)
13	*Fritillaria* spp.	Untargeted metabolomics	21 species specific markers were identified	Liu et al. (2020)
14	*Gleditsia sinensis* Lam.	Targeted metabolomics	728 metabolites identified from epidermis, xylem, and pith of thorn	Ya et al. (2022)
15	*Grammatophyllum speciosum* Blume	Untargeted metabolomics	721 metabolites were identified with vitexin and orientin being the most abundant	Yingchutrakul et al. (2021)
16	*Ligusticum canbyi* (J.M. Coult & Rose)	Targeted and untargeted metabolomics	Detected 34,000 compounds with 70 phthalide metabolites. Ferulic acid was found responsible for antioxidant activity	Turi and Murch (2013)
17	*Maytenus aquifolium* Mart. and *Maytenus ilicifolia* Mart. ex Reiss	Untargeted metabolomics	Differentiated their chemical composition and analyzed the effect of environment in metabolome	Antunes et al. (2020)
18	*Mikania glomerata* Spreng. and *M. laevigata* Sch.Bip. ex Baker	Untargeted metabolomics	Identified that coumarin is present only in *M. laevigata* and volatile compounds like pinenes are more abundant in plants during drought	Ueno and Sawaya (2019)
19	*Persea americana* Mill.	Targeted metabolomics	Quantified 8 acetogenins in peel, pulp, and seeds of the fruit	Rodríguez-López et al. (2015)
20	*Phyllanthus amarus* Schum. & Thonn., *P. acidus* L., *P. emblica* L., *P. urinaria* L., *P. debilis* Linn., *P. virgatus* G. Forst., *P. reticulates* Poir., *P. myrtifolius* (Wight) Mull. Arg. and *P. lawii* J.Graham	Untargeted metabolomics	Identified the differential expression of 14 metabolites from nine *Phyllanthus* spp.	Kiran et al. (2021)
21	*Polygonum multiflorum* Thunb.	Untargeted and targeted metabolomics	Revealed the appropriate processing of its radix for medicinal use	Liang et al. (2018)

(continued)

Table 11.1 (continued)

Sl. No.	Plant name	Metabolomic approach (targeted/ untargeted)	Major metabolites (identified/ discovered)/major findings	Reference
22	*Rehmannia glutinosa* Libosch.	Targeted metabolomics	Detected 228 metabolites from roots of cultivated and wild variety out of which 170 metabolites were unchanged and 58 were differential metabolites	Zhou et al. (2021)
23	*Rosmarinus officinalis* L.	Untargeted metabolomics	Rosmarinic acid, rosmaridiphenol, carnosol, carnosic acid, quercitin, luteolin, etc.	Salem et al. (2020b)
24	*Salsola collina* Pall	Targeted metabolomics	637 metabolites were identified	Li et al. (2021)
25	*Salvia miltiorrhiza* Bunge	Untargeted metabolomics	Identified 23 specific metabolites out of which 11 metabolites changed during "sweating" process	Cao et al. (2020)
26	*Suaeda salsa* (L.) Pall.	Targeted metabolomics	Identified 521 metabolites out of which 165 are differential metabolites of different leaf phenotypes	Wang et al. (2019b)

state, for example, stress response, etc., provided in a living system (Wolfender et al. 2015; Shafi and Zahoor 2021). It mainly finds applications in comparing fingerprints or patterns of metabolites that vary with response to a particular disease condition, environmental changes, etc. (Barderas et al. 2011). The major platforms employed here include NMR spectroscopy, Fourier transform infrared spectroscopy (FTIR), and Raman spectroscopy (Ellis et al. 2007). Gray and Heath (2005) examined cold acclimation effects on the metabolome of *Arabidopsis* using metabolite fingerprinting.

2.3 Metabolite Footprinting

This approach comprises the profiling of extracellular metabolites alone (Pope et al. 2007). It mainly focuses on the biochemical and chemical changes brought about by organisms due to the effect of its immediate environment. Every living cell modifies its medium by the secretion of various enzymes and other metabolites; later, a subsequent interaction of the secreted components with the constituents present in the medium happens. This will generate metabolic profiles that are specific to particular species and/or their genetic makeup (Villas-Bôas et al. 2006). As it

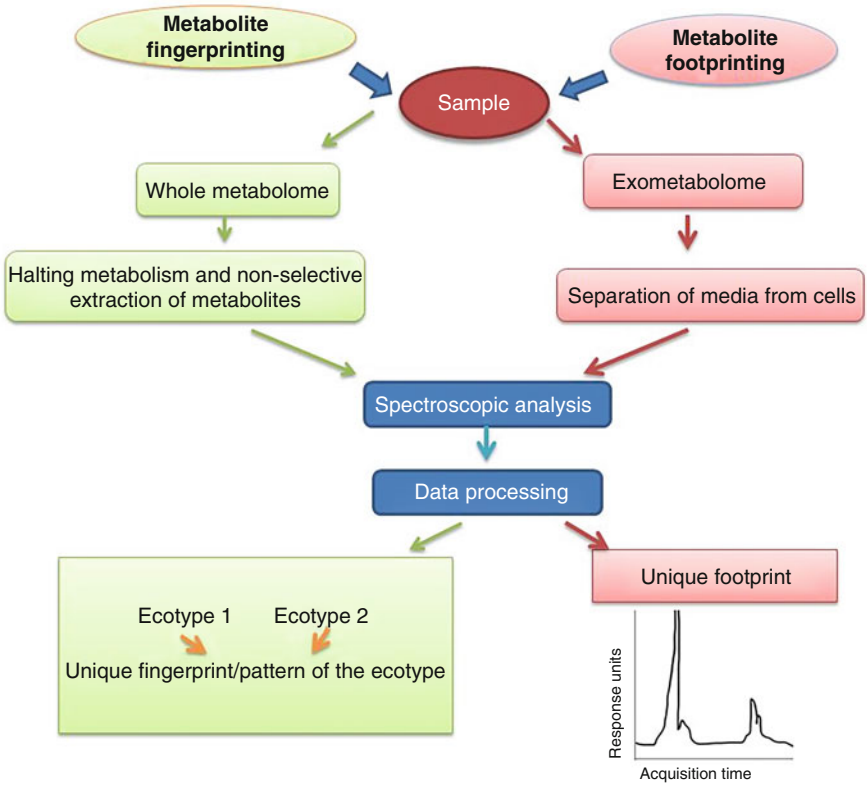


Fig. 11.2 Workflow depicting the procedure and differences of metabolite fingerprinting and footprinting

involves secreted metabolites, sampling is relatively simple in which it only requires the procedure of separating the media from the cell/tissue. Further analysis was done by means of NMR (Filloux and Ramos 2014). The procedure and differences in metabolite footprinting and fingerprinting are outlined in Fig. 11.2.

2.4 Metabonomics

Metabonomics implies extensive evaluation of metabolic changes in a living system. It does quantitative measurement of the metabolic variations of a biological system in response to genetic modification or pathological/physiological stimuli (Bjerrum 2015).

2.5 Chemoprofiling

The chemical constitution of plants needs to be understood to ensure sufficient therapeutic evaluation (Efferth and Greten 2012). Chemoprofiling includes pattern-oriented/multicompound approach and compound-oriented/marker approach. Pattern-oriented approach focuses on all detectable compounds in a plant extract that forms a unique fingerprint, and it does not characterize all the compounds in a plant extract. In the case of compound-oriented approach, some of the bioactive or major constituents are considered for obtaining specific chemoprofiles (Govindaraghavan et al. 2012).

2.6 Metabolic Fluxomics

Rates of metabolic reactions (fluxes) provide information about metabolic phenotypes and cellular regulation mechanisms. The direct measurement of metabolic fluxes is not possible, and it needs to be obtained by measuring other observables like changes in concentration of enzymes, metabolites, carbon balance, etc. (Niedenführ et al. 2015). Flux analysis by means of ^{13}C fluxomics (using ^{13}C, ^{14}C, ^{2}H, ^{15}N isotopic traces) has become a method of choice to decipher the regulation and constitution of metabolic networks (Zamboni 2011; Niedenführ et al. 2015).

3 Technological Advancements in Plant Metabolomics

3.1 Mass Spectrometry Imaging (MSI)

MSI works by ionizing the peptides/protein or metabolites from the biological sample in a two-dimensional or three-dimensional coordinate (Fletcher et al. 2008; Seeley and Caprioli 2012).

3.1.1 Gas Chromatography-Mass Spectrometry (GC-MS)

GC-MS is routinely used for the analysis of organic compounds that are volatile or can be derivatized to make them volatile (Hall 2006). Separation of compounds happens due to the difference in partition coefficients between stationary (solid) phase and mobile (gas) phase (Smedsgaard 2007; Roessner and Beckles 2009). By employing electron impact ionization (EI), analytes are ionized; it results in a unique fragmentation pattern for every constituent phytochemical. Identification of compounds can be made by comparing the GC-MS fragmentation patterns and retention time, along with the available information in the databases of GC-MS (Kopka et al. 2004). GC-MS method gains popularity because it helps in the determination of

amino acids, sugars, and organic acids. One of the limitations of GC-MS is that the overlapping peaks of GC-MS chromatogram render the detection of individual metabolite signals much more difficult (Saito and Matsuda 2010).

3.1.2 Liquid Chromatography-Mass Spectrometry (LC-MS)

LC-MS forms a valuable tool to unveil the immense wealth of phytochemicals including both primary and secondary metabolites. As opposed to GC-MS, LC-MS can handle a wide range of compounds with different chemical properties, for example, they are either volatile or not (Hill and Roessner 2015). In addition to this, partially purified crude extracts can be directly fed into the LC/MS system. This eliminates various steps of sample treatment. The introduction of ultra performance liquid chromatography (UPLC) together with high-resolution Fourier transform (FT) MS, time-of-flight (TOF) MS, and Orbitrap-based MS made beneficial advancements in LC-MS-based metabolomics (Salem et al. 2020a).

3.1.3 Capillary Electrophoresis-Mass Spectrometry (CE-MS)

Capillary electrophoresis employs high voltage for the electrophoretic separation of different ions in a narrow-bore capillary (Ren et al. 2018). The initial separation of metabolites takes place according to their charge-to-size ratio followed by mass-to-charge ratio-based separation (Zhao et al. 2012; Klepárník 2015). Even though CE-MS is considered as a novel method in metabolomics, it suffers from drawbacks of having poor stability, and there are chances for capillary blockage by salt (Ren et al. 2018). However, it performs the ability to separate low volume of biological fluids, simple sample preparation protocol, capability of concentrating analytes and better separation efficiency, making it as a method of choice (Klepárník 2015; Ren et al. 2018).

3.2 Nuclear Magnetic Resonance (NMR) Spectroscopy

NMR spectroscopy is a chief tool for studying plant metabolomics. It gains an upper hand over other techniques by facilitating high-throughput analysis, easy sample preparation, rapid performance, and easy quantitation (Kim et al. 2011). NMR makes possible the analysis of profuse primary metabolites and heterogeneous secondary metabolites such as alkaloids, flavonoids, terpenoids, etc. As the signals in the NMR spectrum are indicative of the molar concentrations of the component compounds, concentrations of these compounds can be compared with other samples, and it eliminates the need for calibration curves for each compound (Kim et al. 2010). NMR also suffers from certain disadvantages. Its low sensitivity demands larger amount of sample than other methods. Also, there is considerable overlapping

of signals in the NMR spectra impeding accurate signal identification and peak integration (Kim et al. 2010; Halabalaki et al. 2014).

3.2.1 2D NMR

Signal overlap is a real problem in 1D NMR, hindering both the identification and quantification of compounds in complex plant extracts. Hence, 2D NMR spectroscopy can be employed, which provides good signal resolution by distributing the resonance in a second axis. One limitation of this method is the lengthy acquisition time as compared to 1D NMR (Kim et al. 2011). *J*-resolved spectroscopy and heteronuclear single quantum coherence (HSQC) are the major useful 2D methods (Ludwig and Viant 2010; Salem et al. 2020a). HSQC is particularly employed as a confirmation tool to detect whether the usual suspected compounds (common primary and secondary plant metabolites) are present or not and for the quantification of metabolites. ^{1}H-^{1}H COSY (correlation spectroscopy), NOESY (nuclear overhauser effect spectroscopy), HMBC (heteronuclear multiple bond correlation), etc. are few important 2D NMR-based methods (Kim et al. 2011; Salem et al. 2020a).

3.2.2 Solid-State NMR

Solid-state NMR allows the examination of semisolid samples such as tissue samples via the use of high-resolution magic-angle spinning (HRMAS). This technique involves mixing of the sample with a minimal solvent volume followed by rotating the sample at 54.74° with high spinning rates (Pérez et al. 2010; Kruk et al. 2017). Better resolution can be achieved by HRMAS. Nevertheless, the faster spinning and high temperature may cause tissue distortion. Also, the quantification of metabolites poses a challenge in HRMAS, which can be overcome by a method called ERETIC (Electronic REference To access In vivo Concentration) (Kruk et al. 2017).

4 Data Processing Methods

Large amount of data is generated in metabolomics analysis through the analytical platforms such as NMR and MS (Liland 2011). Meaningful data can be mined from this complex data set through multivariate analysis (MVA) methods like discriminant analysis, discrimination map analysis, and principal component analysis (PCA). These methods are mainly focused on the reduction of data such as *m*/*z* values from MS analysis, chromatography data, NMR data, etc. The reduced data are presented as discrimination maps, score pots, load plots, etc. according to the MVA method employed. The raw data from the analysis are mathematically and statistically processed, and that is represented in the form of vectors in score plot. These vectors

correspond to the metabolite fingerprint of the tested sample in the case of score plot, whereas in the loading plot, each signal's contribution is represented. The discrimination map, in addition to visualizing the contribution of each signal, also represents the variation in signals. Thus, the marker metabolites can be selected by statistical means by connecting the loaded factor and the data from chemical analysis (Okada et al. 2010).

Another strategy to handle this enormous metabolomic data involves the utilization of software tools. These software packages process the raw data of spectra and also perform statistical analysis in order to find out the metabolites, which are significantly expressed in the sample; later, the metabolites are compared with metabolite databases, and subsequently, multiple "omics" data is integrated and analyzed; finally, it helps to visualize molecular interactions also (Krastanov 2010; Sugimoto et al. 2012). MetaboAnalyst, MetaCore™, and InCroMAP are some of the versatile software tools used for this purpose (Cambiaghi et al. 2016). Among these, only MetaboAnalyst is a comprehensive tool that can perform both the pre-processing of data and its statistical analysis (Xia et al. 2015). MetaCore™ is an integrated database, which makes possible the visualization and functional analyses of various kinds of omics data together with options for biological pathway analyses. This versatile tool also finds applications in biomarker identification to drug discovery process (Cambiaghi et al. 2016). InCroMAP is user-friendly software, which can generate global maps of metabolic processes in cell from the metabolic analysis (Wrzodek 2012; Wrzodek et al. 2012). It suffers a drawback, as it can't perform data pre-processing (Cambiaghi et al. 2016).

5 Databases for Handling Metabolomics Data

The field of plant metabolomics is advancing rapidly, the ultimate aim of which is a holistic understanding of various functions and healing potential of medicinal plants (Afendi et al. 2012). Therefore, to achieve profile management, metabolite identification, effective data mining, and efficient platforms are required (Ferry-Dumazet et al. 2011). For making the metabolomics data meaningful, it has to be organized in a standard form that allows cross-referencing with other datasets. Several specialized metabolomics databases for plants are available, and these databases provide updated and comprehensive information (Shafi and Zahoor 2021). In order to make metabolomics as a valuable tool for functional genomics, it requires the availability of annotated metabolomics data, and it can be accessed through internet. Visualization tools (such as error and ratio plots), if integrated into the databases, would help in the comparison of metabolome at various conditions such as environmental changes, genetic disturbances, and variation in experimental parameters to which the biological system is subjected and would find the effective analytical platform that can effectively communicate the maximum metabolomic changes according to these changing conditions (Bais et al. 2010). The different databases, which handle metabolomics data of medicinal plants, are indicated in Table 11.2

Table 11.2 List of metabolomics databases of medicinal plants (from 2010 onward)

Sl. No.	Database	Description	References
1	CathaCyc	It contains metabolic pathway database of *Catharanthus roseus* containing its RNA-seq data and metabolomics data	Van Moerkercke et al. (2013)
2	HerbalDB 2.0	The updated version of HerbalDB, Indonesian medicinal plant database. It contains 3D structure of 1405 herbal compounds from Indonesian plants to aid in in silico drug design	Syahdi et al. (2019)
3	IMPPAT (Indian Medicinal Plants, Phytochemistry and Therapeutics)	It consists of 1742 (Indian) medicinal plants, 1124 therapeutic uses and 9599 phytochemicals. It includes a library with 9596 phytochemicals	Mohanraj et al. (2018)
4	InDiaMed (Indian Medicinal Plants for Diabetes)	It is a database for the information of medicinal plants in India with antidiabetic activity. Also lists antidiabetic poly herbal formulations	Tota et al. (2013)
5	KNApSAcK family databases	This database describes the connection between the species and the corresponding metabolites they encode. The relation of medicinal plants with their geographical zones is documented along with their activities and their herbal formulations	Afendi et al. (2012)
6	MassBank	First public database containing mass spectral data of small molecules for life sciences (<3000 Da)	Horai et al. (2010)
7	Medicinal Plant Metabolomic Resource (MPMR)	Comprised of detailed RNA-seq and associated metabolomics data from 14 medicinal plants	Wurtele et al. (2012)
8	MED-PDB	Contains details of 147 plants, 53 botanical families and subfamilies, 435 disease types, and 369 active compound types	Sargia et al. (2018)
9	MeRy-B	It consists of ^{1}H NMR metabolic profiles and includes the data from description of the plant to metabolite identification and determining its concentration	Ferry-Dumazet et al. (2011)
10	MetaboLights	It aims at developing cross-species and cross-platform research in metabolomics. It provides both experimental and raw data from metabolomics experiments	Kale et al. (2016)
11	MPD3 (Medicinal Plants Database for Drug Designing)	It provides database merging activities and is a comprehensive database that aids in computer aided drug design (CADD) by providing information about phytochemical bioactivities, targets of phytochemicals and their literature references. Over 5000 phytochemicals reported from ~1000 plants are included with targets over 200 and literature references over 900	Mumtaz et al. (2017)

(continued)

Table 11.2 (continued)

Sl. No.	Database	Description	References
12	Plant Metabolome Database (PMDB)	It helps to visualize the 3D structure of plant metabolites. It contains external and internal links to KEGG, CAS NUMBER, and PUBCHEM	Udayakumar et al. (2012)
13	PlantMetabolomics.org (PM)	It is a combined database and web portal which integrates metabolomics data from multiple laboratories employing various analytical methods. It includes visualization tools such as error and ratio plots	Bais et al. (2010)
14	ReSpect	Repository of MS/MS-based metabolomics data specific for plants which contain 3595 metabolites. Enables the narrowing down of phytochemical structure to candidate structures	Sawada et al. (2012)
15	RIKEN Plant Metabolome MetaDatabase (RIKEN PMM)	Compiles GC-MS-based metabolomics data from plants along with their experimental metadata	Fukushima et al. (2018)
16	SHPIS (Saudi Herbal Plants Information System)	Contains about 120 varieties of unique Saudi Arabian medicinal plants	Syed and Khan (2017)
17	SoyMetDB (The Soybean Metabolome Database)	Metabolomic database for *Glycine max*, which aims to integrate its metabolomics data (LC-MS and GC-MS based data). Include metabolomics data from *Arabidopsis* to enable cross-species comparisons	Joshi et al. (2010)
18	Super Natural II	Consisting of ~326,000 molecules along with their 2D structures, physicochemical and structural properties, probable toxicity (of about 170,000), and vendor information	Banerjee et al. (2015)
19	Uttarakhand Medicinal Plants Database (UMPDB)	Contains details of 1127 medicinal plants belonging to 153 families distributed along 13 Uttarakhand districts	Kumar et al. (2018)

(i.e., from 2010 onward). Various applications of databases in medicinal plant research are schematically represented in Fig. 11.3.

6 Applications of Metabolomics in Medicinal Plant Research

6.1 Investigating the Bioactive Compounds in Medicinal Plants

Inability to efficiently identify potential bioactive compounds from medicinal plants is a bottleneck in their extensive clinical applications, masking the wealth of efficient

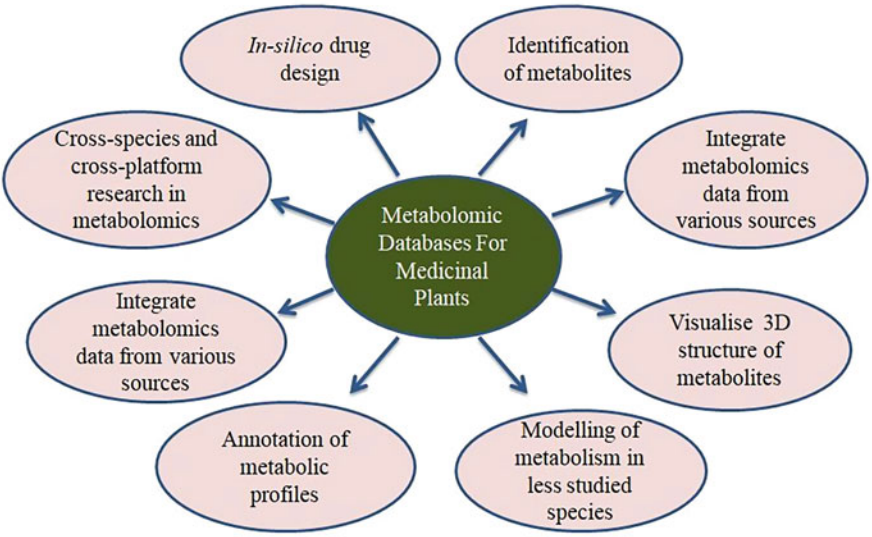


Fig. 11.3 Various applications of plant metabolomics databases

lead compounds for drug industry (Plazas et al. 2019). Metabolomics finds immense applications in unveiling the bioactive compounds responsible for the therapeutic effects of medicinal plants (Yuliana et al. 2013), and some of the examples are briefly outlined. The treatment of Alzheimer's disease is posing a great challenge, because of the absence of efficient drug candidates (Rahman and Choudhary 2015). Only viable option is to focus on drugs that act as cholinesterase inhibitors. To screen for anti-cholinesterase alkaloids found in *Zanthoxylum* members of Rutaceae, metabolomics profiling of its nine species has been performed along with chemometric analysis. This experiment resulted in the detection of 11 isoquinoline alkaloids with potential anti-cholinesterase activity (Plazas et al. 2019). To explore the potential of *Camptotheca acuminata* for antineoplastic treatment, metabolite fingerprinting has been performed, and they identified the alkaloids related to camptothecin having antineoplastic action. Leaves at different growth stages were analyzed to find the optimum stage of growth for harvesting. Various camptothecin-related alkaloids revealed through the study offer promising compounds to be utilized as precursors in the synthesis of semisynthetic derivatives of camptothecin (Montoro et al. 2010). Li et al. (2013) performed metabolic profiling of *Tussilago farfara* to investigate the compounds responsible for its expectorant and antitussive activities. They also analyzed different plant parts to optimize the part with maximum activity; it revealed that flower buds and leaves possessed maximum bioactive principles. The study confirmed the role of 3,5-dicaffeoylquinic acid, rutin, and chlorogenic acid in the therapeutic properties of the plant.

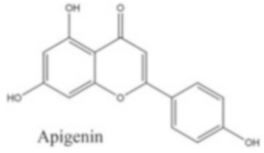
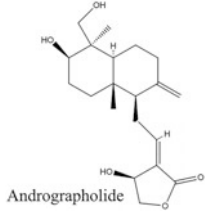
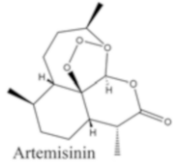
Carica papaya has broad spectrum of therapeutic applications such as anti-inflammatory, antibacterial, anticancer, antioxidant, antimalarial, vasodilatory

properties, etc. Metabolic fingerprinting performed through 1D and 2D ^{1}H NMR revealed that high concentration of secondary metabolites occupied in younger leaves and UPLC-ESI-MS analysis verified and confirmed the active metabolites. Apart from attributing the therapeutic effects to one or two major compounds, the role of synergism of metabolites in its diverse clinical applications was revealed in this study (Gogna et al. 2015). Taha et al. (2020) investigated the antimicrobial, anticancer, and antioxidant activities of a desert medicinal plant *Hyphaene thebaica*. Past studies in this plant were mainly concentrated on the clinical applications of the fruit, while this study utilized leaves, fruit, and male parts of the plant. These three parts were evaluated in three groups according to the anthocyanin, flavonoid, flavonol, phenolic, saponin, and tannin content. Significant increase in antioxidant activity was found in male parts and leaves than fruits. Metabolic profiling by HPLC confirmed the dominance of chrysin, *p*-hydroxybenzoic acid, *p*-coumaric acid, protocatechuic acid, syringic acid, rosmarinic acid, and vanillic acid in male parts; chlorogenic acid in fruit; and apigenin-7-glucosides, catechins, and rutin in leaves. Some of the important bioactive compounds from medicinal plants studied using metabolomics are given in Table 11.3.

6.2 Elucidate the Mode of Action of Herbal Medicine

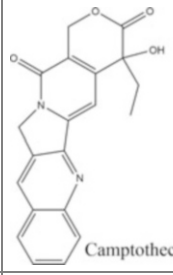
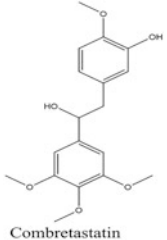
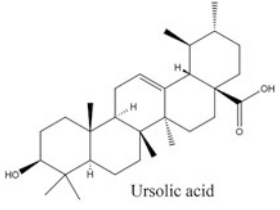
There are concerns over toxicity, efficacy, quality, etc. of many traditional medicinal herbs. Metabolomics is a valuable tool to answer these concerns. Herbal formulations of traditional Chinese medicine (TCM) have wide clinical applications in treatment of liver diseases, which were largely limited due to queries over safety and quality. Metabolomics has been employed to decipher the mode of action of three herbal remedies (yin chen hao tang, xiaozhang tie, and silymarin) for liver disease in TCM through studies in cell culture systems, animal models, and clinical studies. This study provides insights to the efficacy and safety of herbal remedies and also underlines the importance of combinations of herbal medicines in efficient treatment methods (Beyoğlu and Idle 2020). Likewise, the ability of Suanzaoren decoction to treat insomnia has been delineated by assessing the metabolic changes taking place in a model insomnia drosophila after administering Suanzaoren decoction. This decoction is a formulation of five herbal medicines (Poria, seed of *Ziziphus jujuba*; roots and rhizome of *Glycyrrhiza uralensis*, *G. inflata*, and *G. glabra*; rhizome of *Ligusticum chuanxiong*; and rhizome of *Anemarrhena asphodeloides*). The analysis revealed that Suanzaoren decoction could significantly increase sleep activity. In addition to this, the hypnotic effect of this formulation is found to affect the global metabolomics of the test organism (Yang et al. 2012).

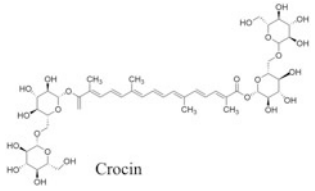
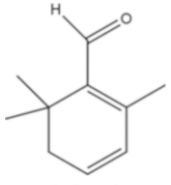
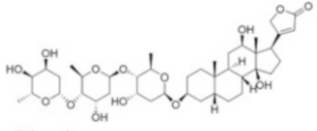
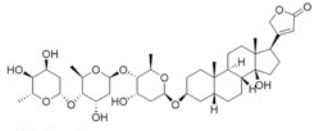
Table 11.3 Major bioactive compounds from medicinal plants studied using metabolomics

Sl. No	Plant name	Major metabolites under study	Medicinal use	Metabolomic technique/s employed	References
1	*Allium sativum* L.	 Apigenin	Its hydroxylated form prevents proliferation of tumor cells and angiogenesis	LC-MS	Afsheen et al. (2018)
2	*Andrographis paniculata* (Burm. f.) Nees	 Andrographolide	Anticancer, anti-inflammatory, anti-HIV, antimalarial activities	^{1}H NMR	Tajidin et al. (2019)
3	*Artemisia afra* Jacq.	 Artemisinin	Anti-plasmodial activity	NMR	Liu et al. (2010)

(continued)

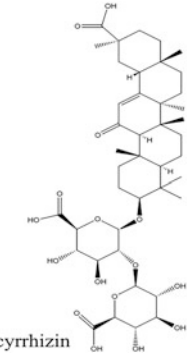
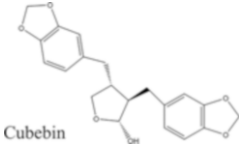
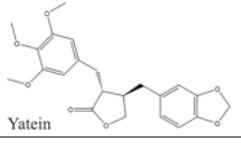
Table 11.3 (continued)

Sl. No	Plant name	Major metabolites under study	Medicinal use	Metabolomic technique/s employed	References
4	*Camptotheca acuminata* Dence.	 Camptothecin	Anticancer activity	HPLC-MS/MS	Cho et al. (2018)
5	*Combretum caffrum* (Eckl. & Zeyh.) Kuntze	 Combretastatin	Anticancer activity	Liquid chromatography high-resolution mass spectrometry platform (LC-HRMS)	Jaroch et al. (2018)
6	*Crataegus oxyacantha* L.	 Ursolic acid	Inhibits angiotensin converting enzyme, cardioactive potential	LC-MS	Afsheen et al. (2018)

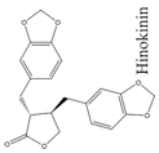
7	*Crocus sativus* L.	 Crocin	Treatment of aging and age associated neurodegenerative disorders	High-resolution MS	Gikas et al. (2021), Rezaee and Hosseinzadeh (2013)
		 Safranal	Antidepressant, anticonvulsant, treatment of withdrawal syndrome		
8	*Digitalis lanata* Ehrh. and *D. purpurea* L.	 Digoxin  Digitoxin	Treatment of atrial fibrillation systolic heart disease	LC-MS/MS	Ravi et al. (2020)

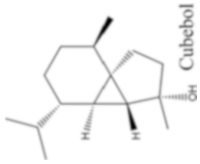
(continued)

Table 11.3 (continued)

Sl. No	Plant name	Major metabolites under study	Medicinal use	Metabolomic technique/s employed	References
9	*Glycyrrhiza glabra* L., *G. uralensis* Fisch. and *G. inflata* Bat.	 Glycyrrhizin	Antioxidant, anti-inflammatory activities	High resolution MS	Rizzato et al. (2017)
10	*Piper cubeba* L.	 Cubebin  Yatein	Anticancerous activity	^{1}H-NMR	Mazlan et al. (2018)

(continued)





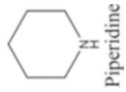
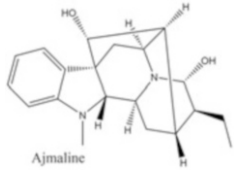
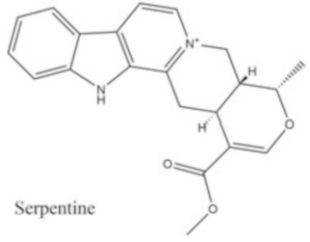
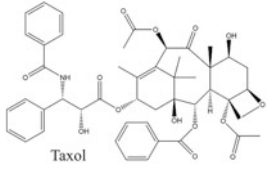


Table 11.3 (continued)

Sl. No	Plant name	Major metabolites under study	Medicinal use	Metabolomic technique/s employed	References
11	*Rauvolfia serpentina* (L.) Benth. ex Kurz.	 Ajmaline	Sodium channel blocker, stimulate intestinal movements and respiration	LC-MS	Afsheen et al. (2018)
		 Serpentine	Used to treat hypertension, neurological and cardiovascular disorders		
12	*Taxus fuana* Nan Li & R.R. Mill and *T. yunnanensis* Cheng et L. K. Fu.	 Taxol	Anticancer activity	Comparative metabolomics via targeted UPLC-MS/MS method to quantify the production of bioactive metabolites	Yu et al. (2018)

6.3 Quality Assessment of Herbal Products

The capabilities of natural product derived drugs can be utilized in full potential only if the chemical composition of herbal products is standardized to check the proper quality of natural product (Heyman and Meyer 2012). Quality of herbal formulations has been assessed earlier based on one or two major compounds present in it. However, herbal medicines comprise multiple compounds, and metabolic profiling can be performed to evaluate the multiple components, thereby meeting adequate quality standards (Lee et al. 2017). Metabolomics is gaining wide acclaim nowadays to assess phytochemical constituents, thus providing an acceptable method for quality control of herbal medicine. By employing chromatographic and spectroscopic methods, a valid metabolite fingerprint can be obtained, and further metabolite profiling helps to identify the individual constituents that form the unique fingerprint (RaoGajula and Nanjappan 2021).

For even properly authenticated plant material, there may be difference in quality between different batches due to a variety of factors such as inter or intra-species variations, environmental factors, harvesting stage, plant parts used, post-harvesting factors, etc. (Nafiu et al. 2017). Xiang et al. (2011) employed metabonomic analysis to characterize ecotypic variation in three species of *Curcuma*, namely, *Curcuma kwangsiensis*, *C. phaeocaulis*, and *C. wenyujin* as part of quality control measures. This study was focused on the essential oil composition, and PCA efficiently distinguished samples according to differences in species and ecotypes. Some of the medicinal herbs are difficult to distinguish between varieties as in the case of *Ficus deltoidea* (a popular medicinal herb of Malaysia), whose seven varieties are laborious to identify morphologically due to extensive heterophylly. The difficulty to identify the desired variety and variations of their chemical constitution is posing challenges in their commercialization. Untargeted metabolomics is performed by ultra-high-performance liquid chromatography time-of-flight mass spectrometry (UHPLC-TOFMS), and the subsequent data analysis was able to distinguish three chemotypes on the basis of differences in flavonoid content. The study also identified 15 glycosylated flavones and 1 furanocoumarin as chemical marker (Afzan et al. 2019). Saposhnikoviae Radix (a common crude drug) obtained from rhizome and root of *Saposhnikovia divaricata* was investigated for the differences in metabolome using the specimens from China and Mongolia. Metabolic profiling confirmed that these two regional groups are clearly distinct with respect to *O*-glucosylcimifugin, being more abundant in Mongolian group. They can also be distinguished based on the differences in content of eight chromones (Batsukh et al. 2020). Rastogi et al. (2020) investigated the interspecies variation among three medicinally important species of *Ocimum*, namely, *O. gratissimum*, *O. kilimandscharicum*, and *O. sanctum* by analyzing the temporal changes in metabolite composition.

Adulteration of medicinal herbs with certain other plants or any other foreign substance is a common practice and a major challenge of natural drug industry (Chanda 2014). Wallace et al. (2018) evaluated the adulteration in commercial plant-derived products by using supplements of *Hydrastis canadensis* (goldenseal) as the

test case. An untargeted metabolomics analysis employing UPLC-MS helped to detect adulteration in 3 test samples among the 35 test samples. This analysis revealed the potential applications of untargeted metabolomics in detecting possible adulteration. Extensive adulteration is observed in highly priced oil of *Serenoa repens* (saw palmetto), which triggered the investigation for methods to detect designer blends of cheap fatty acids as adulterants. The combined use of metabolomic analysis and isotopic fingerprinting suggested the possible adulteration and whose source is mostly animal based fatty acids (Perini et al. 2018).

6.4 Safety and Toxicity Assessment of Natural Products

One of the factors that hinder the clinical applications of traditional phytomedicine into mainstream is their limited molecular level characterization. If there is proper recording of the side effects, reaction with other medicines, safety, hypersensitivity, tolerance, problems of overdose, etc. of natural products, then only the product can be commercialized (Cordell 2015). Nowadays, traditional Chinese medicine (TCM) is attracting wide attention due to debates over safety concerns. Even though TCM contains natural formulations, their complex nature and mechanism of action remain as a barrier to assess it by traditional methods. For this, metabolomics helps to get an idea about the possible toxicity of bioactive compounds in TCM. Cardiotoxicity, hepatotoxicity, nephrotoxicity, and reproduction toxicity have been revealed as the side effects many of the components of TCM by metabolomic studies (Duan et al. 2018). Traditional African medicine (TAM) also has much popularity among African communities with wide acceptance among both rural and urban populations. In this context, metabolomics provides an opportunity to make a holistic analysis of phytochemicals, biomarkers, and the mechanism by which TAM modifies metabolic pathways (Quansah and Karikari 2016).

In order to investigate the different levels of toxicity exhibited by *Senecio scandens* and *S. vulgaris*, a metabolomics study employing UPLC-MS was performed. *Senecio scandens*, which is an approved medicine of Chinese medicine, did not show toxic effects, while *S. vulgaris* belonged to the same genera exhibited significant hepatotoxicity. The metabolomics analysis revealed that senecionine, a particular marker of *S. vulgaris* remained the cause for its toxic effects (Xiong et al. 2012). *Polygonum multiflorum* has been a part of TCM, whose dry roots are widely used for therapeutic properties. Hepatotoxicity is a concern over its use, and different processing methods are documented for its processing to reduce the toxicity. With the aim of finding the most suitable method of its processing, variations in metabolomic profiles of *P. multiflorum* with respect to various processing methods were analyzed by UHPLC/Q-Orbitrap-MS. The study identified emodin-8-*O*-glucoside and torachrysone-*O*-hexose as the toxic markers of *P. multiflorum*. The toxic effects were decreased with every processing method and the best results being obtained by steaming with black soybean; the method was suggested by Chinese Pharmacopoeia (Han et al. 2019).

7 Conclusions and Future Prospects

A large proportion of world's population still relies on medicinal plants for therapeutics and our traditional medicinal systems use herbal formulations, it was tested and evaluated by trial-and-error mechanisms. Even though these medicinal plants have such a history of long-term association with human therapeutics, systematic characterization of their active compounds and the intricate mechanisms underlying their action have been achieved with the advent of metabolomics. Metabolomics is a promising approach, which can revitalize the researches in herbal medicine by characterizing the active compounds in medicinal plants responsible for their clinical action. Metabolomics helps to study the synergism between various compounds in attaining the therapeutic effects, assessing the quality, safety, and toxicity of herbs and herbal formulations, etc. The rapid analysis of large number of metabolites in metabolomics is what makes it distinct from other analytical methods. It can also contribute much to the upcoming era of personalized medicine by metabolic profiling of the concerned individual in response to the herbal drug administration; this helps to monitor the efficacy and toxicity of the drug to each individual. Efficient analytical platforms, data handling methods, and associated databases are crucial to the success of metabolomics. Recent advancements in MS- and NMR-based methods have contributed much to the progress of medicinal plant metabolomics. If these two platforms are employed together, metabolomics has much potential to provide a non-biased quantification and characterization and can provide an integrated picture of component metabolites in the sample. Such a comprehensive knowledge regarding the medicinal plants and herbal formulations can open new avenues in herbal clinical industry. Thus, the treasure trove of phytochemicals as potential drug leads can be explored in future.

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Chapter 12
Metabolomics and Therapeutic Potential of *Ophiocordyceps sinensis*

Nitin Bhardwaj, Sumit Rajaura, Pooja Chauhan, and Ashutosh Singh

1 Introduction

Ophiocordyceps (formerly called *Cordyceps sinensis*) is a genus in the Family Ophiocordycipitaceae of Ascomycete fungi that include more than 400 species. The fungus was first discovered as Ben-Cao-Bei-Yao in 1694 and is usually called Dong-chong-Xia-Cao in China (Hu et al. 2013). It is also known as "winter worm, summer grass" in Chinese literature (Zhang et al. 2012). Most species are parasitic on the pupa of lepidopteran insects (Family: Hepialidae) (Zhang et al. 2012). The fungal spore grows inside the body of the host and produces a fruiting body. The union of caterpillar and fungus makes a parasitic complex that consists of caterpillar and fungus sexual stroma (Fig. 12.1). The stroma is the upper fungal part and usually dark brown or black in color. It is longer than the caterpillar itself, usually 4–10 cm. The fertile part of the stroma is the head. The head is granular because of the ostioles of the embedded perithecia. The stroma emerges out from the head of the caterpillar and kills it through paralysis and mummification (Winkler 2009). *Ophiocordyceps* is distributed on the Tibetan Plateau and its surrounding regions at an altitude above 3500–5000 m including few provinces of China, Bhutan, India (Chamoli and Pithoragarh district of Uttarakhand, North Sikkim in Arunachal Pradesh), and Nepal on the southern flank of the Himalayas (Li et al. 2011). It has optimum growth at 18 °C but can survive up to 40 °C in winter (Li et al. 2018).

O. sinensis (OS) has been well known in Chinese and Tibetan traditional medicine and used for the treatment of a variety of diseases like asthma, bronchitis,

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M. K. Swamy, A. Kumar (eds.), *Phytochemical Genomics*,
https://doi.org/10.1007/978-981-19-5779-6_12

Fig. 12.1 Structure of *Ophiocordyceps sinensis* obtained from the Garhwal region of Uttarakhand is shown here. Stroma represents the fungal part. Sclerotium shows the mummified caterpillar body

pulmonary, renal, and hepatic diseases (Lin et al. 2016). There are many bioactive components in *O. sinensis*, having therapeutic value. Cordycepin, cordycepic acid, polysaccharides, and cyclic peptide proteins are major constituents present in it, which show anticancer properties like, anti-angiogenic, anti-metastatic, anti-proliferative, and apoptotic activity in cancerous cells (Baharara and Amini 2015). Apart from anticancerous effect, its various components also show various pharmacological effects like antiaging, antioxidant, anti-inflammatory, and immunomodulatory effects (Ashraf et al. 2020) (Fig. 12.2). The metabolomics data show that a number of bioactive compounds are present in *O. sinensis*, which can help in treatment of these diseases.

Various herbal plants and fungi contributes to 78% of cash income to the Himalayan rural population (Smith Olsen and Overgaard Larsen 2003). The OS is one of the major sources of income for thousands of farmers in the Himalayas (Hopping et al. 2018). Due to its health benefits and limited supply, the price of *Ophiocordyceps* has risen spectacularly. It is the most outstandingly valued medicinal fungi. Due to habitat loss, change in climate and overexploitation has decreased its production globally. Global increase in requirement of *O. sinensis* in herbal medicine has further making it overexploited (Wei et al. 2021). In 2017, per kilogram price was more than 14,000USD in China, which was three times that of gold (Li et al. 2015).

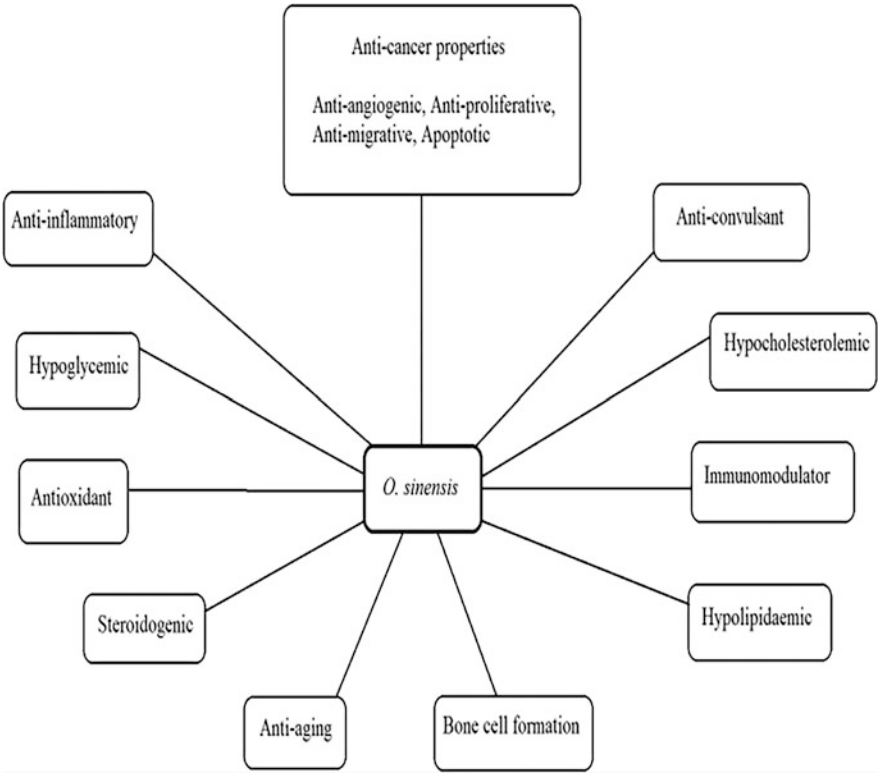

Fig. 12.2 Therapeutics properties of *O. sinensis*: Shown here are the various therapeutics properties of *O. sinensis*

2 Metabolomics of *Ophiocordyceps sinensis*

Metabolomics enables large-scale analysis of tissues, organs, and whole organisms. Various high-throughput metabolomics tools have evolved in the recent years. Application of high-throughput metabolomics to *Ophiocordyceps* has helped to identify many important compounds. Some of them have high therapeutic potential. A variety of compounds including nucleosides and their analogues, carbohydrates, peptides, amino acids, a number of fatty acids, and their derivatives have been reported in *Ophiocordyceps*. The molecular structure of some of the important compounds has been illustrated in Fig. 12.3.

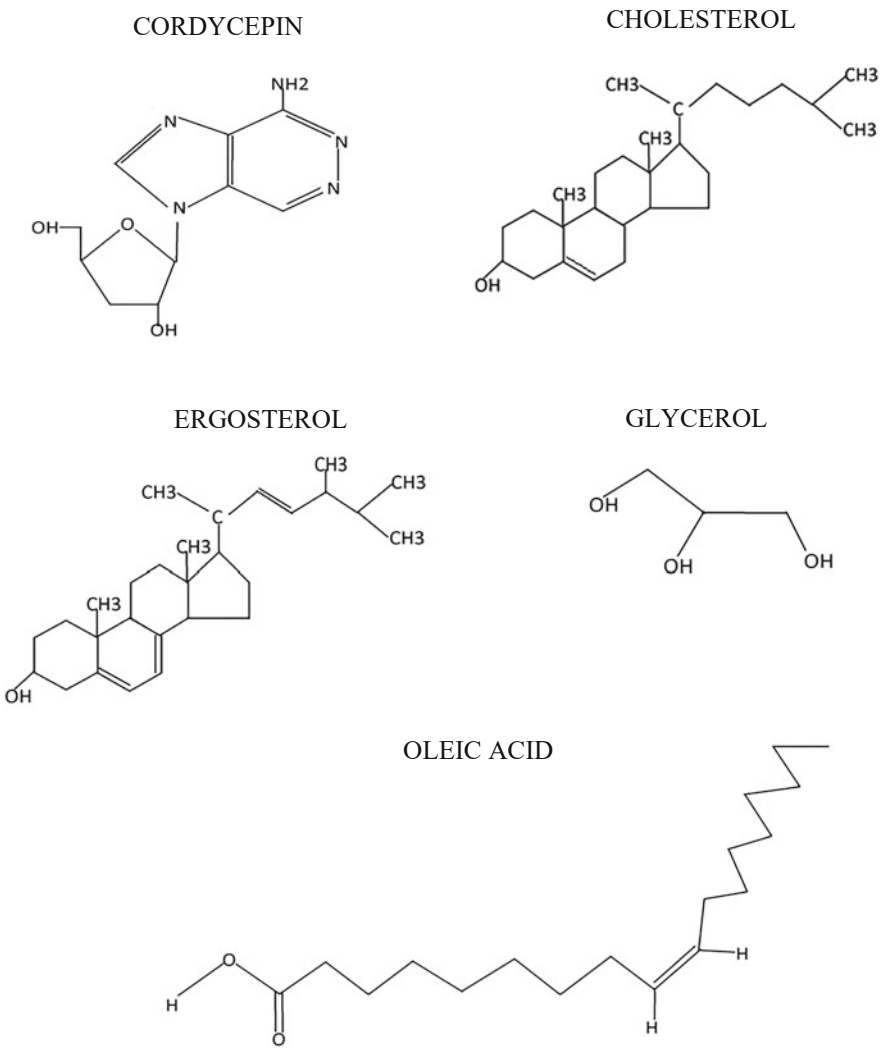

Fig. 12.3 Structure of chemical constituents of *O. sinensis*. Shown here are the chemical structures of important constituents of *O. sinensis*

2.1 Nucleosides and Their Derivatives

Ophiocordyceps is rich in nucleosides, purine, and pyrimidine nitrogen bases and their derivatives (Shrestha et al. 2012). The relative concentrations of cordycepin, thymine, and adenosine are found more in the *O. sinensis* present at 4000 m above than the sea level (Xie et al. 2010). Various nucleosides and their derivatives could be analyzed by using methods like high-performance liquid chromatography (HPLC) (Guo et al. 2018), liquid chromatography-mass spectrometry (LCMS)

Table 12.1 Major nucleosides and nitrogenous compounds present in *Ophiocordyceps*

S. No.	Compound	Empirical formula	Class	Analysis method	References
1.	Inosine	$C_{10}H_{12}N_4O_5$	Nucleoside	NMR, HPLC	Yang et al. (2011), Cheng et al. (2017)
2.	Uridine	$C_9H_{12}N_2O_6$	Nucleoside	HPLC	Yang et al. (2011), Cheng et al. (2017)
3.	Thymidine	$C_{10}H_{14}N_2O_5$	Nucleoside	HPLC	Yang et al. (2011), Cheng et al. (2017)
4.	Cytidine	$C_9H_{13}N_3O_5$	Nucleoside	HPLC	Yang et al. (2011), Cheng et al. (2017)
5.	Guanosine	$C_{10}H_{13}N_5O_5$	Nucleoside	HPLC	Yang et al. (2011), Cheng et al. (2017)
6.	Adenosine	$C_{10}H_{13}N_5O_4$	Nucleoside	HPLC	Yang et al. (2011), Cheng et al. (2017)
7.	Xanthine	$C_5H_4N_4O_2$	Nucleoside	HPLC	Yang et al. (2011)
8.	Guanine	$C_5H_5N_5O$	Purine	HPLC	Yang et al. (2011), Cheng et al. (2017)
9.	Adenine	$C_5H_5N_5$	Purine	HPLC	Yang et al. (2011), Cheng et al. (2017)
10.	Uracil	$C_4H_4N_2O_2$	Pyrimidine	HPLC	Yang et al. (2011), Cheng et al. (2017)
11.	Cytosine	$C_4H_5N_3O$	Pyrimidine	HPLC	Yang et al. (2011), Cheng et al. (2017)
12.	Thymine	$C_5H_6N_2O_2$	Pyrimidine	HPLC	Yang et al. (2011)
13.	Cordycepin	$C_{10}H_{13}N_5O_3$	Adenosine	HPLC	Yang et al. (2011), Cheng et al. (2017)
14.	N^6-(2-hydroxy-ethyl) adenosine	$C_{12}H_{17}N_5O_5$	Adenosine	HPLC	Wang et al. (2019)
15.	20 deoxy-adenosine	$C_{10}H_{13}N_5O_3$	Adenosine	HPLC	Gu et al. (2007)
16.	Hypoxanthine	$C_5H_4N_4O$	Inosine	HPLC	Yang et al. (2007), Cheng et al. (2017)

coupled with electrospray ionization interface method (Xie et al. 2010), and high-performance liquid chromatography fingerprints and quantitative analysis of multi-components by single marker (Chen et al. 2018). A detail description of these molecules has been summarized in Table 12.1.

2.2 Carbohydrates

A number of sugars and their derivatives have been reported in *Ophiocordyceps* species (Shrestha et al. 2012). These include various monosaccharides, polysaccharides, sugar alcohols, sugar acids, and amino sugars (Liu et al. 2015). Pressurized liquid extraction and gas chromatography coupled with mass spectrometry are

Table 12.2 Carbohydrate constituents in *Ophiocordyceps*

S. No.	Compound	Empirical formula	Class	Analysis method	References
1.	Myo-inositol	$C_6H_{12}O_6$	Carbo-cyclic sugar	GCMS	Choi et al. (2010)
2.	Arabinose	$C_5H_{10}O_5$	Monosaccharide	IR	Xiao et al. (2012)
3.	Erythrose	$C_4H_8O_4$	Monosaccharide	LCMS	Wada et al. (2017)
4.	Galactose	$C_6H_{12}O_6$	Monosaccharide	GC	Zhu et al. (2012)
5.	Glucose	$C_6H_{12}O_6$	Monosaccharide	GC	Zhu et al. (2012)
6.	Mannose	$C_6H_{12}O_6$	Monosaccharide	GC	Zhu et al. (2012)
7.	Glucitol	$C_6H_{14}O_6$	Polyols (sugar alcohol)	GC	Zhu et al. (2012)
8.	Glycerol	$C_3H_8O_3$	Polyols (sugar alcohol)	GC	Zhu et al. (2012)
9.	Xylitol	$C_5H_{12}O_5$	Polyols (sugar alcohol)	LCMS	Wada et al. (2017)
10.	Galactonic acid	$C_6H_{12}O_7$	Sugar acid	GC	Zhu et al. (2012)
11.	Gluconic acid	$C_6H_{12}O_7$	Sugar acid	GC	Zhu et al. (2012)
12.	Glucuronic acid	$C_6H_{10}O_7$	Sugar acid	GC	Zhu et al. (2012)
13.	Glyceric acid	$C_3H_6O_4$	Sugar acid	GC	Zhu et al. (2012)
14.	*N*-acetyl glucosamine	$C_8H_{15}NO_6$	Amino sugar	GC	Zhu et al. (2012)

commonly used method to differentiate the natural and cultivated *O. sinensis* based on conjugated and free carbohydrates (Shrestha et al. 2012). A detail description of these molecules has been summarized in Table 12.2.

2.3 Amino Acids, Polyamines, and Cyclopeptides

Amino acids and their derivatives are the important constituents in the *Ophiocordyceps*. Diamine, polyamines, and cyclopeptides have been reported in earlier investigation (Shrestha et al. 2012). Different types of amino acids could be analyzed by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Li et al. 2017). Water-soluble polysaccharide extract also contains more than half percent of protein in it (Chung et al. 2009). A major class of amino acids and related compounds has been shown in Table 12.3.

Table 12.3 Amino acids, polyamines, and cyclopeptides in *O. sinensis*

S. No.	Compounds	Empirical formula	Class	Analysis method	References
1.	Alanine	$C_3H_7NO_2$	Amino acid	GCMS	Zhong et al. (2020)
2.	Asparagine	$C_4H_8N_2O_3$	Amino acid	GCMS	Hyun et al. (2013)
3.	Aspartic acid	$C_4H_7NO_4$	Amino acid	GCMS	Wei et al. (2014)
4.	Glutamine	$C_5H_{10}N_2O_3$	Amino acid	GCMS	Zhong et al. (2020)
5.	Glycine	$C_2H_5NO_2$	Amino acid	GCMS	Zhong et al. (2020)
6.	Histidine	$C_6H_9N_3O_2$	Amino acid	GCMS	Hyun et al. (2013)
7.	Homoserine	$C_4H_9NO_3$	Amino acid	GCMS	Hyun et al. (2013)
8.	Isoleucine	$C_6H_{13}NO_2$	Amino acid	GCMS	Zhong et al. (2020)
9.	Lysine	$C_6H_{14}N_2O_2$	Amino acid	GCMS	Zhong et al. (2020)
10.	Ornithine	$C_5H_{12}N_2O_2$	Amino acid	GCMS	Zhong et al. (2020)
11.	Proline	$C_5H_9NO_2$	Amino acid	GCMS	Zhong et al. (2020)
12.	Serine	$C_3H_7NO_3$	Amino acid	GCMS	Hyun et al. (2013)
13.	Threonine	$C_4H_9NO_3$	Amino acid	GCMS	Zhang et al. (2020)
14.	Tyrosine	$C_9H_{11}NO_3$	Amino acid	GCMS	Yang et al. (2003)
15.	Valine	$C_5H_{11}NO_2$	Amino acid	GCMS	Zhang et al. (2020)
16.	γ-Aminobutyric acid	$C_4H_9NO_2$	Amino acid	GCMS	Cohen et al. (2014), Zhang et al. (2020)
17.	Cystathionine	$C_7H_{14}N_2O_4S$	Amino acid	GCMS	Zhang et al. (2020)
18.	Putrescine	$C_4H_{12}N_2$	Diamine	GCMS	Bhandari et al. (2012)
19.	1,3-Diaminopropane	$C_3H_{10}N_2$	Diamine	–	Shrestha et al. (2012)
20.	Cadaverine	$C_5H_{14}N_2$	Polyamine	–	Bhandari et al. (2012)
21.	Spermidine	$C_7H_{19}N_3$	Polyamine	–	Bhandari et al. (2012)
22.	Spermine	$C_{10}H_{26}N_4$	Polyamine	–	Bhandari et al. (2012)
23.	Cycloaspeptide A	$C_{36}H_{43}N_5O_6$	Cyclo-peptide	NMR	Zhang et al. (2009)
24.	Cycloaspeptide F	$C_{42}H_{53}N_5O_{11}$	Cyclo-peptide	NMR	Zhang et al. (2009)

(continued)

Table 12.3 (continued)

S. No.	Compounds	Empirical formula	Class	Analysis method	References
25.	Cycloaspeptide G	$C_{36}H_{43}N_5O_7$	Cyclo-peptide	NMR	Yang et al. (2011)
26.	Cordyheptapeptide A	$C_{49}H_{65}N_7O_8$	Cyclo-peptide	NMR	Yang et al. (2011)
27.	Cyclo-(Gly-Pro)	$C_7H_{10}N_2O_2$	Cyclo-dipeptide	NMR	Yang et al. (2011)
28.	Cyclo-(Leu-Pro)	$C_{11}H_{18}N_2O_2$	Cyclo-dipeptide	NMR	Yang et al. (2011)
29.	Cyclo-(Val-Pro)	$C_{10}H_{16}N_2O_2$	Cyclo-dipeptide	NMR	Yang et al. (2011)
30.	Cyclo-(Ala-Leu)	$C_{19}H_{34}N_4O_5$	Cyclo-dipeptide	NMR	Yang et al. (2011)
31.	Cyclo-(Ala-Val)	$C_8H_{12}N_2O_2$	Cyclo-dipeptide	NMR	Yang et al. (2011)

2.4 *Fatty Acids, Carboxylic Acids, and Related Compounds*

The O*phiocordyceps* has a number of saturated and unsaturated fatty acids, methyl and ethyl derivatives, phytosterols, sterols, and di, tri, and alkyl and aromatic cyclic carboxylic groups and associated compounds (Yi et al. 2015). Fatty acid assay shows that fruiting bodies of *Ophiocordyceps* comprise around 70% of fatty acids, and among them, the concentration of linoleic acid is present in maximum amount (Hur 2008). Table 12.4 shows the detailed list of these compounds.

2.5 *Aldehydes, Ketones, Phenol, Pyrazines, and Other Compounds*

A major class of aldehydes, ketones, alcohols, aromatic alcohols and phenol, benzene pyrrole, and pyrazine compounds have also been reported in minor quantities (Zhang et al. 2017; Yu et al. 2012; Yang et al. 2011). Headspace solid-phase extraction method and gas chromatography-mass spectroscopy (GCMS) technique are used for identification of these components (Sun et al. 2018). These compounds and related compounds have been shown in Table 12.5.

Table 12.4 Fatty acids, carboxylic acids, and related compounds in *O. sinensis*

S. No.	Compound	Empirical formula	Class	Analysis methods	References
1.	Lauric acid	$C_{12}H_{24}O_2$	Saturated fatty acid	NMR	Yang et al. (2011)
2.	Myristic acid	$C_{14}H_{28}O_2$	Saturated fatty acid	NMR	Yang et al. (2011)
3.	Pentadecanoic acid	$C_{15}H_{30}O_2$	Saturated fatty acid	NMR	Yang et al. (2011)
4.	Palmitoleic acid	$C_{16}H_{30}O_2$	Saturated fatty acid	NMR	Yang et al. (2011)
5.	Palmitic acid	$C_{16}H_{32}O_2$	Saturated fatty acid	NMR	Yang et al. (2011)
6.	Stearic acid	$C_{18}H_{36}O_2$	Saturated fatty acid	NMR	Yang et al. (2011)
7.	Docosanoic acid	$C_{22}H_{44}O_2$	Saturated fatty acid	NMR	Yang et al. (2011)
8.	Lingoceric acid	$C_{24}H_{48}O_2$	Saturated fatty acid	NMR	Yang et al. (2011)
9.	Octanoic acid	$C_8H_{16}O_2$	Saturated fatty acid	GCMS	Elkhateeb et al. (2020)
10.	Decanoic acid	$C_{10}H_{20}O_2$	Saturated fatty acid	NMR	Krasnoff et al. (2005)
11.	Pentadecanoic acid	$C_{15}H_{30}O_2$	Saturated fatty acid	GCMS	Elkhateeb et al. (2020)
12.	Hexanoic acid	$C_6H_{12}O_2$	Saturated fatty acid	LCMS	Chen et al. (2018)
13.	Linoleic acid	$C_{18}H_{32}O_2$	Unsaturated fatty acid	GCMS	Hyun et al. (2013)
14.	Oleic acid	$C_{18}H_{34}O_2$	Unsaturated fatty acid	GCMS	Hyun et al. (2013)
15.	Methyl palmitate	$C_{17}H_{34}O_2$	Methyl ester of fatty acid	GCMS	Hyun et al. (2013)
16.	Methyl oleate	$C_{19}H_{36}O_2$	Methyl ester of fatty acid	GCMS	Hyun et al. (2013)
17.	Ethyl palmitoleate	$C_{18}H_{34}O_2$	Ethyl ester of fatty acid	GCMS	Hyun et al. (2013)
18.	Ethyl myristate	$C_{16}H_{32}O_2$	Ethyl ester of fatty acid	GCMS	Nallathamby et al. (2020)
19.	Ethyl linoleate	$C_{20}H_{36}O_2$	Ethyl ester of fatty acid	GCMS	Nallathamby et al. (2020)
20.	Ethyl oleate	$C_{20}H_{38}O_2$	Ethyl ester of fatty acid	GCMS	Nallathamby et al. (2020)
21.	Ethyl stearate	$C_{20}H_{40}O_2$	Ethyl ester of fatty acid	GCMS	Nallathamby et al. (2020)
22.	β-Sitosterol	$C_{29}H_{50}O$	Phytosterol	NMR	Yang et al. (2011)

(continued)

Table 12.4 (continued)

S. No.	Compound	Empirical formula	Class	Analysis methods	References
23.	Ergosterol	$C_{28}H_{44}O$	Phytosterol	NMR	Yang et al. (2011)
24.	Cholesterol	$C_{27}H_{46}O$	Sterol	NMR	Yang et al. (2011)
25.	Campesterol	$C_{28}H_{48}O$	Sterol	NMR	Yang et al. (2011)
26.	Citric acid	$C_6H_8O_7$	Tricarboxylic acid	GCMS	Hyun et al. (2013)
27.	Fumaric acid	$C_4H_4O_4$	Dicarboxylic acid	GCMS	Hyun et al. (2013)
28.	Succinic acid	$C_4H_6O_4$	Dicarboxylic acid	GCMS	Hyun et al. (2013)
29.	3-Methyl butanoic acid	$C_5H_{10}O_2$	Alkyl carboxylic acid	GCMS	Zhang et al. (2017)
30.	2-Methyl butanoic acid	$C_5H_{10}O_2$	Alkyl carboxylic acid	GCMS	Zhang et al. (2017)
31.	Benzoic acid	$C_7H_6O_2$	Aromatic carboxylic acid	GCMS	Zhang et al. (2017)
32.	ϒ-Nonano-lactone	$C_9H_{16}O_2$	Lactone (cyclic carboxylic esters)	GCMS	Zhang et al. (2017)
33.	Δ-Decalactone	$C_{10}H_{18}O_2$	Lactone (cyclic carboxylic esters)	GCMS	Zhang et al. (2017)

3 Therapeutic Potential of *Ophiocordyceps sinensis*

Ophiocordyceps show multiple health effects such as aphrodisiac, immunomodulatory (Jeong et al. 2010; Lee et al. 2010a, b), anticancer (Cai et al. 2018), antioxidant (Chen et al. 2013), anti-inflammatory (Jeong et al. 2010), neuroprotective (Kong et al. 2015), hypoglycemic (Lo et al. 2006), and antimicrobial activities (Tuli et al. 2014) (Fig. 12.2). A brief description of various health effects has been described here.

3.1 The Antitumor Effects of Ophiocordyceps sinensis

Ophiocordyceps sinensis has been shown as a potent antitumor as it inhibits growth and multiplication of a number of cancerous cells.

Table 12.5 Aldehydes, ketones, phenol, pyrazines, and other compounds in *O. sinensis*

S. No.	Compounds	Empirical formula	Class	Analysis method	References
1.	Furfural	$C_5H_4O_2$	Aldehyde	GCMS	Zhang et al. (2017)
2.	Pentanal	$C_5H_{10}O$	Aldehyde	GCMS	Wu et al. (2019)
3.	Phenylacetaldehyde	C_8H_8O	Aldehyde	HPLC	AL-Shekhany and AL-Khesraji (2012)
4.	Hexanal	$C_6H_{12}O$	Aldehyde	GCMS	Wu et al. (2019), Zhang et al. (2017)
5.	2-Methyl-3-phenyl-2-propenal	C_9H_8O	Aldehyde	GCMS	Zhang et al. (2017)
6.	2,4-Dedecadienal	$C_{10}H_{16}O$	Aldehyde	GCMS	Zhang et al. (2020)
7.	5-Methyl-2-phenyl-2 hexenal	$C_{13}H_{16}O$	Aldehyde	GCMS	Zhang et al. (2017)
8.	2-Heptanone	$C_7H_{14}O$	Ketone	GCMS	Yu et al. (2012), Zhang et al. (2017)
9.	4-Nonen-2-one	$C_9H_{16}O$	Ketone	GCMS	Yu et al. (2012), Zhang et al. (2017)
10.	2-Decanone	$C_{10}H_{20}O$	Ketone	GCMS	Yu et al. (2012), Zhang et al. (2017)
11.	6-Propyl-5,6-dihydro-2*H*-pyran-2-one	$C_8H_{12}O_2$	Ketone	GCMS	Yu et al. (2012), Zhang et al. (2017)
12.	2-Undecanone	$C_{11}H_{22}O$	Ketone	GCMS	Yu et al. (2012), Zhang et al. (2017)
13.	2-Heptadecanone	$C_{17}H_{34}O$	Ketone	GCMS	Yu et al. (2012), Zhang et al. (2017)
14.	2-Furanal methanol	$C_5H_6O_2$	Alcohol	GCMS	Zhang et al. (2017)
15.	Benzyl alcohol	C_7H_8O	Aromatic alcohol	GCMS	Zheng et al. (2015)
16.	Phenylethyl alcohol	$C_8H_{10}O$	Aromatic alcohol	GCMS	Zhang et al. (2017)
17.	*p*-Cresol	C_7H_8O	Phenol derivative	GCMS	Sangeetha et al. (2018), Zhang et al. (2017)
18.	4-Ethylphenol	$C_8H_{10}O$	Phenol derivative	HPLC	Linke et al. (2017)
19.	Butylated hydroxytoluene	$C_{15}H_{24}O$	Phenol derivative	GCMS	Yu et al. (2012)

(continued)

Table 12.5 (continued)

S. No.	Compounds	Empirical formula	Class	Analysis method	References
20.	2-Ethyl-5-methylpyrazine	$C_7H_{10}N_2$	Pyrazine	GCMS	Zhang et al. (2017)
21.	2-Ethyl-6-methylpyrazine	$C_7H_{10}N_2$	Pyrazine	GCMS	Zhang et al. (2017)
22.	2,3,5-Trimethylpyrazine	$C_7H_{10}N_2$	Pyrazine	GCMS	Zhang et al. (2017)
23.	2,3-dimethyl-3-ethylpyrazine	$C_8H_{12}N_2$	Pyrazine	GCMS	Zhang et al. (2017)
24.	2,5-Dimethyl-3-(2-methylpropyl) pyrazine	$C_{10}H_{16}N_2$	Pyrazine	GCMS	Zhang et al. (2017)
25.	2,5-Dimethyl-3-(1-propenyl) pyrazine	$C_9H_{12}N_2$	Pyrazine	GCMS	Zhang et al. (2017)
26.	2-Isoamyl-6-methylpyrazine	$C_{10}H_{16}N_2$	Pyrazine	GCMS	Zhang et al. (2017)
27.	Cordysinins A	$C_{11}H_{18}N_2O_3$	Pyrrolopyrazine	NMR	Yang et al. (2011)
28.	1,3-Dichloro-2-methylbenzene	$C_7H_6Cl_2$	Benzene-halogen derivative	GCMS	Yu et al. (2012)
29.	1,4-Dichloro-4-methylbenzene	$C_7H_6Cl_2$	Benzene-halogen derivative	GCMS	Yu et al. (2012)
30.	1-Ethenyl-3-ethylbenzene	$C_{10}H_{12}$	Alkyl benzene	GCMS	Yu et al. (2012)
31.	1-Ethenyl-4-ethylbenzene	$C_{10}H_{12}$	Alkyl benzene	GCMS	Yu et al. (2012)
32.	Cordysinins C	$C_{13}H_{12}N_2O$	β-Carbolines	NMR	Yang et al. (2011)
33.	Cordysinins D	$C_{13}H_{12}N_2O$	β-Carbolines	NMR	Yang et al. (2011)
34.	2-Acetylpyrrole	C_6H_7NO	Pyrrole	GCMS	Zhang et al. (2017)
35.	Undecane	$C_{11}H_{24}$	Alkane	GCMS	Zhang et al. (2017)
36.	Uric acid	$C_5H_4N_4O_3$	Weak acid	NMR	Yu et al. (2012)

3.1.1 Breast Cancer

A polysaccharide MHP1 isolated from the asexual structure of *O. sinensis*, that is, *Mortierella hepiali*, reduces the metastasis by inhibiting the epithelial-mesenchymal transition (EMT) (Lin et al. 2016). The EMT is inhibited by restricting the transforming growth factor, beta-receptor type (2TGFBRll) expression. The MHP1 transform the phenotype of M2 macrophage to M1 to inhibit the tumor cell growth. The MHP1 increases the expression of epithelial surface markers like E-cadherin and Zona occludens and diminishes the expression of mesenchymal markers, that is, vimentin and fibronectin. It also reduces the level of matrix metalloproteinase like MMP-2 and MMP-4 and prevents the degradation of extracellular matrix and cancer

cell invasion (Lin et al. 2016). Cordycepin decreases the cell viability, inhibited the cell proliferation, and induced the ROS level in human breast cancer cells (Wang et al. 2016a, b). The *Ophiocordyceps* water extract reduced the number of macrophages (F4/80 positive) cells in the tumor. It shows antitumor effect through macrophages as the number of CD86+ and iNOS+ proportion elevate relative to F4/80+ cells (Li et al. 2020). Cordycepin treatment strongly enhanced the activation of caspase-3, caspase-8, and caspase-9 in breast cancer cells as these caspases are the critical elements of the extrinsic and intrinsic apoptotic pathways (Wang et al. 2016a, b).

3.1.2 Hepatocellular Carcinoma

Hepatocellular carcinoma is another common type of malignant tumors, mainly caused by hepatitis B or C, viral infection, and alcohol consumption. The reactive oxygen species (ROS) produced in hepatocytes alters the functioning of various protein and enzymes and leads to the hepatocarcinogenesis. Diethylnitrosamine (DEN), a compound found in tobacco smoke, fast food, cheese, and in alcoholic drinks, considers stimulating hepatocellular carcinoma in rats by increasing oxidative stress by the production of ROS and by carbonylation of certain proteins like endoplasmin, serotransferrin, catechol, and transketolase. DEN enhances the oxidation of many proteins and chaperons like heat shock cognate 71 kDa protein (HSC71C), glucose-regulated protein 75 (GRP75), GRP78, propionyl-CoA carboxylase, catalase, and alpha-enolase (Wang et al. 2016a, b). *O. sinensis* has been shown to alleviate the cellular injuries (Wang et al. 2016a, b). Around 30 protein profiles show significant changes after *O. sinensis* administration. Cordycepin induced apoptosis in human liver cancer cells (HepG2) by inducing the caspase activity. It has also been shown to induce the hepatoprotective effects on alcohol-induced toxicity (Cha et al. 2013). Exopolysaccharide from cultivated *O. sinensis* exhibits a hepatoprotective effect against acute hepatoxicity induced by administration of CCl_4 to rats. The reasons for hepatoprotective effect may be direct free radical scavenging activities, stimulation of the antioxidant systems, and inhibition of lipid peroxidation (Nguyen et al. 2021). The cordycepin administration markedly inhibited the EA.hy926 and HepG2 cell proliferation in a dose- and time-dependent manner (Lu et al. 2014).

3.1.3 Lung Cancer

Cordycepin, a potential biomolecule present in *Ophiocordyceps*, has been reported to inhibit the growth and proliferation of many cancerous cell lines. It is also reported that cordycepin helps in the treatment of human's non-small cell lung cancer (NSCLC) by inducing autophagy and extrinsic apoptosis by downregulating the expression of cellular-FLICE inhibitory protein (c-FLIPL). The cordycepin treatment leads to a significant increase in initial and late apoptotic cells of human

NSCLC cell lines, H1299 and H460 (Yu et al. 2017). Cordycepin can cause growth arrest in tumor cells in vivo by inducing the expression of CAV1 and p-JNK, which results in the downregulation of Foxo3a phosphorylation in human lung adenocarcinoma (Joo et al. 2017). Cordycepin treatment induced the expression levels of caspase-3 in human lung cancer A549 cells, which may lead to apoptosis of cancer cells. Cordycepin exerted antimigratory effects on human lung cancer cells via regulating the expression of E-cadherin, vimentin, and MMP-9 (Tao et al. 2016). Hwang et al. (2017) reported that cordycepin inhibits ERK/Slug signaling pathway through the activation of GSK3β, which, in turn, upregulates Bax, which leads to the apoptosis of the lung cancer cells.

3.1.4 Oral Cancer

Cordycepin and *Ophiocordyceps* treatment showed anticancer properties in an in vivo experiment on a mouse model of oral cancer and an in vitro experiment on a cell line 4NA group (4NAOC-1). The extract preparation inhibits the malignant tumor transformation and tumor development by lowering the level of a monoclonal antibody ki-67, epidermal growth factor receptor (EGFR), interleukin-17A (IL-17A) cytokine, and programmed death ligand-1 (PD-L1) signaling molecules (Hsu et al. 2017). The immune response against cancerous cells is developed by increasing the apoptosis of cancerous cells by raising the expression of interferon gamma (INF-ϒ) and tumor necrosis factor α (TNF-α) cytokines and by reducing the multiplication of cancerous cells (Hsu et al. 2017). Cordycepin treatment leads to early translocation of phosphatidylserine (PS) from the internal to external leaflet and induced cell apoptosis in OEC-M1 human oral squamous cancer cells (Wu et al. 2007). Cordycepin causes the upregulation of ATG5 and trigger autophagy through the upregulation of p21 in an autophagy cascade-dependent manner and is related to cell cycle arrest in G2/M phase to induce cell death in oral squamous carcinoma cells (Ho et al. 2019).

3.1.5 Murine Leukemia

Cordyceps militaris protein (CMP) (extracted from the fruit bodies of *Cordyceps militaris*) treatment reduced the viability of primary cells like macrophages, splenocytes, and a normal hepatocyte cell line BNL, and RAW264.7 cell lines. The *Ophiocordyceps* treatment with BNL 1ME.7R1 cells (primary murine cells) results in cytotoxicity and leads to the cellular damage by the increment of lactate dehydrogenase. CMP causes cell death by apoptosis via mitochondrial-mediated pathways (Bi et al. 2018). Cordycepin treatment induced apoptosis in human HL60 leukemia cells by DNA laddering, caspase 3 mediated, and by the cleavage of PARP3 protein (Chou et al. 2014).

3.1.6 Human Bladder Cancer

The data shows that *Cordyceps* extract decreases the survival rate of human urinary bladder carcinoma cell line (T24). The treatment with the aqueous extract activates the A3 adenosine receptor of T24 cells, which declines the expression of the protein kinase B (PKB)/Akt protein kinase pathway. This decreased level of Akt activates caspase3 and leads to the apoptosis of cancer cells (Cao et al. 2017).

3.1.7 Human Colorectal Carcinoma

The efficiency of ethanol extract of *Ophiocordyceps* was investigated on human colorectal carcinoma (RKO) cells. The ethanol extract contains many bio-compounds like cordycepin, cordycepic acid, sterol nucleoside, and polysaccharides, which exhibits anticancer properties. The treatment with ethanol extract leads to activation of phosphoprotein p53, which stimulates pro-apoptotic B-cell lymphoma2 (Bcl2) family proteins and triggers the release of cytochrome C and breakdown of poly(ADP-ribose) polymerase-1. Cyto-C activates caspase 9 and 3 that further leads to the apoptosis of cancerous cells (Lee et al. 2015a, b). Cordycepin treatment causes G2/M cell cycle arrest and modulated the p53-mediated pathways in human colon cancer cells (Lee et al. 2010a, b). It induced apoptosis through DR3 pathway in human colonic cancer cells (HT-29) (Lee et al. 2013).

3.1.8 Brain Cancer

Glioblastoma multiforme (GBM) represents one of the human brain cancer. The temozolomide (TMZ) is commonly used drug for the treatment of glioblastoma multiforme (Zhang et al. 2010). The TMZ usually activates the AMP-activated protein kinase (AMPK)) and endogenous protein kinase B (AKT). AMPK contributes to apoptosis by inhibiting mTOR, but AKT, in contrast, promotes resistance against drugs in the glioma cells. The cordycepin enhances the activity of pro-apoptotic proteins like Bax (BCL2 Associated X, apoptosis regulator) and lowers the expression of anti-apoptotic proteins. Cordycepin resists the transfer of glioma cells from the G2 to M phase of the cell cycle and leads to cell arrest. The combination therapy increased the expression of AMPK, arrests the cells in the G2 phase, inhibits G2-M phase transition, and helps in the reduction of the level of AKT (Bi et al. 2018). Cordycepin suppressed the migration of human glioblastoma cells by lysosomal degradation. It decreases the expression of integrin, FAK, and paxillin protein expression (Hueng et al. 2017). Cordycepin inhibited the cell growth and induced apoptotic pathway in SH-SY5Y and U251 cells, which is model to mark human neuroblastoma and glioblastoma, respectively. Cordycepin along with

chloroquine, an inhibitor of autophagy, further ceases the growth and induces the death of cancer cells of brain (Chaicharoenaudomrung et al. 2018).

3.1.9 Human Liver Cancer

Cordycepin treatments inhibit the growth and multiplication of a human liver cancer cell line HepG2. The cordycepin treatment leads to the morphological changes in cells like chromatin compaction and condensation, fragmentation and shrinking of nuclear membrane and nucleus, formation of apoptotic body, changes in mitochondrial permeability, and storage of sub G1 cells. The cordycepin modulates the expression of Bcl2 proteins and initiates the primary and secondary signaling pathway of apoptosis (Shao et al. 2016).

3.2 Human Gastric Cancer

The role of cordycepin was studied in the regulation of apoptotic pathway in the SGC-7901 cell line of human gastric cancer. The study shows that cordycepin regulates the proliferation of cells through an intrinsic and extrinsic apoptotic pathway by activating the caspase 3, caspase 8, and p53 protein, respectively. The cordycepin administration enhances the level of ROS in these cells, which imbalance the mitochondrial membrane potential. Cordycepin treatment also inhibits the cell cycle by arresting the cells in the S phase. Cordycepin inhibits the adenosine A3 receptor (A3AR), which might have some role in the activation of apoptosis (Nasser et al. 2017).

3.2.1 Pancreatic Cancer

The impact of *Ophiocordyceps* in the treatment of pancreatic cancer was investigated both in vivo and in vitro experiments by using MIAPaCa-2 and Capan-1 cancer cell lines. The cordycepin regulates metastasis via mitochondrial-mediated intrinsic pathway by reducing the mitochondrial membrane potential (MMP). The level of BAX, a splinter of caspase 3 and 9, increased, while the expression of Bcl2 and cleaved poly ADP-ribose polymerase (PARP) declined. Cordycepin also arrests the cell cycle in the S-phase to prevent the proliferation and accumulation of cancerous cells (Zhang et al. 2018).

3.2.2 Osteogenesis

The cordycepin treatment stimulates osteoblast formation and reduces the differentiation of osteoclast cells in murine pre-osteoblastic cells (MC3T3-E1). The

cordycepin administration does not have any cytotoxic effects on MC3T3-E1 and murine macrophage cell line, RAW264.7. The osteoclast differentiation pathway involves the binding of receptor activator of nuclear factor-kappa b ligand (RANKL), with the macrophage receptor RANK. Cordycepin attenuates this process and regulates the osteoclast maturation, resorption, and differentiation. Cordycepin also initiates the osteoblast differentiation by enhancing the action of bone morphogenetic protein (Yu et al. 2018).

3.3 Role of Ophiocordyceps *in the Improvement of Kidney Functioning*

Berger's disease or IgA nephropathy (IgAN) is a kidney disorder caused by the increased level of a lineage T helper cells type 22 (Th22), which enhances the inflammatory responses by elevating the level of interleukin 22 (IL-22). The therapeutic effects of *Ophiocordyceps sinensis* on kidney functioning were investigated in a mice model. The *Ophiocordyceps* treatment reduces the proliferation and IL-22 expression in IgAN mice. This indicates that *Ophiocordyceps* regulates the division of mesangial cells and lowers the activation and functioning of Interleukin 22 (IL-22). OS treatment reduces the effect of Th22 and has some medicinal properties to control the Berger's diseases (Xiao et al. 2018). The OS administration shows antifibrotic effects in the renal fibrosis. The polysaccharides of OS mediate its effect by modulating the expression of TGFβ1 receptor (Zhang et al. 2012). N^6 hydroxyethyl adenosine isolated from *Cordyceps* induced the antioxidant level in kidney and reduced the blood glucose level (Wang et al. 2019).

3.4 Ophiocordyceps *Enhance the Steroidogenesis in Males*

Ophiocordyceps sinensis extract and cordycepin administration activate the purified Leydig cells to increase the testosterone secretion (Huang et al. 2004). The OS treatment has a dose-dependent impact on the activation of Leydig cells through the cyclic adenosine monophosphate-protein kinase-A (cAMP-PKA) signaling pathway (Hsu et al. 2003). The cordycepin treatment enhances the testosterone level by stimulating the adenosine receptors, which further activates protein kinase A (PKA) and protein kinase C (PKC) cascade. The steroidogenesis stimulation by cordycepin occurs via mitogen-activated protein kinase (MAPK), PKA, and PKC/phospholipase C (PLC) in a model cell line derived from the mouse Leydig cell tumor (MA-10) (Chen et al. 2017).

3.5 *Immunomodulatory Properties of* Ophiocordyceps

O. sinensis also acts like an immunomodulator, and it can enhance the production of macrophages and activation of cells of bone marrow via Peyer's patches cells. The hot water extract activates macrophages, which further stimulate interleukin 6 (IL6) and interleukin 2 (IL2) production, which helps in the proliferation of T lymphocytes and hematopoietic stem cells. The OS treatment also activates the granulocyte-macrophage-colony-stimulating factor (GMCSF) and enhances the proliferation of bone marrow cells in C3H/HeJ mice (Koh et al. 2002).

3.6 *Hypoglycemic and Hypolipidemic Effects of* Ophiocordyceps

The Ophiocordyceps water extract contains almost 50% protein around 30% carbohydrates and a small amount of uric acid. The orally administrated water extract causes mild hypoglycemic effects. It reduces the activity of α-glucosidase and enhances the uptake of peripheral glucose by energizing the receptor activity of insulin hormone (Chung et al. 2009).

Many cardiac and vascular diseases are related to the higher levels of total cholesterol (TC), low-density lipoprotein (LDL), and triglycerides (TG). The experiment data suggest that the cordycepin treatment helps in the prevention of hyperlipidemia by decreasing the levels of TC, TG, and LDL (Koh et al. 2003). The cordycepin treatment elevates the level of AMP-activated protein kinase (AMPK), which decreases the level of glycerol 3 phosphate acetyltransferase (GPAT) and HMG-CoA reductase (3-hydroxy-methyl-glutaryl-coenzyme A reductase). The GPAT and HMG-CoA reductase play an important role in the formation of TC and TG, respectively. The AMPK also phosphorylates acetyl coenzyme-A carboxylase (ACC), which helps in the formation of fatty acids (Guo et al. 2010).

3.7 *Anti-inflammatory Roles of* Ophiocordyceps

The anti-inflammatory roles of cordycepin were studied in SGC-7901 cells. The data suggests that cordycepin phosphorylates Janus kinase signal transducer and activator of transcription proteins (JAK-STAT). This activation helps in the translocation of these protein factors from the cytoplasm to the nucleus to initiate the pro-inflammatory gene expression (Nasser et al. 2017). Cordycepin treatment inhibits the LPS-induced lung injury by suppressing the expression of NF-κB, p65, NRF2, and HO-1 expression (Lei et al. 2018).

4 Conclusion and Future Perspective

Several natural and therapeutic medicines are available for the treatment of cancer. These drugs have been shown to induce various side effects, including genotoxicity, carcinogenicity, and cellular toxicity. There is an urgent need for the discovery of new pharmaceutical molecules for the treatment of cancer and other diseases. Due to the toxicity of allopathic drugs, the research focus has been shifted to identified natural compounds (Ayurvedic formulations) having minimum side effects. *O. sinensis* is used as traditional medicinal herbs in many hilly regions from past decades. The *Ophiocordyceps* is used for the treatment of several diseases like aphrodisiac, asthma, bronchitis, pulmonary, renal, and hepatic diseases. Scientific researches show that components present in *O. sinensis* play a major role in providing it therapeutic values. The experimental data suggest that the *O. sinensis* inhibits the growth of many tumorigenic cells by regulating or affecting various mechanism, mainly by stimulation or enhancement of apoptotic pathways. It can also be used in the treatment of various other disease, helps in improvement of various organs, and promotes the immune response and can be seen as a potent medicine for treatment of some disorders.

Acknowledgments The grants, No. 56/2/Hae/BMS to NB and No. 52/08/2019/BIO-BMS to AS, received from Indian Council for Medical Research (ICMR), New Delhi are highly acknowledged. NB also thanks to University Grant commission (UGC) (30-496/2019) for providing the Start-up grant.

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Chapter 13
Genomics and Metabolomics: A Strategy for Elucidation of Metabolic Pathways in Medicinal Plants

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1 Introduction

Significant development in "omics" technologies over the past two decades has led to an increased research interest in acquisition of biological data from diverse plant species. When it comes to genome size, land plants stand out for diversity, duplication and expansion in genome size primarily accounted to polyploidy and accumulation of repetitive DNA sequences. Each genome sequencing event has uncovered species-specific novel genes besides the vast amount of non-coding sequences. Explicitly, the first ~50 plant genomes have provided information on the gene number, types and numbers of repeats, and how genomes grow and contract. However, it becomes challenging to functionally annotate the vast sequence information generated particularly due to the existence of epigenetic landscapes in plant genomes. It has been identified that in some plants like tomato this epigenome is tissue and developmentally regulated (Zhong et al. 2013), while in some others it is plant specific such as in maize (Eichten et al. 2013). Furthermore, size of the plant genome adds complexity to genome assembly. This is one of the reasons for the availability of complete genome sequence information for only a few model plants. For majority of the medicinally important plants, very few genome sequencing has been undertaken. This lack of genetic information for medicinal plants limits research on understanding molecular mechanisms of secondary metabolites biosynthesis and thereby hinders development of platforms for its pharmaceutical applications.

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M. K. Swamy, A. Kumar (eds.), *Phytochemical Genomics*,
https://doi.org/10.1007/978-981-19-5779-6_13

Contrary to this, transcriptome analysis is a powerful technology for elucidating gene-expression profile and the associated regulatory mechanisms. Very often the biosynthesis and accumulation of secondary metabolites are tissue specific and so as the expression of genes encoding enzymes of the biosynthetic pathways (Guo et al. 2021). Such spatial variations necessitate the need to profile the metabolome and integrate the same with transcriptomic data to get a comprehensive insight into the biosynthesis and regulation of secondary metabolites. Though there exists no direct link between the transcriptome and metabolome unlike that with the proteome, a transcriptome–metabolome-integrated study will anchor the metabolomics data which provides metabolic phenotype to the transcriptome data against the backdrop of known biosynthetic pathways. Several studies have integrated the transcriptomic and metabolomics data (Cavill et al. 2016). Such studies encompassing the functional genomics approach thus rely on the pathway databases to correlate the metabolite(s) variations determined following metabolomic analysis with differential gene(s) expression obtained from the transcriptomic study. Such an approach has helped in elucidating the underlying molecular mechanisms following various abiotic and biotic stress stimuli in various plants.

2 Transcriptomic Analysis in Secondary Metabolism: Advances and Progress

Lack of genomic information for medicinal plants can be overcome by transcriptome sequencing which enables investigation of both functional as well as differentially expressed genes (Jia et al. 2015). Furthermore, it provides comprehensive information on the overall transcriptional activity of the organism without any reference genome (Guo et al. 2021). Complementary DNA-amplified fragment length polymorphism (cDNA-AFLP), expressed sequence tag (EST), hybridization-based chip technology, and serial analysis of the gene (SAGE) are some of the traditional methods used for transcriptomic data accession and analysis (Chen 2004; Zhang and Sheng 2008; Wang et al. 2009; Simkin et al. 2011). The lack of reference genome makes these techniques often expensive and time-consuming. As most of the medicinal plants are non-model organisms, these methods are quite complicated. With the progressive advancement in sequencing technology, transcriptomic analysis has advanced from traditional chip hybridization technique to the well-advanced RNA sequencing technology usually described as RNA-Seq (Mironova et al. 2015).

The next-generation sequencing platform developed in 2006 is a high-throughput, rapid, low-cost, deep coverage technique that can sequence simultaneously millions of nucleic acid molecules and covers several drawbacks of first-generation sequencing. NGS technique is broadly used by the research world in the assembly of many models, non-model plant, and animal genomes. The application of NGS in obtaining the whole genome sequence of *Chosenia arbutifolia* (Chen et al. 2014; Mei et al. 2016; Feng et al. 2019) and SARS-COV-2 (Li et al. 2020) shows the supreme utility of NGS in the expanding research society. Illumina, Roche 454, Solexa, and ABI SOLiD are some of the common and widely used NGS

platforms (Zhang et al. 2016). The development of third-generation sequencing is another landmark progress in the field of sequencing technologies. Third-generation sequencing overthrowing the common challenges from NGS is widely used in transcriptome sequencing, genome sequencing, structural variation detection, and methylation detection attributing to the long-read length. Oxford Nanopore sequencing and PacBio single-molecule real-time (SMRT) sequencing technologies are the two third-generation sequencing technologies (Ma et al. 2019a, b). The SMRT sequencing works on the principle of sequencing by synthesis, the attachment of fluorescent group to the phosphoric acid accosts the issue of background noise, non-essentiality of amplification and accurate quantification by self-correction makes SMRT highly advanced and precise (Li et al. 2018). Nanopore sequencing technology uses variations in electrical signals to identify the base composition (Niedringhaus et al. 2011). Low cost, high throughput, and long-read length are some of the advantages of Nanopore sequencing. NGS and third-generation sequencing with high throughput is prevalently used for transcriptomic research.

After sequencing, transcriptome assembly is the fundamental procedure for subsequent analysis. Based on the availability of the reference sequence, de novo assembly or reference sequence-based assembly can be selected. Considering the non-availability of corresponding genomic sequence such as in the case of most medical plants, de novo-based assembly is usually performed. It is the most suitable method for non-model plants. Software packages for the genome de novo assembly include Trinity (Grabherr et al. 2013), Rnnotator (Martin et al. 2010), Oases (Schulz et al. 2012), and Soapdenovo-Trans (Xie et al. 2014) and those for sequence-based assembly include Scriptura (Guttman et al. 2010) and Cufflinks (Trapnell et al. 2013). Further, based on the error rate, data complexity, and the quality of the result, Trinity is the most widely used software for the de novo assembly. Trinity software obtains high-quality assembly with operating efficiency and maintaining higher accuracy (Strickler et al. 2012; Lu 2013). Additionally, for reference-based assembly Cufflink software obtains assembly results with better quality and higher accuracy but the number of transcripts obtained with Scripture is far higher than that obtained with Cufflinks. Gene function annotations are carried out using bioinformatics tools to determine the function of unknown genes by comparing the sequence with the data available from the public databases. Gene ontology (GO) function classification and Kyoto Encyclopedia of Genes and Genomes classification are the two primarily used methods for gene function annotation. The commonly used databases for unigene annotation include GO, KEGG, NCBI Nucleotide Sequence Database, Clusters of Orthologous Groups of Proteins, Non-Redundant Protein Sequence Database, and Swiss-Prot database (Liu et al. 2018a, b). Flow chart representing the important steps of transcriptome analysis is shown in Fig. 13.1.

Transcriptomic research has a wide range of applications in the study of medicinal plants in the areas of mining of novel functional genes, identification of plant development pathways, and investigation of several uncharacterized secondary metabolite pathways. Secondary metabolites are one of the most important plant natural products having high-medicinal properties and helping in the adaptation of plants to different types of external stress. They are also used as flavors, fragrances, and other fine value chemicals. They have differential expression according to the

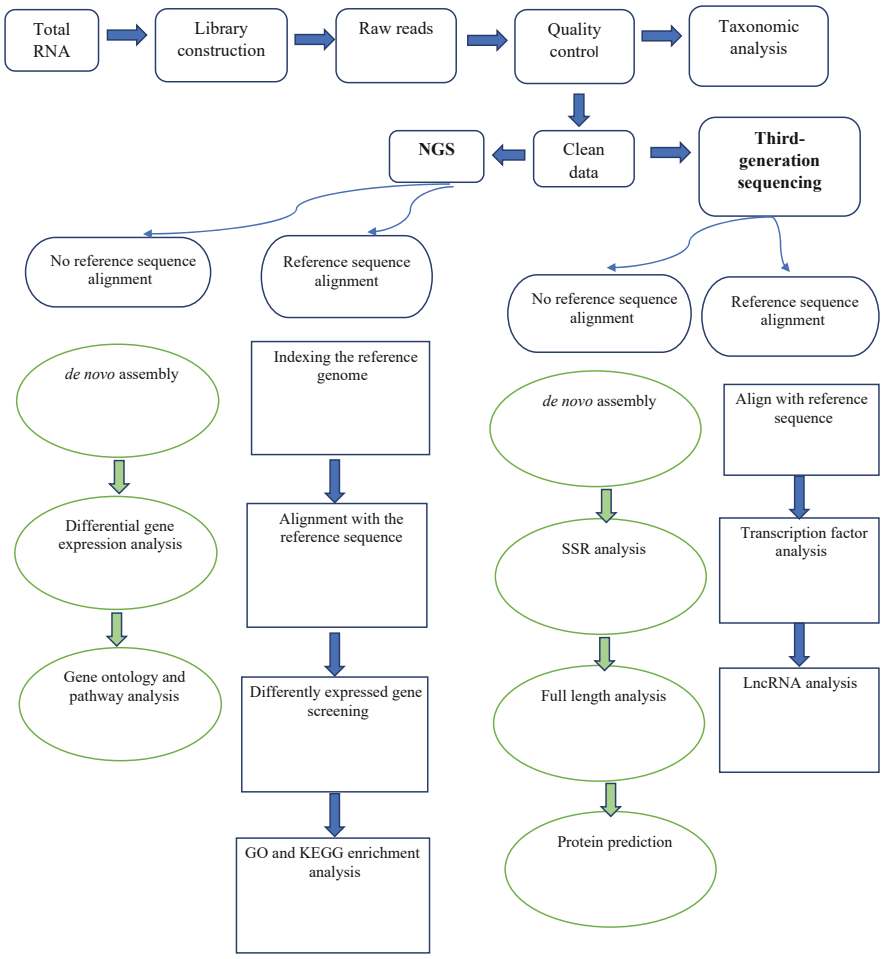

Fig. 13.1 Flow chart representing key steps of transcriptome analysis

growth stages, organs, and environment of the plants. Transcriptome data from these resources can form a scientific basis for the accumulation and effective utilization of these active molecules. For instance, *Entada phaseoloides* is a medicinal plant widely used in traditional medicine because of its anti-inflammatory activity and wind dampness eliminating effect. Saponins, belonging to the class of triterpenes, are the primary active compound in *E. phaseoloides*. The transcriptome data analysis of root, stem, and leaf tissue of the plant resulted in the identification of 26 cytochrome P450 and 17 uridine diphosphate glycosyltransferase candidate genes, which are involved in saponin biosynthesis (Liao et al. 2020). These identifications contribute to the functional genomics of triterpene biosynthesis. Extracts from *Tetrastigma hemsleyanum* are used as antibiotic for the treatment of sore throat and fever. Metabolome and transcriptome analysis of purple and green leaves of *T. hemsleyanum* identified a total of 209 metabolites and 4211 transcripts that were

differentially expressed in the purple and green leaves. Sixteen compounds were identified related to 14 transcripts involved in the anthocyanin biosynthesis pathway (Yan et al. 2020). *Lantana camara* is another medicinally important plant with diverse biologically active phytochemicals such as steroids, phenylpropanoid glycosides, and flavonoids. A total of 72,877 and 513,985 unigenes were identified by transcriptome sequence analysis of leaves and roots of *L. camara*, respectively. Among these unigenes, 229 and 943 genes from leaf and root tissue, respectively, were involved in phenylpropanoic acid biosynthesis (Shah et al. 2020). Flavonoids are high-potent plant secondary metabolites used in the treatment of cancer, HIV, and dengue because of their anti-oxidant and anti-inflammatory properties. Transcriptome sequencing of *Arisaema heterophyllum* Blume obtained 35,686, 43,363, and 47,783 unigenes from the root, tuber, and leaf tissues, respectively. From these data, 87 genes related to the isoflavone biosynthesis pathway were identified and experimentally verified (Wang et al. 2018). Similarly, *Saussurea lappa* is another pharmacologically potent plant wherein the sesquiterpene lactone is major bioactive compound. Transcriptome analysis of leaf samples from *S. lappa* pinpointed proteins that are involved in the sesquiterpene and flavonoid biosynthesis pathway (Bains et al. 2019). These data can create huge revolutions in functional genomics research as the number of transcripts encoding for genes related to alkaloid biosynthesis is least identified and characterized. Further, studies on the flavonoid biosynthesis pathway and its precursors were conducted by using NGS technology for de novo transcriptome sequencing and analysis of *Abrus mollis* (Yuan et al. 2018). The RNA sequencing of leaf, root, and stem tissue of *Artemisia argyi* identified 99,807 unigenes. Multiple genes encoding enzymes or transcription factors related to terpenoid biosynthesis were also identified (Liu et al. 2018a, b). Interestingly, applications of transcriptome sequencing include the utilization of 454 sequencing technology for transcriptome sequencing of root tissue of *Panax ginseng*, which identifies cytochrome P450 and UDP-glycosyltransferase genes involved in the biosynthesis of saponin (Jayakodi et al. 2014). *Ginkgo biloba* is a highly important medicinal tree because of the rich percentage of flavonoid content it possesses. Transcriptome sequencing of *G. biloba* samples with varying concentrations of flavonoid was carried out, and 37,625 unigenes were identified from the data obtained. Among these, several genes identified were annotated for the biosynthesis, transportation, and regulation of flavonoids (Wu et al. 2018). PhytoMetaSyn Project (www.phytometasyn.ca) combined next-generation sequencing and computational algorithms to investigate specialized metabolic pathways in non-model plants. Seventy-five non-model plants that produce phytochemicals with wide biotechnological applications were selected. After sequence assembly and annotation, 800,000 recognized transcripts were obtained. Further, candidate biosynthetic genes associated with six metabolic pathway were also described (Xiao et al. 2013).

Even though third-generation sequencing has several advantages over NGS, the comparative high-sequencing cost and low throughput at this stage limit its application. However, a combined approach of third-generation sequencing with NGS can be done for genotyping recognition and to reduce errors. It is envisaged in future that the reduced cost for third-generation sequencing along with its advantages such

as higher accuracy, short time, long-read length, full transcript sequencing, etc., is likely to be extensively used for medicinal plant transcriptome research. Multi-omics combining proteomics, metabolomics, and transcriptomics will be the future of medicinal plant research (Guo et al. 2021).

The transcriptome analysis is an emerging tool in the case of medicinal plants to identify and characterize many functional genomes and metabolic pathways that regulate the synthesis of various secondary metabolites. This will help for subsequent research on the unexplored, highly valuable medicinal plants which have an incredible role in pharmacological industries. Improved cultivation techniques and variant selection for medicinal plants by the functional gene and regulatory mechanism analysis are milestone accomplishments of transcriptomic analysis where the database is still very limited.

3 Metabolome Analysis: Tools and Techniques

The term metabolome was introduced by Oliver (1998) during their study on yeast genome (Burgess et al. 2014; Pereira Braga and Adamec 2019), where they analyzed the changes in the relative concentrations of metabolites that were associated with the deletion or overexpression of gene. The discipline metabolomics is the study and analysis of metabolome (complete set of low-molecular weight compounds) in a cell, tissue, or an organism. These small molecules in a biological sample are often analyzed through a combination of separation and detection tools such as GC-MS, LC-MS, HPLC-MS, NMR, etc. The most commonly used techniques are listed in Table 13.1.

The field of plant metabolomics has been identified as a widely exploited technology in recent years. Metabolomics mainly comprises three major steps: metabolite extraction, separation, and detection. In the first phase of extraction, there is no single protocol universally applicable for all types of samples. The extraction methods are depended on the nature of compounds of interest and it should be done without interfering with the chemical structure of the molecules. The extraction procedures can be divided into hydrophilic (methanol or a combination of methanol and water as solvents), lipophilic (chloroform as solvent), a combination hydrophilic–lipophilic extraction (combination of methanol–water–chloroform as solvents), and polar/semipolar lipophobic extraction (hot water or alcohol/water mixtures as solvent) methods. After extraction, the metabolites are separated by chromatographic techniques including gas chromatography (GC), liquid chromatography (LC), high-performance liquid chromatography (HPLC), ultra-performance liquid chromatography (UHPLC), etc., or by non-chromatographic platform named capillary electrophoresis (CE). The chromatographic techniques are based on the polarities and volatility of metabolites, while CE works based on their mass to charge ratio (m/z). Separation techniques are usually coupled with the detection techniques such as mass spectrometry (MS), nuclear magnetic resonance (NMR), etc., and these coupling often termed as "hyphenated-approaches" (Carrera et al. 2021; Hall 2011).

Table 13.1 List of most commonly used tools for metabolite profiling in plants

Name of method	Principle	Characteristics
1. **GC-MS** (Gas chromatography-mass spectrometry)	Based on the partition of specific molecules between gas and liquid phases at a given temperature using a specific GC column GC capillary columns are selected based on the metabolite polarity and volatility	Separate and detect thermally stable and volatile metabolites (alcohols, aldehydes, esters, etc.), semi-volatile metabolites (amides, amines, amino acids, sugars, organic acids, peptides, and lipids), chemical derivatization procedures to make them volatile Applicable to low molecular weight compounds (~500 Da) High reproducibility and relatively high throughput Relatively inexpensive
2. **LC-MS** (Liquid chromatography-mass spectrometry)	Reversed phase chromatography is the most common separation technique	Used for high molecular weight (>500 kDa) plant metabolites Separates based on hydrophobicity Nonpolar compounds are eluted more slowly than polar compounds Wider metabolite coverage Easy sample preparation No derivatization High sensitivity
3. **CE-MS** (Capillary electrophoresis-mass spectrometry)	Based on the proportion of mass to charge ratio (m/z) or separation is based in differences in electrophoretic mobilities (neutral compounds are not separated)	Fast and high-resolution of charged compounds or ionic metabolites Suitable for polar analysis in aqueous samples No derivatization and recovery of samples not possible High sensitivity Low cost
4. **NMR** (Nuclear magnetic resonance)	Based on spin behavior of atomic nuclei in a magnetic field which is represented by the resonance frequency	Used for the structural elucidation of metabolite Minimal sample preparation Absolute quantification Independent of analyte polarity Highly reproducible spectra, low sensitivity, expensive
5. **FT-ICR–MS** (Fourier transform-ion cyclotron resonance–mass spectrometry)	Based on mass to charge ratio (m/z) of ions within the fixed magnetic field supported cyclotron frequency	Separation of similar molecular mass compounds Can detect fragmented metabolite ions Chemical structure can generate from peak analysis Expensive due to the use of superconducting magnets and computational analysis of large amounts of data High sensitivity

The relationship between metabolites and quality traits in fuji apples and also the comparison of fuji apple metabolites from different regions of China were analyzed by LC-MS followed by correlation analysis. Fifty different metabolites belonging to 19 categories were analyzed, and it has been found that flavonoids and isoflavonoids positively contribute to the fruit-eating quality such as odour intensity, texture, and the total sensory score of fuji apple. However, its acidity and firmness are negatively influenced by glycerophospholipids. It is noticed that the origin of fuji apple also influences its metabolite production that finally contributes to its physiological and sensory qualities (Xie et al. 2021). Ultra-high-performance liquid chromatography coupled to quadrupole time-of-flight mass spectrometry (UHPLC-Q-TOF/MS) was used by Ding et al. (2022) for the identification of metabolites correlation with the oil production in Sea buckthorn (*Hippophae* L.). Sea buckthorn berry pulp (SBP) oil is known for its abundant palmitoleic acid (C16:1) content, nutritional and health properties. From the metabolomic and gene expression studies, it has been shown that metabolites play critical role in SBP development and oil biosynthesis, especially glycerol-3-phosphate (G3P) found to be crucial in the accumulation of oil during the mid-early developmental stages of sea buckthorn (Ding et al. 2022). The metabolomic study in five *Curcuma* species by ultra-performance liquid chromatography-mass spectrometry/mass spectrometry (UPLC-MS/MS) found that accumulation of medicinally important compounds varies among different species. This study shows that metabolomic study is important in the field of medicine that allows users to select different species with specific metabolites so that we can differentiate their use in food and medicine (Ye et al. 2022). Many of the metabolites contribute flavor and odor to fruits and vegetables. The rapid advances in metabolite profiling have made it easier to identify specific metabolites that contribute to flavor and odor and thereby to evaluate the cultivars based on their metabolites and nutritional qualities. An untargeted metabolic approach was conducted in five tomato cultivars using GC-TOF-MS (gas chromatography time-of-flight mass spectrometry) and UHPLC-LTQ-Orbitrap-MS/MS (ultrahigh performance liquid chromatography–linear trap quadrupole orbitrap–tandem mass spectrometry) platforms to differentiate the cultivars according to their metabolite profiling. The study showed varied metabolite concentrations among five cultivars and provided information that can be useful for the improvement of tomato cultivars (Mun et al. 2021). Many of the plants are used as fresh or in a dried state. Drying plant parts for various commercial uses can cause loss of volatile metabolites as well as the flavor of the product. For example, *Arctium lappa* L. (burdock) is a nutritious vegetable that also possesses medicinal properties such as antihyperglycemic, antioxidant, and other pharmacological properties. Dried plant roots of burdock are used as herbal tincture and tea constituent in many Asian countries. To check the effect of drying on the volatile composition of burdock, a metabolomic study with the help of headspace (HS)-GC-MS method was carried out by Xia et al. (2021). They used different drying methods such as natural drying, sunlight drying, hot-air and vacuum drying at different temperatures, and vacuum freeze-drying. It was shown that main volatile components like aldehydes, ketones, heterocycles, terpenes, etc. are influenced and varied in their concentrations upon different drying methods. The flavour was mostly maintained in traditional drying methods including sunlight drying and hot-air

drying than vacuum drying (Xia et al. 2021). The GC-MS approach was also applied in many plants to identify the metabolite profile change upon different drying methods. This aroma profile change has been studied in *Thymus vulgaris* (known for its essential oil) and *Anoectochilus roxburghii* (Wall.) Lindl (known for its medicinal properties). Results showed that high-temperature drying and lyophilization caused loss of essential oil content in *Thymus vulgaris*, while metabolite recovery on drying varied according to the method used for drying in *A. roxburghii*. These results also revealed that the drying methods should be selected based on the nature of plants because drying affected differently in different plants (Sárosi et al. 2013; Ye et al. 2019).

The untargeted or non-targeted metabolomics studies are dealing different tools to gather possible chemical information without focusing on any specific metabolite or group of compounds. The genus *Copaifera* (*Fabaceae*) contains important medicinal species but lack information about chemical data. An untargeted metabolomics study with the help of UHPLC-HRMS/MS (ultra-high-performance liquid chromatography coupled to high-resolution mass spectrometry) and data analysis using GNPS platform and chemometric tools was conducted in five *Copaifera* species using different organs. This study identified 29 metabolites that were analyzed through their bioactivity and 19 chemical markers by chemometric analysis (Antonio et al. 2021). As mentioned, there is no single extraction procedure standardized for an untargeted metabolomics study, till date.

4 Secondary Metabolism and Environmental Factors

It is often noticed that changes in secondary metabolism in response to stress are often species-specific. The plants have been identified with metabolite alteration under stress conditions that may lead to a particular physiological response or a phenotype (Arbona et al. 2013). Biomarker identification by metabolomic analyses can be used for the diagnosis of various plant diseases. The "olive quick decline syndrome" of olive tree caused by bacteria was analyzed by an untargeted metabolomic approach using high-performance liquid chromatography coupled to quadrupole-time-of-flight high-resolution mass spectrometry (HPLC-ESI-Q-TOF-MS). This study showed upregulation and downregulation of various defense responsive metabolite production, and many of them belong to the flavonoid family. This suggested that imbalanced regulation of some of these metabolites could be the reason for decreased defense mechanisms in infected plants (Di Masi et al. 2022). The most serious disease named Huanglongbing (HLB) of citrus plant is caused by bacterium *Candidatus Liberibacter asiaticus* (*Las*). The effect of infection on the metabolome of the citrus plant was determined by ^{1}H nuclear magnetic resonance (NMR) spectrometry. Results showed that infection badly affected its juice quality. This was the first study on this infection that shows how this pathogen hinders the plant's natural defense mechanism as well as provides infection-related alteration in nutrient composition (Slisz et al. 2012). A GC-MS-based untargeted metabolomic research was conducted by Galbiatti et al. (2021) to identify the changes in the

volatile composition of *Plectranthus neochilus* in response to the environmental changes. *P. neochilus* is a medicinal plant used as digestive, antispasmodic, and analgesic purposes. Moreover, essential oil of the plant is known for its antimicrobial, antiparasitic, and antioxidant activities. GC-MS analysis was targeted to the volatile composition where it showed that sampling time differences (morning and afternoon) on the same day do not affect the volatile profile. However, the seasonal changes and environmental factors can change the volatile composition. For example, the winter season drops the intensity of most of the compounds that ultimately leading to the variation in the bioactivity of *P. neochilus* (Galbiatti et al. 2021). Analysis of biomarkers of the growth period and different drying methods in *Citrus wilsonii* Tanaka (CWT) was done by UPLC-Q-TOF/MS-based non-target analysis. Abundancy analysis of all the metabolites suggested that middle period of the growth is the best choice of fruit harvesting in CWT where it possesses high levels of active ingredients. Metabolite profile also revealed that the most abundant ingredient naringin in CWT is significantly decreased during maturation (significant decrease when outer skin turned yellow). Therefore, considering the physical and chemical properties of naringin that reflect the maturity of CWT suggested that naringin can be used as a potential biomarker of CWT. A comparative study of important metabolites of *Citrus wilsonii* Tanaka (CWT) with different drying processes was also conducted. The samples were dried using oven-drying and freeze-drying methods resulting in high VIP (variable importance in the projection in the partial least square discriminate analysis (PLS-DA)) values for metabolites like citric acid and naringin. This indicates that their relative content was affected by different drying methods. Interestingly, limonoids were not affected by drying methods but showed relatively stable nature. The metabolite naringin (4′,5,7-trihydroxyflavanone 7-rhamnoglucoside) changes during different drying methods of CWT concluded that naringin can be a good judgmental tool for different drying methods of CWT (Yan et al. 2021). Metabolomics has been used to know whether long-term protection of a suburban forest from herbivory will change the metabolome of plant species from the unprotected one or not. Selected indigenous and non-indigenous species in a suburban forest in New Jersey, USA, were subjected to metabolomics analysis. The plot was divided into two where one plot opened for deer (unfenced) and the other one closed for deer (fenced), for 5.3 years. LC-MS/MS followed by data analysis tools such as principal component analysis (PCA), partial least squares-discriminant analysis (PLS-DA), and hierarchical cluster analysis (HCA) were used for the metabolome analysis. Results showed significant divergence in global metabolome profile between fenced and unfenced plots. It was also identified that some of the upregulated metabolites in fenced groups are involved in defense-regulated metabolic pathways (Morrison and Woldemariam 2022). A comparative metabolites study in normal and discolored red pepper using non-targeted metabolomic analysis was carried out by Feng et al. (2022). Using UHPLC-QE Orbitrap/MS analysis, it was revealed that the carotenoid composition has no change in these two types of pepper, whereas compared to normal pepper overall carotenoid content is reduced in discolored pepper. Also identified 408 differentially accumulated metabolites and those expressions are different in discolored and normal pepper (Feng et al. 2022). Environmental stress-related metabolite profile change

in dandelion (*Taraxacum mongolicum* Hand.-Mazz.) was revealed using HPLC-Q-TOF-MS analysis. Dandelion is rich in flavonoids and is used as an antidiabetic, anticarcinogenic, and anti-inflammatory agent. Metabolomic analysis showed noticeable difference in the chemical composition of dandelion from four different geographical regions, and the difference is mainly related to flavonoid biosynthesis. It was concluded that the environmental differences and stress conditions in those four geographical regions contribute to the change in metabolites in dandelions (Zhang et al. 2021). Another environmental change-related metabolite profile change has been studied in *Phaseolus vulgaris* L. (common bean) germplasm collection. The untargeted metabolomics by UHPLC-Q-Orbitrap-MS and targeted metabolomics by UPLC-Q-TOF-MS were carried out to identify the metabolite change (Mecha et al. 2022). The use of untargeted and targeted metabolomics to identify the metabolome changes under exposure to diclofenac (DCF), one of the environmental risk factors, in *Lemna minor* (an aquatic plant model) was studied by Wahman et al. (2022). The study was performed by using the RPLC-HILIC-ESI-TOF-MS platform and results showed metabolite variation in control and treated samples. Out of the two platforms used for the metabolome analysis, the untargeted metabolomics approach provided more information about changes in metabolites such as organic acids, lignin, sugars, amino acids, dipeptides, flavonoids, bioflavonoids, fatty acids, etc.

5 Metabolomics and Fluxomics: Tools for Metabolite Pathway Engineering

Metabolomics is growing along with another area of a study named fluxomics. While metabolomics deals with the quantification and analysis of all metabolites, fluxomics deals with the fluxome or the total set of fluxes in the metabolic pathway. Current approaches of fluxomics rely on the use of an isotope-labeled or ^{13}C-labeled precursor of metabolic pathways (Barrales-Cureño et al. 2021; Cascante and Marin 2008). Cocuron et al. (2019) conducted a comparative metabolomic and fluxomic study in the embryo of two maize varieties named alex and LH59, which are observed with 48% and 34% oil production, respectively. For metabolite and biomass extraction, HPLC-coupled mass spectrometry and GC-MS analysis were carried out. The isotope-labeled fluxome analysis and quantification were helped with NMR, GC-MS, and LC-MS/MS. The metabolic pathway contribution to FAS (fatty acid synthesis) in terms of carbon, reductant, and energy provision was identified by ^{13}C-metabolic flux analysis (MFA). The alex embryo was observed with altered metabolism without any change in their carbon conversion efficiency (CCE). It was noticeable that the increased production of oil in alex is achieved through the increased entry of carbon into the plastids where the plastidic malic enzyme plays an important role in the overall process. They revealed that maize achieved increased oil production by rerouting carbon through specific metabolic pathways (Cocuron et al. 2019). Some of the other recent research works in plant metabolomics are listed below (Table 13.2). Thus, change in carbon flux could also

Table 13.2 Targeted and untargeted approach of metabolite profiling in selected plants

Category	General uses	Target source	Method	Inference	Reference
Targeted metabolomics • Accurate and precise quantification of metabolites • Requires prior knowledge of target of interest	Identification and extraction of specific metabolites	*Vicia villosa* (Hairy vetch)	HPLC-MS/MS	Interspecific competition of hairy vetch with rye (*Secale cereale* L.) altered flavonoids accumulation in the root exudate of hairy vetch	Hazrati et al. (2021)
		Different diverse crop plants	LC-MS/MS	Provided targeted metabolites assay for the analysis of metabolites in crop plants samples Possible to quantify up to 206 plant metabolites	Zheng et al. (2021)
		Fruits and beverages	UPLC/QqQ-MS/MS	Targeted metabolomics assay developed to identify 135 phenolics	Vrhovsek et al. (2012)
Untargeted/non-targeted Metabolomics • Measurement and comparison of all detectable signals in a series of unknown samples • No prior knowledge needed	Biomarker development Track bioactive compounds Metabolite identification Identification of adulteration in botanical samples	*Medicago sativa, Pisum sativum, Trifolium pratense,* and *Vicia faba*	UHPLC-MS	Host plants metabolite profile has changed upon infection with pea aphid (*Acyrthosiphon pisum*) insect Major infection responsive metabolites identified are flavonoids, saponins, non-proteinogenic amino acids, and peptides	Sanchez-Arcos et al. (2019)
		Plants from *Betulaceae* family	UHPLC-LTQ-IT-MS/MS	Analyzed antioxidant compounds in the *Betulaceae* family and identified 19 candidate metabolites Ethyl gallate was identified as the most active antioxidant in one of the plant species	Lee et al. (2019)
		Nicotiana tabacum	GC-MS	Identified association of plant metabolomes with natural climate and geography Annotated 171 non-polar and 225 polar metabolites	Ma et al. (2019a, b)

		Sorghum bicolor	UHPLC-MS	Change in metabolites during *Burkholderia andropogonis* infection was analyzed Identified metabolites like salicylic acid, jasmonic acid, and zeatin as biomarkers of sorghum defense	Mareya et al. (2019)
		Fragaria × *ananassa* (Strawberry)	LC-MS/MS and GC-MS	Annotated differentially expressed metabolites of strawberry under osmotic stress	Antunes et al. (2019)

result in enhanced production of the desired metabolite and hence an active area of research in secondary metabolite pathway engineering.

6 Conclusion

It is apparent that integration of transcriptomics with metabolomics can contribute to a deeper understanding of gene-to-metabolite pathways in plants. The detection of metabolite changes occurring during the different stages of plant development like leaf maturation, flowering, post-flowering phase, stages of fruit setting is always accompanied with differential expression of genes and activation of enzymes. By superimposing the metabolic with that of transcriptomic data, one would be able to shortlist the candidate genes which are up and downregulated during the physiological process. The role of bioinformatics tools and database cannot be undermined as they play pivotal role in the whole process of secondary metabolite pathway analysis. Therefore, the integration of genetic, transcriptomic, and metabolic data is key to pathway analysis and engineering in future.

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Chapter 14
Databases Relevant to Phytochemicals and Genes That Govern Biosynthesis of the Phytochemicals

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1 Introduction

Phytochemicals have been known to possess pharmacological, biocidal and therapeutic properties and a market value which makes them more important for research and application. Numerous scientific investigations have been conducted for the discovery, characterization and isolation of phytochemical components giving rise to a massive amount of information and resources for further investigation. Phytochemical databases have considerably organized the vast amount of information concerning the natural compounds and their derivatives which have significantly aided the modern drug discovery and development process. The useful information collected from a wide variety of biological experiments and systems on a single platform is the purpose of metabolomics databases. The extraneous efforts made by developers to make these databases publicly accessible have turned the job easier for potential researchers. The data type that has been accumulated from different data sources varies and thereby often needs manual filtering and investigation to obtain an analytical form. Sometimes databases offer a conceivable form of data and other times they leave it to the user for customization.

Pest and pathogen infections cause huge crop losses threatening food security, endangering the food supply for more than a billion people on earth and indirectly lead to environmental damages (Tilman et al. 2011; Godfray et al. 2010; Alaniz et al.

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M. K. Swamy, A. Kumar (eds.), *Phytochemical Genomics*,
https://doi.org/10.1007/978-981-19-5779-6_14

2020). Around the globe, the highest potential crop loss was due to weeds (34%), followed by animal pests (18%) and pathogens (16%) during the period 2001–2003 (Oerke et al. 2012). The losses scale up to a whopping $470 billion annually by arthropods alone (Culliney 2014). A total of 40% of crop produce worldwide is destroyed by insects, pests, weeds and plant pathogens, out of which 14% is due to arthropods (Pimentel 2009). In the US, the invasive pests and pathogens cause crop production losses of over $40 billion every year (Paini et al. 2016). The concerns of environmental safety, biodiversity conservation and negative impacts of pesticides on human health and well-being have raised the call for a sustainable intensification (SI) of agricultural production and safer pest management to meet the demands and cover the losses (Waterfield and Zilberman 2012). The development of pest resistant plants is important to meet the burgeoning demands for food by the world population. Plant natural active substances like Antimicrobial peptides (AMPs), Natural Products (NPs) and medicinal plant- and plant Essential Oils (EOs) play an important role in the resistance mechanism against pest and pathogens and offer a natural and economically efficient method to counter pathogen infections and diseases. Few such natural amino acid derivatives which target fungi, insects and weeds have been discussed with their mechanism of action and application in crop protection (Lamberth 2016). Ouyang et al. (2014) and Zhang et al. (2012) have reviewed the phytochemicals that possess anti-cancer properties and can induce autophagy. A number of web resources and databases for these natural agents have facilitated their exploitation for pest management and control. Their anti-microbial activity and applicability has been assessed in order to make them accessible for sustainable development of pest resistant crops (Gurr and You 2016). The web portals available for these natural active agents are of special interest for scientists and researchers working in the field of botany, agriculture, biomedical industry and pharmaceutical companies.

Antimicrobial resistance is another major threat to agriculturists and the human health sector, and must be addressed urgently before the pathogens outplay us in all possible strategies. Current intensive non-environmentally favorable agricultural expansion has unintentionally led to the evolution of antimicrobial resistance (Tomasetto et al. 2017). Machine-based prediction tools based on NMR spectra and molecular descriptors have been designed for discovering new NP leads with accuracy of more than 70% in order to overcome antibiotic resistance (Dias et al. 2019). Bacterial pathogens often serve as a host of a plethora of antimicrobial agents and active compounds that can be exploited in the form of druggable molecules (Maglangit et al. 2021). Bacterial anti-biofilm agents and derivatives can prove to be natural therapeutics in combination with conventional drugs for biofilm forming bacterial infections (Melander et al. 2020). Web servers like Auto *In Silico* Macromolecular Mutation Scanning (AIMMS) help predict the free energy changes upon mutations in view of drug resistance for any combination of ligand and protein to help develop new antibiotics rationally (Wu et al. 2020).

In an increasingly stringent regulated world, the impetus for alternative pest management strategies is heightened with focus shifting towards biological control for a more regulated and effective control of pests and insects. Biological

insecticides, antifeedants and repellents are a cost-effective method for long-term refuge from pest attacks for commercial crops (Isman 2006). Human and animal health is the topmost priority globally, however, in countries with stricter norms, the commercialization of botanical products has considerably lagged behind (Isman 2020). In countries with relaxed regulatory norms and where pest infestation causes massive losses to the overall yield and production, combination of botanical insecticides and "reduced risk" conventional pesticides can be practical solutions (Isman 2008).

Biopesticides, plant-incorporated protectants (PIPs) and other related plant products are becoming more and more important in the current scenario of organic farming and sustainable agriculture (Seiber et al. 2014). Growing resistance to chemicals has pushed the bar for effective insecticides with augmented efforts being made in the direction of bioherbicides, bionematicides and biorational weedicides. More sustainable plant protection strategies like botanical insecticides have an immense potential in organic agriculture (Isman 2017).

The spider venom has been used as a successful peptide-based insecticide with a wide range of targets and can be used as stand-alone or with chemical insecticides (King and Hardy 2013; Windley et al. 2012; Herzig et al. 2014). Latest versions of a manually curated database ArachnoServer (www.arachnoserver.org) manage nucleotide and protein sequences, 3D-structures and functions of over 1400 mature spider toxins with diverse medicinal and agricultural applications (Herzig et al. 2010; Pineda et al. 2018). Recombinant baculoviruses, transgenic plants, toxin-fusion proteins and peptidomimetics are potent bioinsecticides with reduced health concerns and risks.

MassBank provides mass spectra of about 2337 primary and secondary plant metabolites for easy identification of chemical compounds (Horai et al. 2010). Similarly, Mery-B is an online platform useful for extraction, identification and visualization of 1H-NMR spectra of plant metabolites (Ferry-Dumazet et al. 2011). A highly efficient analytical database for comparing, visualizing and downloading metabolite data is PlantMetabolomics (PM.org) which even provides detailed protocols and manuals for conducting various experiments (Bais et al. 2010). A specialized database for visualizing, graphics and editing 3D structures of plant metabolites is in the form of Plant Metabolites Database (PMDB) with additional links to Kyoto Encyclopedia of Genes and Genomes (KEGG), PubChem and Chemical Abstract Service numbers (Udayakumar et al. 2012). Another comprehensive database for useful phytochemicals is KNApSAcK Core DB which comprises about 101,500 entries of plant species and the respective metabolites expanding over their molecular formulae, biological activities, geographical locations etc. An open access repository MetaboLights by European Bioinformatics Initiative (EMBL-EBI) allows the users to deposit and access raw and experimental metabolomics data types identifiable with a unique Id (Kale et al. 2016). Another repository RIKEN Plant Metabolome MetaDatabase (RIKEN PMM) stores metabolite profiles of plants based on gas chromatography-mass spectrometry (GC-MS) (Fukushima et al. 2018). Software packages like Xconnector provide a unified platform for retrieval and visualization of metabolomics data from at least nine databases namely, Yeast

Metabolome Database (YMDB; Ramirez-Gaona et al. 2017), Livestock Metabolome Database (LMDB; Goldansaz et al. 2017), Human Metabolome Database (HMDB; Wishart et al. 2022), KEGG (Kanehisa and Goto 2000), ReSpect Phytochemicals database (ReSpectDB; Sawada et al. 2012), Toxin and Toxin Target Database (T3DB; Wishart et al. 2015), The Blood Exposome Database (Barupal and Fiehn 2019), Small Molecule Pathway Database (SMPDB; Jewison et al. 2014) and Phenol Explorer Database (Rothwell et al 2013; Anwar et al. 2021). It is extremely efficient software which retrieves and seamlessly re-formats the information for a target metabolite from single or multiple databases to form an excel file using a python script. It uses keywords to search for the metabolite of interest and even generates a publication-ready graphical summary of the data retrieved for the convenience of the user. This software is available as an application compatible for different operating systems. With an enormous amount of data and research material on phytochemicals and related compounds, the development of databases becomes more meaningful and essential for outcome integration. Thus, we discuss the state-of-the-art of the phytochemicals databases and review their unique features, tools, integrations and limitations in view of their application in the design and discovery of novel therapeutic drugs. Also, this chapter will provide a trend line for the improvement and development of better databases and web portals catering to the needs and requirements in the modern scenario.

2 Antimicrobial Peptides (AMPs) Databases

Antimicrobial peptides are widely known low-molecular weight peptides which have protective properties against microbes, pests and pathogenic organisms. They have been employed for plant molecular farming where the insecticidal peptide is expressed in a plant-based alternative host for pest management and control (Holaskova et al. 2015). Structurally annotated therapeutic peptides (SATP; http://crdd.osdd.net/raghava/satpdb/) combine 22 publicly available peptide databases covering 19,192 unique natural, non-natural and modified peptides, with major functions in cancer, microbial infections, hypertension and drug delivery (Singh et al. 2016). PlantPepDB is another plant peptide database which systematically records about 3848 peptides which have been validated experimentally or predicted using homology (http://14.139.61.8/PlantPepDB/index.php) (Das et al. 2020). PlantAFP is a database of 2585 experimentally validated plant-based antifungal peptides curated from public databases, research publications and patents (http://bioinformatics.cimap.res.in/sharma/PlantAFP/) (Tyagi et al. 2019). Another database PhytAMP provides easy access to 271 valuable peptides of agricultural and pharmaceutical importance (http://phytamp.pfba-lab.org) (Hammami et al. 2009). Three updated versions of Antimicrobial Peptide Database (APD; http://aps.unmc.edu/AP) have been released over the years and it currently hosts the richest variety of 2619 natural AMPs from various natural sources such as bacteria, fungi, protists, plants and animals (Wang et al. 2016). Another update to CAMP Database,

$CAMP_{R3}$, has been added which includes sequences, structures and sequence-specific signatures of over 4000 AMPs (http://www.camp3.bicnirrh.res.in/) (Waghu et al. 2014, 2016). Compared with CAMP and APD, the new updated version of data repository of antimicrobial peptides (DRAMP; http://dramp.cpu-bioinfor.org/) contains 14,040 new entries of AMP sequences (Kang et al. 2019). In comparison, DRAMP2.0 covers the most number of antifungal (1761) and insecticidal (98) peptides. APD3, $CAMP_{(R3)}$ and DRAMP2.0 have been incorporated with prediction tools like iAMP-2L (Xiao et al. 2013), AVPpred (Thakur et al. 2012), AMPer (Fjell et al. 2007) and AntiBP2 (Lata et al. 2010) that mainly predict whether the peptide of interest is AMP or not, its class, post-translational modifications (PTMs), target pathogens or enzymes and lay out its functional and anti-microbial activity. The second class of tools integrated into databases are search tools like Basic Local Alignment Search Tool (BLAST), peptide mapping and similarity search which help identify potential AMP homologs to facilitate the design and discovery of new AMPs.

A specially developed database InverPep provides primary and physicochemical information of about 702 AMPs from invertebrate sources with 33 new AMPs that have not been reported earlier (Gómez et al. 2017). Other databases like DADP (http://split4.pmfst.hr/dadp/) and MilkAMP (http://milkampdb.org/), especially dedicated to AMPs from anuran tissues and dairy products, respectively, provide relevant structural, physicochemical and functional information on AMPs to be used in pharmaceutical and food industry (Novković et al. 2012; Théolier et al. 2014). AVPdb records the detailed information of about 2683 antiviral peptides targeting over 60 medically important viruses like HIV, SARS, HCV and HSV (http://crdd.osdd.net/servers/avpdb/) (Qureshi et al. 2014). Several prediction models based on the N and C terminal amino acid residues are developed for the accurate prediction of antibacterial peptides (AntiBPs) (Lata et al. 2007). An advanced model allows to predict and design novel AMPs based on the 3D structures (Liu et al. 2018).

However, there are certain limitations associated with the currently available AMP databases like lack of activity data and functional studies that may lead to false prediction. Despite ample databases based on other kinds of AMPs, there are still a scant number of databases that collect insecticidal peptides and their related information. Overall, there are a significant number of AMP databases which could serve as a source of inspiration for the development of novel peptide-based antimicrobial agents and compounds. The databases have been summarized and compared in Table 14.1.

3 Natural Products (NPs) Databases

Besides AMPs, there is a growing interest and research towards the NPs as potential pesticides and biocidal compounds for plant protection. NPs are defined as compounds that are derived from natural sources and have biological activities (Baker

Table 14.1 Some of the public-integrated databases for phytochemicals of insecticidal, fungicidal and therapeutic properties

Databases	Entries	Description	Web link
Antimicrobial peptides (AMPs) databases			
Structurally Annotated Therapeutic Peptides (SATP)	19,192	Unique AMPs from 22 publicly available databases	http://crdd.osdd.net/raghava/satpdb/
PlantPepDB	3848	Experimentally validated or predicted peptides	http://14.139.61.8/PlantPepDB/index.php
PlantAFP	2585	Experimentally validated AFPs	http://bioinformatics.cimap.res.in/sharma/PlantAFP/
PhytAMP	271	Peptides of agricultural and pharmaceutical importance	http://phytamp.pfba-lab.org
Antimicrobial Peptide Database (APD)	2619	AMPs of natural origin	http://aps.unmc.edu/AP
$CAMP_{R3}$	4000	Sequences, structures and sequence-specific signatures	http://www.camp3.bicnirrh.res.in/
Data Repository of Antimicrobial Peptides (DRAMP)	14,040	Most number of antifungal (1761) and insecticidal (98) peptides.	http://dramp.cpu-bioinfor.org/
InverPep	702	AMPs from invertebrate origin	http://ciencias.medellin.unal.edu.co/gruposdeinvestigacion/prospeccionydisenobiomoleculas/InverPep/public/home_en.
DADP	2571	AMPs from anuran tissues	http://split4.pmfst.hr/dadp/
MilkAMP	371	AMPs from dairy products	http://milkampdb.org/
AVPdb	2683	Antiviral peptides	http://crdd.osdd.net/servers/avpdb/
Natural products (NPs) databases			
Super natural	3,00,000	Pathway information and the mechanism	http://bioinformatics.charite.de/supernatural
Therapeutic Target Database (TTD)	1400	NP-derived drugs	http://bidd.nus.edu.sg/group/ttd/ttd.asp
Naturally Occurring Plant-based Anti-cancer Compound-Activity-Target Database (NPACT)	1500	Experimentally tested anti-cancer NPs	http://crdd.osdd.net/raghava/npact/
Three-dimensional structure database of Natural Metabolites (3DMET	8581	3D structures	http://www.3dmet.dna.affrc.go.jp/
CamMedNP	2500	Natural medicinal compounds from	

(continued)

Table 14.1 (continued)

Databases	Entries	Description	Web link
		the Cameroonian flora	
Natural Product Activity and Species Source (NPASS)	35,000	Quantitative activity records	http://bidd2.nus.edu.sg/NPASS/
AfroDb	1000	NPs from Africa	
North African Natural Products Database (NANPDB)	4500	NPs from Northern Africa	http://african-compounds.org/nanpdb/
South African Natural Compounds Database (SANCDB)	600	NPs from South Africa	https://sancdb.rubi.ru.ac.za/
Eastern Africa Natural Products Database (EANPDB)	1870	NPs from East Africa	http://african-compounds.org
Nuclei of Bioassays, Ecophysiology and Biosynthesis of Natural Products Database ($NuBBE_{DB}$)	2000	NPs from Brazilian biodiversity	https://nubbe.iq.unesp.br/portal/nubbedb.html
ETM-DB	1054	NPs from Ethiopian biodiversity	http://biosoft.kaist.ac.kr/etm
Alkamid	300	N-Alkylamides (NAAs) database	http://alkamid.ugent.be/
BIAdb	846	Benzylisoquinoline alkaloids database	http://crdd.osdd.net/raghava/biadb/
Collection of Open Natural Products (COCONUT)	406,076	Largest number of NPs	https://coconut.naturalproducts.net
Insecticides Physicochemical-Properties Analysis Database (InsectiPAD)	495	Insecticide likeness analysis	-NA-
Fungicides Physicochemical-Properties Analysis Database (FungiPAD)	-NA-	Fungicide likeness analysis	http://chemyang.ccnu.edu.cn/ccb/database/FungiPAD/
StreptomeDB 3.0	6524	NPs from Streptomyces	http://www.pharmbioinf.uni-freiburg.de/streptomedb
Plant Secondary Compounds Database (PSC-db)	2853	Enable Target prediction of PSCs	http://pscdb.appsbio.utalca.cl
Medicinal plants and essential oils databases			
Essential Oils Database (EssOilDB)	123,041	Volatile patterns of Terpenoids	http://nipgr.res.in/Essoildb/
	2000	Essential oils of Amazon Forest	-NA-

(continued)

Table 14.1 (continued)

Databases	Entries	Description	Web link
Database of the Amazon aromatic plants and their essential oils			
AromaDb	1321	Medicinal and therapeutic properties of aromatic compounds	http://bioinfo.cimap.res.in/aromadb/
Collective molecular activities of useful plants (CMAUP)	5654	Biological activities of medicinal plants	http://bidd2.nus.edu.sg/CMAUP/

et al. 2007). Among the biopesticides registrations with the Environmental Protection Agency (EPA), NPs constitute more than 50% of the registrations during the period 1997–2010 (Cantrell et al. 2012). Xie et al. (2015) had previously reviewed the development of three NP databases that compile the information on traditional herbal medicine in the Chinese provinces and two NP databases for natural and artificial toxins and available natural compounds, respectively (Dunkel et al. 2006; Schmidt et al. 2009; Chen 2011; Xue et al. 2012; Huang and Wang 2014). An extended version of Super Natural Database also provides the pathway information and the mechanism of action of around 300,000 NPs (Banerjee et al. 2015). New update of Therapeutic Target Database (TTD) covers the species-origin and families of almost 1400 NP-derived drugs (Zhu et al. 2012). Other databases which predict protein targets of herbal active ingredients and nutraceuticals are Herb Ingredients' Targets (HIT) and DrugBank (Wishart et al. 2008; Ye et al. 2010). About 1500 anticancer NPs that have been experimentally tested are summarized in Naturally Occurring Plant-based Anti-cancer Compound-Activity-Target Database (NPACT) (Mangal et al. 2013). To help in structure-based drug design, the 3D structures of most of natural compounds in the KEGG COMPOUND (https://www.genome.jp/kegg/compound/) can be accessed through an automatically and manually curated three-dimensional structure database of Natural Metabolites (3DMET) (Maeda and Kondo 2013). CamMedNP is specially developed for the structure and properties of the natural medicinal compounds from the Cameroonian flora (Ntie-Kang et al. 2013a). Natural Product Activity and Species Source (NPASS) is a freely available database which provides the quantitative activity records like half-maximum inhibitory concentration (IC_{50}) and minimum inhibitory concentration (MIC), sources and physicochemical properties of over 35,000 NPs from vast majority of species which target microbial species and proteins (Zeng et al. 2018). Other databases like AfroDb (Ntie-Kang et al. 2013b), North African Natural Products Database (NANPDB) (Ntie-Kang et al. 2017), South African Natural Compounds Database (SANCDB) (Hatherley et al. 2015), Eastern Africa Natural Products Database (EANPDB) (Simoben et al. 2020) and Nuclei of Bioassays, Ecophysiology and Biosynthesis of Natural Products Database ($NuBBE_{DB}$) (Valli et al. 2013; Pilon et al. 2017) gather systematic taxonomical and ethnopharmacological information of NPs from the

natural biodiversity in Africa and Brazil. SANCDB helps to visualize structures in four formats namely, Structure-Data File (SDF), Simplified Molecular-Input Line-Entry System (SMILES), Tripos Molecule Structure Format (MOL2) and Protein Data Bank (PDB) and also allows submitting new isolated NPs through their submission pipeline. $NuBBE_{DB}$ helps to predict NMR spectra of the natural compounds through its NMR Predictor tool. Another database focuses on Ethiopian herbal medicines and phytochemicals providing their physicochemical properties, molecular activity and toxicity levels (Bultum et al. 2019). Many natural herbs have applications in the treatment of cardiovascular diseases and are included in the Cardiovascular Disease Herbal Database (CVHD) with easy access and utility (Gu et al. 2013). Manually curated databases like Alkamid and BIAdb summarize the structural and functional information of *N*-Alkylamides (NAAs) and benzylisoquinoline alkaloids, respectively, which are important leads for therapeutic drugs and functional foods (Deepak et al. 2010; Boonen et al. 2012). Both BIAdb and ANPDB are laced with similarity and structure prediction tools. While BIAdb offers the largest number of physicochemical properties, ANPDB provides the richest collection of antibacterial and antiviral NPs. Collection of Open Natural Products (COCONUT) collects literary and functional information for the largest number of NPs from different open and web sources at one place (Sorokina et al. 2021). Two online platforms for determining the physical and chemical properties and the potentiality of new insecticides and fungicide candidates with an accuracy of 75% and 82.5%, respectively, are Insecticides Physicochemical-properties Analysis Database (InsectiPAD) and Fungicides Physicochemical-properties Analysis Database (FungiPAD) (Jia et al. 2019; Wang et al. 2019). A specially designed online database of predicted and tested NPs from the genus Streptomyces is StreptomeDB 3.0, which is the only database of its kind till date to be used by scientists and pharmacists (Moumbock et al. 2021). Another recent Plant Secondary Compounds database (PSC-db) facilitates estimation of Quantitative Structure–Activity Relationship (QSAR), enabling docking and target binding studies to enhance the discovery of new bioactive compounds (Valdés-Jiménez et al. 2021).

In short, a huge improvement is seen in the functionality of the phytochemicals databases with respect to their features and applicability. A few disadvantages still remain, for example, a large number of NP entries in these databases are not substantiated through controlled experiments. Another concern is the region specificity of some of the databases like AfroDb, NANPDB, SANCDB, etc., which are dedicated to collect information on NPs from the local biodiversity. Overall, the current databases are highly equipped in providing wholesome information about the useful NPs building a way for further investigation and research. Although NPs and their derivatives constitute more than half of the clinically used drugs and have huge potential in the pharmaceutical industry, newly discovered NPs are relatively barred from market approval over the past three decades (Paterson and Anderson 2005).

4 Medicinal Plants and Essential Oils Databases

Essential oils and aromatic compounds are highly useful in industries such as cosmetics, phytopharmaceutical and agribusiness in the form of insecticides and fungicides. Several plant essential oils, flavonoids, alkaloids and aromatic compounds have insecticidal and fungicidal properties and can serve as "reduced-risk" pesticides during the crop protection and preservation (Isman 2000; Hikal et al. 2017; Campos et al. 2019; Ma et al. 2020). KOVATS.DB is a ready to use software that requires a copyrighted Paradox Software as a prerequisite prior to installation and has a collective literature on MS spectra of the components of essential oils and other volatile food aromatic compounds (Libbey 1991). Several essential oils from higher plants possess anticandidal properties and were collected from an Antimicrobials Database (Amicbase) (Pauli 2006). A special database for the essential oils and aromas of the plant species of the Amazon Forest has been developed which lists general information of at least 350 plants (Maia and Andrade 2009). Another Essential Oils Database (EssOilDB) provides the volatile patterns of 123,041 essential oils specially highlighting the diversity and range of terpenes from 92 plant taxonomic families (Kumari et al. 2014). EssOilDB serves as a literature-based resource for the presence of skin allergens in essential oils in order to assist in making safe cosmetics (Dornic et al. 2016). AromaDb was developed as a comprehensive resource for the structural and biological properties of medicinal and aromatic compounds (Kumar et al. 2018b). Collective Molecular Activities of Useful Plants (CMAUP) organizes activity levels, gene ontologies (GO), pathway associations and protein targets of multiple plant active ingredients (Zeng et al. 2019). Medicinal Plants Database for Drug Designing (MPD3) offers a freely accessible and downloadable docking platform for phytochemicals and their targets, along with their activities and literature references (Mumtaz et al. 2017). Another universal database hosts pharmacological information on 147 medicinal plant species and 369 bioactive compounds from around the world (Sargia et al. 2018). Some specialized databases like CathaCyc and SoyMetDB incorporate tissue specific metabolite profile and transcriptome data of Catharanthus roseus and Glycine max, respectively, for pathway networking and visualization (Joshi et al. 2010). Metabolomics and transcriptomic data of twenty different growth/developmental tissues from fourteen medicinal plants were compiled and analyzed in Medicinal Plant Metabolomics Resource for contributing towards drug development and design (Wurtele et al. 2012). Indian Medicinal Plants Phytochemistry and Therapeutics (IMPPAT) and InDiaMed are dedicated to offering the structural properties and drug likeness of therapeutic phytochemicals from medicinal plants of the Indian biodiversity (Tota et al. 2013; Mohanraj et al. 2018). Uttarakhand Medicinal Plants Database (UMPDB) specifically collects basic and literary information of about 1127 medicinal plants from the districts of the state of Uttarakhand (Kumar et al. 2018a). Other databases like HerbalDB2.0 provide the 3-D structures of 1405 chemical compounds from Indonesian biodiversity. Eleven Native American medicinal plants were assessed for the qualitative and quantitative analysis of essential oils

and terpenoids and reported in a literature resource (Lawson et al. 2021). Another literary source compiles the essential oil composition of the aromatic medicinal plants of Uzbekistan (Mamadalieva et al. 2017). Saudi Herbal Plants Information System (SHPIS) integrates local and community information on 120 natural Saudi herbal varieties which may aid in development and research.

5 Conclusion

Databases are essential for managing and curating humongous data nowadays. With the advancements in computational programs and software, we can handle large datasets and take useful information from it. The phytochemicals have huge importance for humans as well as drug discovery, testing and commercialization. With hundreds of plants sequenced and thousands of genes and metabolites identified, the development of the databases is natural and must be encouraged. The databases on the medicinal plants, their bioactive compounds, the mode of the action of bioactive compounds and any side effects should be attempted. Even though the empirical evidence for the phytochemicals is increasing at a massive rate, there is still a long way for them to have much impact in the marketplace. This may be due to several reasons like scarcity of the resource, time and extraneous efforts in isolation and extraction of the active ingredients, quality control and standardization, reduced stability and bioavailability and the extensive registration process. These limitations have been tried to overcome through high-throughput approaches like library generation, synthesis and virtual screening using NP as lead structures (Pascolutti and Quinn 2014; Hao et al. 2016; Chen et al. 2020). The future studies related to databases on medicinal plants and their phytochemicals must focus on the limitations associated with the currently available databases and those limitations should be addressed. The databases should be easily accessible, easy to use and user-friendly.

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Chapter 15
Trichomics: Trichomes as Natural Chemical Factories

Rakhi Dhankhar, Karuna Regmi, Anubhuti Kawatra, and Pooja Gulati

1 Introduction

Trichomes are tiny hair-like structures which extend from the epidermis of aerial parts of plants and roots hairs (Zhang et al. 2021). These structures can be unicellular or multicellular and are generally not connected to the plant vascular system (Wagner et al. 2004). Trichomes are tremendously diverse in terms of size, shape, density, morphology, composition, functions, and type of secreted compounds which depend on the plant species they are present on (Glas et al. 2012). On a single species or even a single plant, different types of trichomes can co-exist (Huchelmann et al. 2017). The size of trichomes can vary from few microns to centimeters; for example, the mature trichomes in an *Arabidopsis thaliana* length in millimeters whereas the length of cotton trichomes can be as long as 20 cm (Wang et al. 2019). Trichomes are classified into glandular and non-glandular categories; the glandular trichomes secrete and store various secondary metabolites.

Trichomes act as first line of defense for plants against various environmental stresses. The immobile nature of plants makes them more vulnerable to various abiotic and biotic stresses. The various secondary metabolites secreted by trichomes protect the plants against herbivory, various phytopathogens, and insect pests (Wagner et al. 2004). Besides providing a physical barrier against harmful radiations and adverse temperatures, trichomes also enhance plant's fitness by reducing transpiration rates and thus drought stress (Łaźniewska et al. 2012). The stickiness, fuzziness, and the smell (like in mint and basil leaves) on various plant leaves or stems are due to phytochemicals produced by the trichomes. However, certain evidence also suggests their negative roles as they can serve as sites for microbial

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M. K. Swamy, A. Kumar (eds.), *Phytochemical Genomics*,
https://doi.org/10.1007/978-981-19-5779-6_15

infections but the benefits offered by trichomes overweigh their negative impact (Imboden et al. 2018).

In the past two decades, trichomes have attracted substantial attention due to their ability to synthesize, store, and secrete a wide variety of secondary metabolites which can be exploited for human use (Schuurink and Tissier 2020). The plant-based secondary metabolites are used for the synthesis of essential oils, natural pesticides, fragrances, pharmaceuticals; e.g., the cotton trichomes are one of the most widely used fibers; similarly nutrient-rich trichomes in tea are crucial in tea quality (Wang et al. 2021). Although some of these plant secondary metabolites can be chemically synthesized, however many of them are highly complex with regard to their structure and complex precursors (Huchelmann et al. 2017). The complexity of the synthetic process makes it uneconomical; additionally, chemical synthesis is also a not environment-friendly process. Thus, trichomes are commercially exploited for the production of these important plant secondary metabolites (phytochemicals) and hence they are often termed as 'biofactories' (Wang 2014). Synthetic biology approaches have been carried out to engineer the metabolic pathway for the synthesis of many secondary metabolites (Wang 2014). Thus, the knowledge is translated from plants to the microbes like baker's yeast (*Saccharomyces cerevisiae*) which have been used for the heterologous production of these compounds (Wang 2014).

The 'omic exploration' has significantly impacted trichome research. The pathways and genes concerning the growth and development of trichomes are now well elucidated. This knowledge has helped to develop a new branch of science that is termed as 'trichomics'. The advances in mass spectroscopy and metabolic profiling techniques have now facilitated to study hundreds of metabolites in even a single cell (Huchelmann et al. 2017). This resulted in the discovery of many metabolites from glandular trichomes.

Considering the wide importance of trichomes for plants as well as humans, a detailed knowledge in this field is indispensable. The present chapter discusses various aspects related to trichomes. Their classification and biological functions along with different phytochemicals produced by trichomes are discussed in detail. The recent advancements involving engineered pathways for developing trichomes as natural chemical factories have also been elaborated.

2 Trichomes: Classification and Structure

Trichomes are unicellular or multicellular appendages that are present in almost all vegetative and reproductive organs in the angiosperm plants (Celedon et al. 2020). Based on the characteristics and functions, trichomes have been classified into non-glandular trichomes and glandular trichomes (Werker 2000). Non-glandular trichomes are unicellular or multicellular, branched, or unbranched. They do not participate in synthesis and secretion of specialized compounds. Although the non-glandular trichomes are devoid of secretory glands, they can store large amounts of phenolics (without secreting them). The non-glandular trichomes are involved in

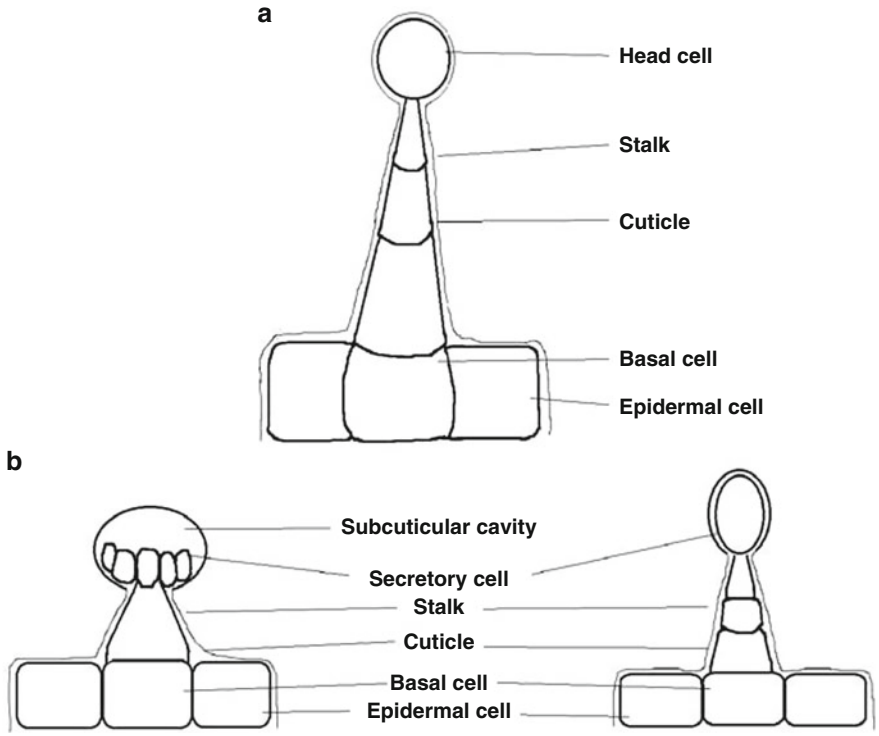

Fig. 15.1 Simplified structure of glandular trichome, (**a**) Glandular Trichome, (**b**) Peltate Glandular Trichome, (**c**) Capitate Glandular Trichome

protective and defensive roles against various abiotic and biotic stresses (Karabourniotis et al. 2020). The focus of the present chapter will be on glandular trichomes as they are the sources of various plant metabolites.

Glandular trichomes (GTs) are usually multicellular and found on about 30% of all vascular plants (Fahn 2000). They are characterized by synthesis, storage, and secretion of secondary (specialized) compounds. Their structure involves basal cell, stalk, and apical secreting cells (Fig. 15.1). Head is the site for production of specialized compounds by secretory cells. On the basis of the head size and stalk length, glandular trichomes can be subdivided as peltate and capitate trichomes (Fig. 15.2). Peltate GTs consist of one basal cell, small stack, and few secretory cells which make up a big head. The secreting cells of the head are arranged in one or two concentric circles and on its top is a subcuticular storage space that stores compounds produced by secretory cells and due to this the trichomes appear as bulb-like structures (Turner et al. 2000). On the other hand, capitate GTs possess a single basal cell, few stalk cells, and head with secreting cells (Maffei 2010). The basic structure of peltate and capitates is depicted in Fig. 15.1. Exudates released from these GTs are non-volatile compounds (Tissier et al. 2012).

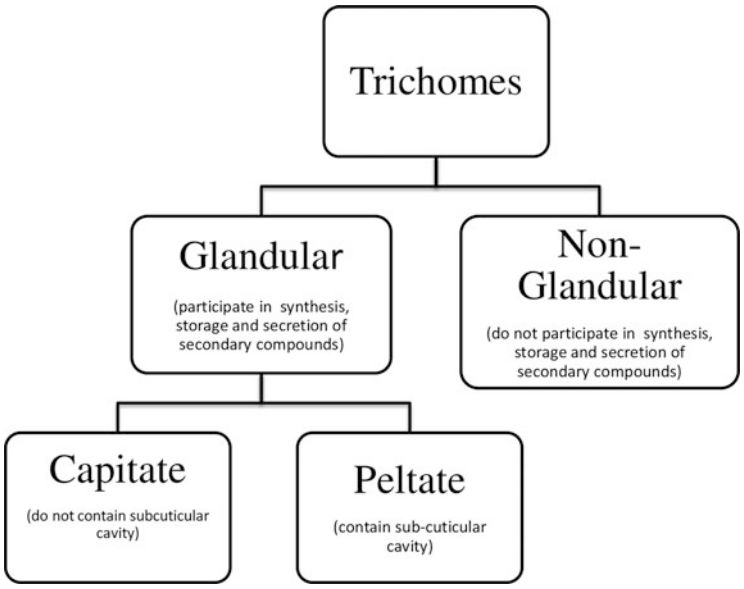

Fig. 15.2 Different types of trichomes

3 Development of Trichomes

In order to exploit the glandular trichomes by metabolic engineering, a complete understanding of their development is needed. Also, trichome development can serve as an excellent model to study plant cell differentiation. Trichome development initiates when signals sent by neighboring cells of an epidermal cell drive it to acquire trichome identity. This epidermal cell then divides in a controlled manner (Huchelmann et al. 2017). The model for the studies of non-glandular trichome development has been *A. thaliana.*

A. thaliana has been widely used as a model because its various mutants are available which help in studying about trichome development. The cells giving rise to trichomes undergo endoreduplication; i.e., the DNA of these cells divides one to four times without nuclear and cell division (Walker and Marks 2000). About 70 genes have been identified which take part in trichome development (Pattanaik et al. 2014). Regulation of these genes is done by positive and negative regulators (Hülskamp 2004). Various transcription factors such as bHLH, MYB, WDR, C2H2 act as positive regulators in *Arabidopsis* (Kirik et al. 2005). A MBW complex plays a significant role in trichome development in *Arabidopsis* (Fambrini and Pugliesi 2019) (Fig. 15.3). MBW (MYB/bHLH/WD) is formed by the products of genes *GLABROUS1 (GL1), TRANSPARENT TESTA GLABRA1 (TTG1),* and *GLABRA3/ ENHANCER OF GLABRA3 (GL3/EGL3)* (Walker et al. 1999; Zhang et al. 2003). *GL1* codes for MYB23 and functions in trichome initiation. *GL3* and *EGL3* are involved in trichome development (Kirik et al. 2005). Mutants *gl3* and *egl3* give rise

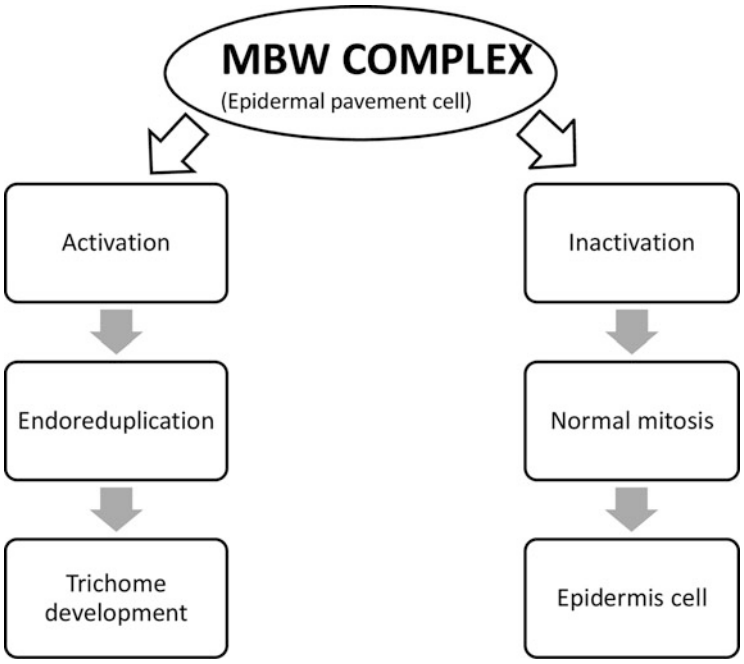

Fig. 15.3 A simplified diagram for trichome development in *Arabidopsis thaliana*. Activation of MBW (MYB/bHLH/WD) causes trichome development via endoreduplication. Inactivation of the MBW complex gives rise to epidermal cell via normal mitosis

to glabrous phenotype (Pattanaik et al. 2014). *TTG1* codes for WD40 (W-Trp, D-Asp) repeat which regulates various processes in trichome development such as cell signaling, cell cycle, determination of cell fate (Zhang and Schrader 2017). Mutants such as *gl1* and *ttg1* give rise to hairless phenotype (Hülskamp 2004). If the MBW complex is activated, trichome development occurs via endoreduplication process but in case of its inactivation, normal mitosis of epidermal pavement cell occurs (Zhang and Schrader 2017). Various hormones such as gibberellins, cytokinins, and jasmonic acid also help in trichome development (An et al. 2011; Pattanaik et al. 2014).

4 Biological Functions of Trichomes

Trichomes play a major role in the plant's first line of defense and they produce secondary metabolites which are not only essential in growth and development of plants but also needed by the plants during stress conditions and help in attracting predators of herbivores and repelling harmful pathogens (Glas et al. 2012). Trichomes help to cope with environmental stresses by acting as a physical and

chemical defense system (Wang et al. 2021; Li et al. 2018). Physical protection against environmental stresses can be induced by forming a mechanical barrier like development of thick and dense covering of trichome. This covering protects the plant from low humidity, high temperature, insect damage and also reduces the rate of transpiration (Wagner et al. 2004).

Two types of plant defense systems have been identified. One is constitutively active and the second is an inducible defense system which gets activated by herbivore or pathogen attack. Jasmonic acid (JA) is very important for inducible defense (Glas et al. 2012). Octadecanoid pathway gets activated by interaction of plants with pathogens or herbivores, thereby resulting in an increase in the synthesis of JA which in turn induces the expression of defense genes (Kant et al. 2004). Plant defense system can also be divided into direct and indirect defense. Direct defense includes production of compounds by plants, specifically trichomes to directly attack the predators or reduce herbivory. Indirect defense system involves the production of compounds which attract natural enemies of the attackers or predators of the plant which indirectly reduces herbivory. The phytochemicals produced by the GTs act as chemical barriers and hence major effectors of a plant's defense. Metabolic engineering has been applied to manipulate secondary metabolite production so that plant's protection can be enhanced and the plant can survive for a longer period of time. The role of GTs in protection against abiotic and biotic stresses is described below.

4.1 Abiotic Stress Tolerance

Abiotic stress causes negative impact on living organisms by nonliving variants. Abiotic factors include heat, cold, temperature, drought, heavy metals, and ozone, which influence the living beings and have adverse effects on them. Evidence suggests that GTs have a major role in providing tolerance to heavy metal and ozone stress (Ciriaková 2009) (Ainsworth et al. 2012).

Due to human activities, heavy metals (HM) have been accumulating in the biosphere which are harmful to the living beings. These are non-biodegradable and cause disastrous effects on plants and animals (Ciriaková 2009). If the levels of HMs are high, it can lead to damage of cellular components such as DNA and enzymes which can end up in formation of reactive oxygen species (Ali et al. 2013; Zengin and Munzuroglu 2005). The excessive ROS formation can cause serious damage to plant cells including DNA damage, protein degradation, disruption of cell components and membranes, leakage of ions, redox imbalance thereby leading to programmed cell death (Sharma et al. 2012). With the help of trichomes, plants fight against HM stress by using various defense strategies such as storing the heavy metals, secreting secondary metabolites to reduce the toxic effects of heavy metals, and by expressing genes which encode for proteins which counteract the effects of heavy metals (Küpper and Kroneck 2005). Researches on *Nicotiana tabacum* have shown the role of trichomes in HM detoxification. Trichomes exuded cadmium

(Cd) crystals when a tobacco plant was exposed to high levels of Cd (Harada and Choi 2008). It has been observed in case of tobacco that the trichomes expressed genes encoding proteins such as glutathione peroxidase and T-phylloplanin like proteins which are antipathogenic. In *Vicia faba*, high expression of metallothionein which helps in metal tolerance has been reported (Foley and Singh 1994). Further, it has been reported in case of *Leontodon hispidus* that high levels of metals such as calcium cause the plant to accumulate this HM in its trichomes to sequester its negative effects on plant (de Silva et al. 1996).

GTs have also been shown to provide resistance to the significantly increasing levels of tropospheric ozone (O_3) which is damaging to all living beings (Luckwill 1943). The mechanisms which infer this resistance are still not well understood yet (Ainsworth 2017). O_3 encourages ROS formation in plants which deteriorates the cell and induces cell death (Cho et al. 2011; Kanagendran et al. 2018). GTs have more resistance to high O_3 levels while non-glandular trichomes are not resistant toward it. Density of GTs is also related to O_3 resistance as plants with low GT density showed more vulnerability to O_3 (Li et al. 2017).

4.2 Biotic Stress Tolerance

Trichomes also provide a chemical defense system against various pathogens (Peiffer et al. 2009; Tian et al. 2012). The role of GTs in protecting the plants against attack by insects is well elucidated. It has been reported that O-acyl sugars which are sticky compounds secreted from GTs of Solanaceae plants act as defense mechanisms against many insects When an insect try to attack the plant, the stickiness or high density of trichomes renders its movement which eventually leads to the death of insect due to its inability to move and feeding inhibition Weinhold and Baldwin 2011). Tobacco NtLP1 is a protein which causes the GTs to secrete lipids, when this protein was overexpressed in transgenic tobacco lines, it elevated the aphid tolerance (Choi et al. 2012). Hairless (Hl) gene in *Solanum lycopersicum* L. plays a significant role in trichome development and accumulation of secondary metabolites such as terpenes and phenolics which infer resistance against insect attack (Kang et al. 2010). Recessive hairless (hl) mutation resulted in reduction in terpenes and phenolics secretion and also showed reduction in resistance against insect attack (Kang et al. 2010).

GTs also resist attack against various fungal species. GTs of *Solanum berthaultii* secrete compounds which make these plants resistant to fungal species such as *Phytophthora infestans* (Lai et al. 2000). Extremely acidic exudates of GTs in *Cicer arietinum* when present in low concentration facilitate germination of fungus *Ascochyta rabiei* but high concentration of exudates inhibits fungal spore germination (Armstrong-Cho and Gossen 2005). *Peronospora hyoscyami f.sp. tabacina* causes blue mold in tobacco and its germination is hindered by T-phylloplanin, a trichome-specific glycoprotein (Kroumova et al. 2007).

In case of *A. thaliana*, the trichomes were observed to be able to create response against different waves such as chewing sounds of caterpillar which triggered the trichomes to synthesize secondary compounds which act against the pathogen (Appel and Cocroft 2014). Contact urticaria is a very common disease caused by stinging nettle (*Urtica sp.*). When the trichomes of these plants are touched, they are broken down and the toxic compounds present inside them penetrates our skin which causes allergy like symptoms such as hives and irritation (Levin 1973). The immediate irritation caused by these trichomes is due to histamine production which is responsible for inflammatory response and itching (Thangam et al. 2018). In ash gourd (*Benincasa hispida*), high trichomes density helped in providing resistance against viruses. Aphids (*Aphis gossypii* and *Myzus persicae*) acted as vectors for viruses such as papaya ringspot, watermelon mosaic virus, cucumber mosaic virus and destroyed the ash gourd by transmitting the viruses (Khan et al. 2000).

In several model plants like tobacco, tomato, cotton, and corn, GTs have been related to indirect defense mechanisms in protecting the plants against herbivory (De Moraes et al. 1998; Kant et al. 2004; Schnee et al. 2006). This indirect defense mechanism includes the production of volatile compounds such as terpenoids which attract predators of the herbivores. For instance, production of sesquiterpene (E)-β-farnesene from GTs of *S. berthaultii* attracts parasitoids (*Diaeretiella rapae*) of aphids (Beale et al. 2006) but it also exhibits direct mechanism by repulsion of this aphid (*Myzus persicae*) (Gibson and Pickett 1983). Zingiberene is a sesquiterpene which repels silverleaf whiteflies (*Bemisia tabaci*) (Bleeker et al. 2009, 2011) and tobacco spider mite (*Tetranychus evansi*) (Maluf et al. 2001) and is also toxic to larvae of Colorado potato beetle (*Leptinotarsa decemlineata*). Infection of *S. habrochaites* foliage with beet armyworm larvae (*Spodoptera exigua*) was increased when sesquiterpene was removed by using menthol (Eigenbrode et al. 1994). Phenylpropenes also contribute to defense against herbivory. For example, eugenol is a phenylpropene which protects the plants against coleopteran species, nematodes, and fungi like *Cladosporium herbarum* (Obeng-Ofori and Reichmuth 2010; Adams et al. 1996; Sangwan et al. 1990). 2-Tridecanone is a methyl ketone which contributes to plant protection against various herbivorous arthropods such as *Manduca sexta, Aphis gossypii, Helicoverpa zea* (Williams et al. 1980; Dimock and Kennedy 1983), *Macrosiphum euphorbiae* (Musetti and Neal 1997), and 2-undecanone destroys two-spotted spider mite (Chatzivasileiadis and Sabelis 1997). Acylsugars produced by GTs exhibit both direct and indirect defense mechanisms. Direct defense includes capturing of pests and preventing their movement due to stickiness of these acyl sugars (Wagner et al. 2004) and it also includes repulsion of pathogens (Goffreda et al. 1989). Indirect defense has been shown in case of larvae of lepidopteran herbivore species which eat trichomes containing acyl sugars, and due to this, they give out a certain type of smell making them appear as enemy of their whereabouts (Weinhold and Baldwin 2011).

5 Emergence of Trichomics

The advancement of technology has revolutionized trichome research. With the rapid development in high-throughput techniques and 'omics' approaches along with reduced cost of sequencing, novel genes and pathways related to trichomes have been discovered. The various modern approaches like CRISPR/CAS9-mediated knockout and siRNA-mediated knockdown techniques have aided in studying the various genes involved in initiation, growth, and development of trichomes (Zhang et al. 2021). A collaborative approach involving genomics with transcriptomics followed by proteomics and metabolomics along with the knowledge of system biology has led to striking revelations related to trichomes and thus a new branch termed as 'trichomics' has evolved.

The phytochemical analysis of the trichomes has surprised the scientists due to the tremendous diversity of its molecules. The trichome cells of the leaves are entirely different from the leaf epidermal cells in terms of shape, structure, and biochemistry. This has startled scientists. The scientists have tried analyzing the trichome cells for their metabolites. Further genomics and transcriptomics and metabolomics have been specifically targeted to understand the genomic basis of the trichome secondary metabolites. The analysis of trichomes for transcriptomes has helped identify many specific genes that are not found in other cells suggesting the unique genetic makeup of the trichomes. This understanding of genes involved in synthesis of various secondary metabolites has helped biotechnologists to design processes and engineer various metabolic pathways for their large-scale industrial production. Besides, on the basis of this knowledge, many trichome-based databases like www.planttrichome.org and http://bioinfo.bch.msu.edu/trichome_est have been developed that further help in advancing trichome research (Wang 2014).

Although an apparent progress in trichomics has been achieved in past few years, still there are many dimensions that remain unexplored. First and foremost, trichome biochemistry has not studied so far. The growth and development of trichomes have been largely studied in model plants like *Arabidopsis*, but the knowledge about trichomes in non-model plants is almost nil. Secondly, the role of trichome development genes in stress resistance in plants is not widely studied. There are reports that suggest dual role of such genes for example, with the help of modern techniques like chromatin immunoprecipitation (ChIP) assays, RNA-seq analysis, and RT-qPCR assay, it has been revealed that the genes like RGL2 which are involved in trichome differentiation also play role in seed dormancy and germination by integrating light perception, GA metabolism, and GA signaling pathways (Yang et al. 2020). This knowledge is crucial to facilitate stress resistance in economically important plants.

6 Phytochemical Diversity of Trichomes

GTs are regarded as natural chemical factories as they synthesize, store, and secrete a wide variety of chemicals, which were earlier assumed to be secreted by leaves or stems (Fahn 2000; Schilmiller et al. 2008). These compounds involve terpenes, methyl ketones, acyl sugars, phenylpropenes, etc. Further, high-throughput techniques have been employed in turning these GTs into chemical factories by amplifying the secretion of the chemicals which can be used for various purposes such as in bio-control activities, food, and pharmaceutical industries. Some of the economically important phytochemicals produced within the GTs are described below.

6.1 Terpenes

Terpenes are unsaturated hydrocarbons and natural compounds with formula $(C_5H_8)_n$, where n is the number of linked isoprene units. Small isoprene units (Fig. 15.4) are joined to one another to form terpenes and oxygen-containing terpenes are called terpenoids. There are two biosynthetic pathways for terpenes—MVA (mevalonic acid) and MEP (methylerythritol phosphate) pathway. The MVA pathway occurs in cytosol and MEP pathway occurs in plastids (Tholl 2006;

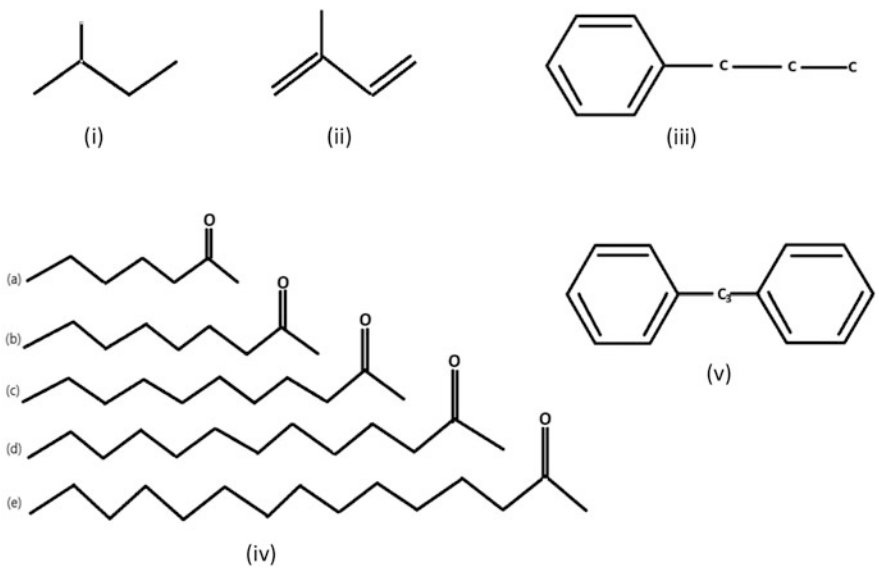

Fig. 15.4 Structure of phytochemicals produced by trichomes, (i) isoprene, (ii) isoprene unit, (iii) phenylpropanoid carbon skeleton, (iv) common methyl ketones found in plants. (a) 2-Heptanone, (b) 2-nonanone, (c) 2-undecanone, (d) 2-tridecanone, (e) 2-pentadecanone, (v) basic flavonoid carbon skeleton

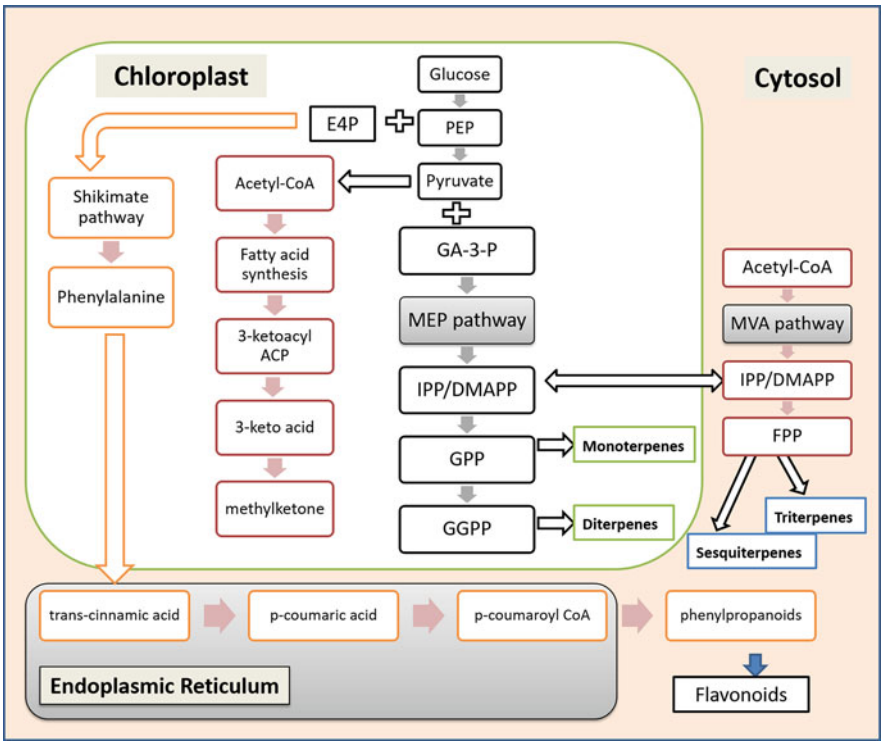

Fig. 15.5 Simplified representation of major metabolic pathways in glandular trichomes (GTs). *MVA* mevalonic acid, *MEP* methylerythritol phosphate, *IPP* isopentenyl diphosphate, *DMAPP* dimethyl allyl diphosphate, *GA-3-P* glyceraldehydes-3-phosphate, *GPP* geranyl pyrophosphate, *GGPP* geranylgeranyl pyrophosphate, *FPP* farnesyl pyrophosphate, *PEP* phosphoenolpyruvate, *E4P* erythrose-4-phosphate

Vranová et al. 2012). The building blocks of terpenoids are 5-carbon isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) and isopentenyl diphosphate isomerase (IDI) catalyzes their interconversion. In MVA pathway, pyruvate and glyceraldehyde-3-phosphate combine to form an intermediate which is eventually converted into IPP and DMAPP (Hoeffler et al. 2002; Rodíguez-Concepción and Boronat 2002), and in case of MEP pathway, three molecules of acetyl-CoA join together to form IPP (McGarvey and Croteau 1995) (Fig. 15.5). In further steps, head-to-tail condensation reaction takes place catalyzed by prenyltransferases (Wang and Ohnuma 2000), and in this reaction, geranyl pyrophosphate (C10), neryl pyrophosphate (C10), farnesyl pyrophosphate (C15), geranylgeranyl pyrophosphate (C20), and polyprenyl terpenoid precursor molecules are converted to cyclic and acyclic terpenoids with the help of enzyme terpene synthases (Chen et al. 2011; Falara et al. 2011). Further secondary transformations like hydroxylation, reduction, glycosylation occur which gives rise to terpenoids like monoterpenes, sesquiterpenes, triterpenes, diterpenes, and tetraterpenes (Croteau et al. 2005;

Degenhardt et al. 2009). Terpenoids are important in direct and indirect plant defense (Dicke and Sabelis 1987; Schnee et al. 2006).

6.2 Phenolics

A phenolic compound consists of an aromatic ring structure having one or more hydroxyl groups. Phenolics include phenylpropanoids, flavonoids, and tannins. Phenylpropanoids consist of a benzene ring and three carbon side chains (Fig. 15.4) and are formed from the products of shikimate pathway (Fig. 15.5) (Herrmann 1995). The shikimate pathway involves the biosynthesis of aromatic amino acids by erythrose-4-phosphate and phosphoenolpyruvate. The phenylalanine produced from the shikimate pathway undergoes non-oxidative deamination with the help of enzyme phenylalanine ammonia lyase (PAL) and forms trans-cinnamic acid. Hydroxylation of trans-cinnamic acid occurs and para-coumaric acid is formed and catalyzed by enzyme cinnamate 4-hydroxylase. Activation of para-cinnamic acid occurs and this activation reaction is catalyzed by 4-coumarate CoA ligase which results in the formation of para-coumaroyl CoA and this compound is precursor of various phenolic compounds (Fig. 15.4). Phenylpropanoids have been reported to play a role in attracting pollinators and plant defense (Obeng-Ofori and Reichmuth 2010; Tan et al. 2002). Phenylpropenes are present in essential oils produced by the species of Lamiaceae family (Croteau et al. 2005). The intermediate compound present in phenylpropanoid biosynthetic pathway, trans-cinnamic acid can be modified to produce compounds like benzenoids and methyl cinnamates. The former is secreted in low amounts and the latter is synthesized in large amounts by GTs (Kapteyn et al. 2007; Sumner et al. 2011). In basil peltate GTs, phenylpropenes such as eugenol and methyl chavicol and their corresponding enzymes chavicol o-methyltransferase (CVOMT) and eugenol o-methyltransferase (EOMT) have been reported to decrease in quantity when trichomes mature (Deschamps et al. 2006).

Another type of phenolics is flavonoids, which consist of two aromatic rings connected by a three-carbon bridge (Fig. 15.4). Pigments in flowers, fruits, and seeds are the most well-known flavonoids. Flavonoids biosynthesis involves the condensation of 4-coumaroyl CoA (intermediate compound of phenylpropanoid pathway) and malonyl-CoA and this reaction is catalyzed by chalcone synthase (CHS) and an additional cyclization reaction occurs. In further reactions, variety of flavonoids are formed by modification of basic flavones structure by hydroxylation, methoxylation, prenylation, or glycosylation reaction and these reactions give rise to different flavonoid groups such as anthocyanins, flavones, flavonols, isoflavones, and flavonones (Ferrer et al. 2008). Flavonoid glycosides production has been reported in GTs of *Phillyrea latifolia* when these plants were exposed to sunlight indicating that flavonoids play a role in UV protection (Tattini et al. 2000). Cannabinoids are also phenolic compounds produced by hemp (*Cannabis sativa*) exhibiting psychoactive and medicinal properties (Ross and ElSohly 1996).

6.3 Methyl Ketones

Methyl ketones are fatty acid-derived compounds which are synthesized and stored in GTs of various plants. Numerous studies have indicated that GTs density, amount of methyl ketone production, and plant resistance to pathogens are related to each other (Antonious 2001). Methyl ketones are synthesized by de novo fatty acid biosynthetic pathways in chloroplast. The steps involved in this pathway in case of *Solanum habrochaites* include the hydrolysis reaction in which 3-ketoacyl-acyl carrier (intermediate compound of fatty acid biosynthetic pathway) is converted to 3-keto acids which undergoes decarboxylation in the second step. The first step is catalyzed by methyl ketone synthase 2 (MKS2) (Ben-Israel et al. 2009; Falara et al. 2011) and the second one by methyl ketone synthase 1 (MKS1) (Fridman et al. 2005; Yu et al. 2010). Methyl ketones have been reported to protect plants against plant pathogens and pests such as *Manduca sexta, Aphis gossypii, Helicoverpa zea* (Zhu et al. 2018; Kashyap et al. 1991), two-spotted spider mite (Chatzivasileiadis and Sabelis 1997), and *Macrosiphum euphorbiae* (Musetti and Neal 1997).

6.4 Acyl Sugars

Acyl sugars are non-volatile compounds and their structure includes fatty acids esterified to the sugar backbone. Secretion of these compounds has been observed in plants of the Solanaceae family (Kroumova et al. 2016; Moghe et al. 2017). Acyl sugar biosynthesis involves two stages. In the first stage, fatty acyl chains are synthesized, and in the second stage, the acyl chains are esterified to glucose or sucrose (Fan et al. 2016, 2017; Schilmiller et al. 2015). Acyl sugars have been reported to play a role in direct and indirect defense.

7 Applications of Phytochemicals from Trichomes

Phytochemicals synthesized within the GTs have been extensively exploited for human use and several biotechnological applications. For example, the Lamiaceae family includes aroma-producing plants such as basil, oregano, mint, lavender, thyme, and all these plants can be used for essential oil production (Schilmiller et al. 2008). *Artemisia annua* produces a sesquiterpene called Artemisinin which is used as a drug for malaria treatment (Weathers et al. 2011). Cannabinoids produced by GTs of *Cannabis sativa* exhibit various activities. For instance, a psychoactive cannabinoid namely tetrahydrocannabinol (THC) shows anticancer and anti-nausea properties (Pellati et al. 2018; Taura et al. 2007). A non-psychoactive cannabinoid namely cannabidiol (CBD) works against neurodegenerative and cardiovascular diseases.

Fungicidal properties are displayed by sesquiterpene secreted from GTs of *Gossypium hirsutum* (cotton) (Dayan and Duke 2003; Mellon et al. 2014). GTs of *Cistus creticus* produce labdane-type diterpenes which are effective in prevention of gastric ulcer, bacterial and fungal infection, and inflammation (Demetzos et al. 1997; Costas Demetzos et al. 2001). The taste and smell of various food and pharmaceutical products come from GTs of *M. piperita* which produces monoterpenes (like menthol and menthonic) and *M. spicata* which produces carvone (Chauhan et al. 2009). Ambroxan, which is used in flavor and fragrance industries, is formed from the accumulation of sclareol (a labdane diterpene) produced by GTs of *Salvia sclarea* (Frija et al. 2011; Moulines et al. 2004). Some more industrially important chemicals produced by trichomes of different plants are listed in Table 15.1.

8 Metabolic Engineering to Convert Trichomes into Natural Chemical Factories

The previous sections discuss few examples of high economical value phytochemicals produced by trichomes. However, the tiny biomass of plant trichomes limits the production of these crucial metabolites (Wang 2014). Metabolic engineering has been used to enhance the secondary (specialized) metabolite production by glandular trichomes and convert these trichomes as natural chemical factories.

Once the gene and biosynthetic module for trichomes are defined in plants, the knowledge can be transferred in microbes for large-scale production. The eukaryotic host *Saccharomyces cerevisiae* is best suited for the production of many trichomes. *Escherichia coli* has also been employed for the production of trichomes. The common fragrance ingredient ambergris which is derived from diterpenoid sclareol, which is obtained from plant clary sage (*Salvia sclarea*). Caniard and coworkers cloned and characterized two diterpene synthase enzymes for biosynthesis of sclareol. The biosynthetic pathway was reconstructed in yeast and sclareol was produced (Caniard et al. 2012). In another study, the sclareol biosynthetic pathway was engineered in *E. coli* and bioprocess conditions were optimized to produce 1.5 g/L of sclareol (Schalk et al. 2012).

Besides the heterologous expression in microbial hosts, the production of some important plant metabolites is induced in the host plants itself by metabolic engineering methods. These methods change the fate of metabolic fluxes in favor of enhanced production of these metabolites. These approaches are generally carried out with the metabolites which are not involved in the growth and development of plants and are thus not involved in complex metabolic pathways (Huchelmann et al. 2017). Metabolic engineering in tobacco and tomato plants for enhanced production of important phytochemicals is well described.

Tobacco plants naturally produce enormous amounts of terpenoids (Tissier et al. 2012). Methods used for trichome engineering in tobacco plants include gene silencing and use of trichome-specific promoters as ubiquitous promoters have

Table 15.1 Industrially important chemicals produced by glandular trichomes of various plants

Sl. No.	Class of the metabolite	Chemical	Origin	Application	Reference
1.	Terpenoids (sesquiterpene lactone)	*Artemisinin*	*Artemisia annua*	Antimalarial drug	Czechowski et al. (2019)
2.	Phenylpropanoids (Phenylpropenes)	Eugenol	*Ocimum basilicum, Eugenia caryophyllata*	Used in cosmetology, medicine, and pharmacology.	Ulanowska and Olas (2021)
3.	Phenylpropanoids (Phenylpropenes)	Chavicol	*Ocimum basilicum*	Ordant in perfumes, flavoring agent	Sharmeen et al. (2021), Wang (2014)
4.	α-acids	α-bitter acid	*Humulus lupulus*	Impart bitterness in beer	Eyres and Dufour (2009)
5.	Prenylated Flavonoid	Xanthohumol	*Humulus lupulus*	Adds unique aroma in beer, anticancerous agent	Jiang et al. (2018)
6.	Monoterpene	Menthol	*Mentha canadensis*	Used in pharmaceutical, cosmetic, tobacco, food	Kamatou et al. (2013)
7.	Terpenoids	R-curcumene	*Solanum Habrochaites*	Repellent to herbivores and insects	Bleeker et al. (2011)
8.	Diterpene	Sclareol	*Salvia sclarea* (clary sage)	Fragrance	Wang (2014)
9.	Sesquiterpene lactone	Pyrethrin	*Tanacetum cinerariifolium*	Botanical insecticide	Wang (2014)
10.	Polyketide	Cannabinoids	*Cannabis sativa* (Hemp)	Psychoactive and medicinal properties	Wang (2014)
11.	Sesquiterpene	Gossypol	*Gossypium hirsutum*	Fungicidal properties	Mellon et al. (2014)
12.	Diterpene	Labdanum	*Cistus creticus*	Medicinal properties	Demetzos et al. (1997); Costas Demetzos et al. (2001)
13.	Monoterpene	Thymol and Carvacrol	*Thymus vulgaris*	Essential oil production	Dauqan and Abdullah (2017)
14.	Monoterpene	Carvacrol and Thymol	*Origanum vulgare*	Essential oil production	Sivropoulou et al. (1996)
15.	Sesquiterpene	(E)-β-Farnesene	*Solanum berthaultii*	Repulsion of herbivores	Gibson and Pickett (1983)

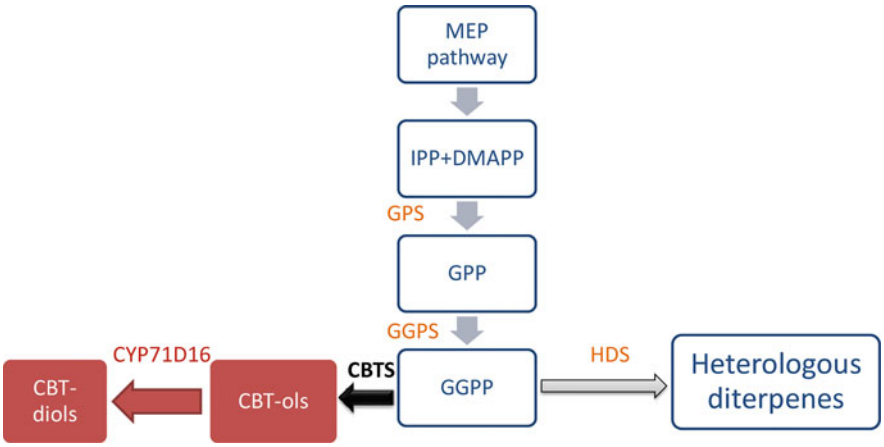

Fig. 15.6 The figure shows the inhibition of CMBT-diol pathway by using metabolic engineering. The site where the pathway was inhibited has been shown in black and the pathway which no longer propagates has been shown in red. *GPS* geranyl diphosphate synthase, *GGPS* geranylgeranyl diphosphate synthase, *HDS* heterologous diterpene synthase, *CBTS* cembratrien-ol synthase, *CYP71D16* a cytochrome P450 oxygenase

shown to cause distorted development of plants (Besumbes et al. 2004). Terpenoid biosynthesis has been chosen for engineering because of its simple metabolic pathway and derivation of various terpenoids from the same precursors such as IPP and DMAPP. The cembratriene-diol (CBT-diol) pathway involves an intermediate compound namely geranylgeranyl pyrophosphate (GGPP) and this compound has been targeted for engineering (Fig. 15.6). The CBT-diol pathway was inhibited at the enzyme cembratriene-ol synthase (CBTS), and heterologous expression of diterpene synthase was encouraged by trichome-specific promoter. Expression of casbene and taxadiene (both of them are diterpenes) was increased by using trichome-specific promoters but the levels of these compounds were still less than endogenous expression. Also, it was reported that elimination of CBT-diol production had no effect on casbene production (Tissier et al. 2012). Monoterpenes (C10) and diterpenes (C20) are derived from the (plastidial) MEP pathway, while sesquiterpenes (C15) and triterpenes (C30) are derived from the (cytosolic) MVA pathway but this is not always true because it has been observed that the intermediates of MVA and MEP pathway can cross membrane of plastid, i.e., exchange of prenyl diphosphates between the cytosol and the plastids (Hemmerlin et al. 2012) and this crosstalk between the MVA and MEP pathways can be used for engineering of terpenoid synthesis (Dudareva et al. 2005). But the inner workings of crosstalk are still not fully known. The enzymes farnesyl pyrophosphate synthase (FPS) and sesquiterpene synthase (STS) which are normally present in cytosol of *N. tabacum* were targeted toward plastid which resulted in rise of sesquiterpene production (Wu et al. 2006). Engineering in *N sylvestris* resulted in production of a compound which is normally not produced in this plant, that is, Z-abienol (Sallaud et al. 2012).

Triterpenes can also be produced in GTs of tobacco by targeting the IPP/DMAPP from plastid to cytosol. Triterpenes are of great use because of their high carbon content so they can be used for biofuel production (Khan et al. 2014). Squalene is an intermediate molecule in triterpene biosynthesis pathway and its production in cytosol by plastidial enzyme squalene synthase (SQS) and farnesyl diphosphate synthase (FPS) was achieved by the use of CMBT promoter (Wu et al. 2012). But the plants with high expression of squalene showed distorted morphology which may be because the promoter was not entirely specific to trichomes.

Tomato plants have also been engineered to serve as chemical factories. GTs of tomato are able to synthesize both cis- and trans-precursors. The cis-precursors include Neryl pyrophosphate in *S. lycopersicum* and cis-FPP in *S. habrochaites* (Sallaud et al. 2009; Schilmiller et al. 2009). Trichome-specific promoters have been used for increased production of methyl ketones (Yu and Eran 2014). Engineering has been done to enhance resistance to herbivores in case of tomato by fusing 7-epi zingiberene with MTS1 promoter and fusing Z-Z-farnesyl-diphosphate synthase with MKS1 (Meckel syndrome type 1) promoter (Bleeker et al. 2012; Yu et al. 2010).

9 Conclusion and Future Perspectives

Trichomes are unicellular or multicellular hair-like epidermal structures that are present in most parts of the plants. These structures provide protection to plants against various biotic and abiotic stresses. Glandular trichomes synthesize various specialized metabolites which are economically important. The advent of new technologies in the field of molecular genetics has paved the path of metabolic engineering in plants. With the help of these technologies, the tiny biomass of trichomes can be transformed into cell factories for the production of industrially important metabolites. Engineered crops can be grown whose trichomes synthesize metabolites which can make them more resistant to pests, pathogens, and herbivory. However, in spite of having such immense applications, all the benefits of the phytochemicals from trichomes have not been exploited due to lack of detailed information regarding their development and biosynthetic pathways. Only those metabolites are synthesized whose pathways are simple and well elucidated. The research in the field is still limited and the development of glandular trichomes and various factors involved in it along with the cell-stage specific data is yet to be explored. Majority of the research is done on model plants such as Arabidopsis, and there is a knowledge gap about various other non-model plants with industrially important metabolite producing trichomes. These trichomes are very potent alternatives for the environmentally hazardous chemical synthesis processes. Recent advances in omics technologies, genome sequencing, and genome editing offer high hopes for the advanced studies of these fascinating plant organs.

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Chapter 16
Transgenic Medicinal Plants for Improved Plant Metabolites Production

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1 Introduction

Since antiquity, man has relied on plants, one of the richest sources of physiologically health-promoting activate compounds, in his battle against sickness. The plant kingdom, which has over 250,000 species, is a reservoir for thousands of complex secondary metabolites with a low molecular weight. Now, pharmaceutical industries are facing an increasing need for a diverse spectrum of physiologically active molecules that are natural in origin that may be used to prevent or treat major reasons for mortality for instance, heart disease, cancer, diabetes, or respiratory illness. Many

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M. K. Swamy, A. Kumar (eds.), *Phytochemical Genomics*,
https://doi.org/10.1007/978-981-19-5779-6_16

phytochemicals employed in this way are bioactive substances. These are very diverse molecules in terms of structure and function that are synthesized in plant cells, these variety of primary and secondary metabolites on their own or in response to diverse stimuli. They serve a varieties of roles in nature, including shielding plants from infections, UV radiation, and herbivores, as well as giving unique smells and colours to aid in pollination and seed dissemination by animals. Additionally, they serve as significant signal and regulatory molecules for main metabolic processes. Secondary metabolites from plants are important in modern medicine. Green biotechnology, which includes the commercialisation of transgenic plants or other photosynthetic organisms, is becoming more popular because of the importance of plants as a source of secondary metabolites. These methods enable the manufacture of a diverse array of goods, recombinant proteins, secondary metabolites, physiologically active chemicals, papers, biofuel, etc. They may also be utilised to enhance a plant's nutritional quality and to create ecologically friendly agricultural methods (Pham et al. 2019). Numerous molecules now employed in medicine are derived from plants, and the evidence indicates an increasing trend towards natural biologically active substances. In comparison with chemically manufactured molecules, many compounds of natural origin exhibit increased biosafety, have fewer negative consequences, and are often connected with lower production charges (Jadaun et al. 2017). When climate change and human environmental degradation take their toll on medicinal plant species in their native environments, they are unable to adapt as quickly as they should. Because of this and the increasing need for plant-derived substances, new and more efficient in vitro techniques are being developed to produce plant material.

Research analysis revealed that, when combined with currently available precision technologies in molecular genomics and proteome engineering, high-throughput in vitro plant cultures may be utilised to produce a large number of naturally occurring secondary metabolites under specific circumstances. Green biotechnology, which allows for the manipulation of cellular processes on several levels, has the potential to be a viable alternative to conventional techniques to produce biologically active substances. The capacity to create and integrate multiple genetic constructs into the plant genome allows for the efficient manufacture of a wide variety of chemicals utilised in medicine, diagnostics, and industries. Significant research is currently being conducted on novel biotechnological solutions and long-term alternative techniques for improving plant metabolite production.

1.1 Metabolic Engineering's Significance in Terms of Excessive Secondary Metabolite Synthesis

By allowing for the manipulation of biosynthetic pathways, metabolic engineering offers a novel perspective on the expression of genes involved in secondary

metabolite synthesis (Verpoorte and Alfermann 2000). This involves studying enzymatic reactions and biosynthetic processes at the gene, transcriptome, and proteome level, as well as modifying genes encoding critical and percentage enzymes in biosynthesis pathway (Cusido et al. 2014). In theory, plant cell cultures' secondary metabolite output may be increased by overexpression of regulating enzyme-encoding genes engaged in their biosynthesis processes. Though overexpression of specific gene does not necessarily result in increased output (Lu et al. 2016). Additionally, metabolic engineering techniques use the suppression of competing pathways to boost the metabolic flow of biosynthetic pathways intermediate for increased performance through a number of strategies. Numerous bioactive compounds may be suppressed to promote the accumulation of previous intermediates. The most effective and recent application is the knowledge of the phenylpropanoid biosynthetic pathway, which is involved in the manufacture of various secondary metabolites of plants (Nanda et al. 2016).

A comprehensive understanding of metabolic pathways remains a hurdle making use of this technique for enhanced production. To fulfil industrial demand for key secondary metabolites on a wide scale, further research is required to uncover rate-limiting processes and regulatory constraints, as well as bottlenecks due to the lack of transparency in their biochemical routes (Oksman-Caldentey and Arroo 2000).

1.2 Transgenic Plants as Biopharma Factories

Transgenic plants' biopharmaceuticals must fulfil the same performance and safety criteria as conventional biopharmaceuticals. Transgenic systems such as mammalian cells, bacteria, and fungus have conventionally been used to manufacture biopharmaceuticals (Vaccaro et al. 2014). There will be a huge increase in demand for current biopharmaceuticals as well as novel therapeutic proteins found via genomics. It is important to investigate alternative transgenic production techniques and identify cost-effective ways to secure future supply of safe recombinant biopharmaceuticals. In addition to lower health hazards from pathogen contamination, therapeutic protein synthesis in plants offers significant economic and quality advantages (Singh et al. 2016). Transgenic crops might be grown using existing infrastructure with no financial input, opening the door to commercial production of biopharmaceuticals. Plants may be a low-cost source of recombinant DNA. Depending on the crop, the cost of generating recombinant proteins in plants might be 10- to 50-fold less than fermentation in *Escherichia coli* (Giddings et al. 2000).

1.3 Plant Genetic Engineering Using Nanotechnology

Recent advances in nanotechnology have created a new and advantageous technique for genetically modifying plants. Nanomaterials as carriers of genes have been

produced and are already being used in tobacco, maize, *Arabidopsis thaliana*, and onion (Li et al 2021). The types of gene carriers utilized in plant genetic transformation, the methodologies for combining them with foreign genes, and the distinctions and advantages of these technologies compared to previous traditional transgenic processes are all explored in brief. The challenges and potential connections associated with nanomaterial-mediated gene delivery system are considered in order to provide novel ideas for optimizing the design and development of more advanced plant genetic transformation technologies (Jat et al. 2020).

1.4 Metabolism Modulation by Phyto miRNAs in Medicinal Plants

Secondary metabolites in plant may be modified in dynamic methods to:

1. Increase the production of helpful compounds,
2. Decrease the production of hazardous metabolic pathways, and
3. Generate newly discovered metabolite.

The methods, RNA interference (RNAi) advanced technologies, that also involve the regulation of genes specifically by sncRNAs, have already emerged as a viable tool for plant biotechnologists, not only for comprehending the function of plant genes, but also for producing enhanced and unique features in plants by manipulation of both favourable and undesired genes. Among sncRNAs, miRNAs have been implicated in a variety of regulatory functions in plants, including development, signal transduction, stress response, and metabolism. Without a doubt, the application of miRNAs in bioengineering needs the search of miRNAs engaged in the manufacture of metabolite and also a knowledge of the biosynthetic routes, and the identification of critical places along the pathways where the miRNAs act. Thus, researchers begin by addressing these three challenges with medicinal plant metabolic engineering. The use of miRNAs may provide a unique perspective on the metabolic engineering of medicinal plants (Sabzehzari and Naghavi 2019).

2 CRISPR Cas9 in Plants

CRISPR/Cas9 technique has a few appealing characteristics, such as highly efficient, easiness of use it, adaptability, and the ability to perform multiplexed improvements; as a result, it has emerged as one of the good potential genome editing tools and brags a good prospect in causing preferred genetic changes in plants (Li et al. 2017). At the moment, the CRISPR-Cas9 method has been implemented in only a few plant species due to a lack of adequate gene sequences for many MAPs, though researchers are confident that additional research will fully leverage the possibility

of using the CRISPR-Cas9 system in other medicinal plants to recognise the genetic traits and enzymatic activity involved in the biosynthesis of bioactive molecules. The latest research reveals that CRISPR/Cas9 technology is rapidly emerging into the ideal biological tool for genetic manipulation and that its strength in genetic manipulation in plants, particularly folk medicine plants, has been revolutionised. Multiplex genome editing, which involves the selective deletion of a few genes or the concurrent up- or down-regulation of numerous genes, may result in the development of beneficial agricultural features in target plants. With a greater toolkit of CRISPR tools, we may anticipate that complex features will be able to be tweaked at will soon. Therefore, CRISPR-Cas9 can modify the biosynthesis route in heterologous medicinal plants using an arbitrarily structured and precisely regulated genetic circuit to enhance pharmaceutical output. To provide a far higher influence on medical plant biology, however, more efforts are required to enhance the CRISPR/Cas9 methods, rendering them quite user-friendly and widely available for study and find optimal solution.

2.1 Regulating the Transcription Factor by Advanced Genetic Tools

Plant genetic engineering is not a novel method; it has been around for more than three decades. Plant biotechnology and agricultural genetics will benefit from several new genetic technologies in the years to come. Genetic modification is the technique of dynamically adjusting the genome of an organism, either by introducing one or more genetic variations and regulatory elements or by decreasing the expression of indigenous alleles (Chahel et al. 2019). A DNA construct is randomly inserted into one or more chromosomes and one or more loci for each of these outcomes. This strategy has been shown to be beneficial in instances when basic characteristics such as herbicide tolerant and resilience to insects have been bred into plant. The random pattern of gene insertions, on the other hand, could have unintended consequences, and these approaches are not well suited for large-scale coordinated modifications, such as introducing an entire metabolic pathway to a plant. Translational and applied plant biology faces a lot of complicated problems, like how to feed and clothe a growing world population while protecting the environment. Enhancing current plant traits is necessary for improved crop production. This is particularly true for crops that need to increase their production and stress tolerance in order to adapt to environmental changes (Sohrabi 2018).

2.2 *Several Strategies Have the Following Applications in Plant Biotechnology*

- It is possible to control the precise expression of transgene and endo-genous gene using innovative synthetic promoters and repressors for greater spatio-temporal control.
- It is now possible to produce lengthy DNA constructs and vectors that are required for multigene transformation into plants because of recent advances in DNA synthesis and assembly technologies.
- Several approaches, including plant artificial chromosomes, allow the transformation of plants with massive constructs required for metabolic pathway engineering. There is no apparent 'winner' among the many methods.
- Using a wide range of new technologies, including ZFNs, TALENs, and CRISPR, editing of the plant genome might have the largest impact on precisely altering DNA sequences in crops.
- Commercialisation of some crops and crop–transgene combinations would be impossible without effective tools for removing and containing transgenes.
- Gene activation or gene repression in plants has been achieved by attaching zinc finger proteins (ZFPs) to transcriptional activation or repression domains, appropriately.

2.3 *Agricultural Crops Improved through CRISPR/Cas9-Mediated Genetic Engineering*

CRISPR/Cas9 is a revolutionary genome editing tool that has made crop breeding more accessible and successful owing to its simplicity and convenience of use. Other nucleases are more sophisticated and difficult to use, such as zinc finger nucleases and transcription activator-like effector nucleases. CRISPR/Cas9 is a method that employs a non-specific Cas9 nuclease and a single-guide RNA to direct Cas9 to a specified genome sequence and cause it to cut two strands of DNA. The repair process then adds or removes mutations. This is the most widely utilised reverse genetics and crop improvement technique in a broad variety of crops (Karkute et al. 2017).

Plant genome editing technique based on the type II CRISPR/Cas9 system has been integrated effectively with *Agrobacterium rhizogenes*-mediated transformation in a number of plant species, including *A. thaliana*, rice, and wheat (Feng 2013, 2014). 2014 (Ron). Hairy roots changed with Ri from *A. rhizogenes* display fast growth, reduced apical dominance, increased branching, and improved stable secondary metabolite production, making them an appealing paradigm for researching secondary metabolite biosynthesis, especially in medicinal plants (Guo et al. 2013).

Genome editing has exploded in popularity within the biological sciences, particularly the use of site-directed nucleases such as the CRISPR system. Genome editing is a technique that may be used in crop breeding programmes might have a big impact. Improved production, insect resistance, climate change adaption, and industrial and medicinal uses might all be aided by this research.

3 *Agrobacterium* in Plant Metabolic Engineering Strategies

It has been more than a decade since *Agrobacterium tumefaciens* was isolated and later shown to be a functional genomics engineer of plant genetic material (Kado 2014). *A. tumefaciens* is a naturally occurring phytopathogen that produces neoplastic diseases (crown gall) on a wide range of plants. The bacterium gains an intrinsic potential to infect wound sites in plants by transferring T-DNA from the bacterial cell to the plant genome through a type IV secretion system (T4SS), leading to the creation of various tumours (Nester 2015). *A. tumefaciens* infection resulted in the first transgenic plants in 1983, heralding the dawn of a new era in science and technology (Aguilar et al. 2010). With a better knowledge of the mechanics of genetic material transfer to plant cells, *Agrobacterium* transfection has become the most widely used approach of plant genetic manipulation. T-DNA is transported from bacterial cells to the nucleus of the plant cell during transformation, where it is incorporated into the chromosomal DNA. Surprisingly, *Agrobacterium* has the potential to hinder the plant's natural defensive reaction when it infects plant tissue (Pitzschke 2013).

Agrobacterium's biological technique for transforming DNA is well recognised. It is commonly known that genes are transmitted from a bacterial cell to a plant cell through T-DNA, a component of the Ti mega-plasmid. These transposable elements have a role in tumour formation and opine secretion in plant tissue. It contains two regions involved in bacterial–plant interactions: virulence genes (virA, B, C, D, E, G, F, and H) codec proteins involved in transgene transfer and integration into the plant genome, and a region encoding genes involved in opines synthesis, which bacteria use as a source of carbon and nitrogen (Guo et al. 2019; Balasubramani et al. 2021). This procedure allows for the creation of transgenic plants through either stable transformation, in which the new trait is inherited by subsequent generations, or transient transformation, in which the genetic material remains in the cell nucleus but does not permanently integrate with the genetic material (Xia et al. 2016). Since *Agrobacterium*'s initial successful attempts at plant genetic transformation in the early 1980s, the system has shown significant promise for transforming dicotyledonous and monocotyledonous plants. T-DNA, a component of the Ti mega-plasmid, is commonly used to transport genes between bacteria and plants. This transposable element is involved in tumour formation and the release of opine in plant tissue. It contains two bacterial–plant interaction regions: virulence genes (virA, B, C, D, E, G, F, and H) codec proteins involved in transgene transfer and integration into the plant genome, and a region encoding genes involved in the

synthesis of opines, which bacteria use as a source of carbon and nitrogen (Guo et al. 2019; Balasubramani et al. 2021). This method allows for the creation of transgenic plants via stable transformation, in which the new characteristic is passed down through successive generations, or temporary transformation, in which the genetic material remains in the cell nucleus but does not permanently integrate with the genetic material (Wei et al. 2019; Rahimi et al. 2021). Since *Agrobacterium*'s first success in genetically changing plants in the early 1980s, the technology has shown significant promise for genetically manipulating dicotyledonous and monocotyledonous plants.

However, there are more strategies for modifying plant DNA. Among these chemical approaches is protoplast PEG treatment, which causes both steady and temporary change (Ikeuchi et al. 2017). Electroporation may improve the efficacy of transformation even further by producing transient micropores in the cell membrane with an electrical impulse, enabling DNA to enter protoplasts. Finally, microprojectile bombardment, in which gold or tungsten particles are coated with the appropriate DNA molecules and propelled into the cell using high voltage or compressed gas, may be used (Gao et al. 2018).

Plant genetic transformation has numerous applications in the expression of recombinant proteins (Buyel 2018); these proteins could be used for a variety of treatment and diagnostic purposes, to increase plant resistance to biotic and abiotic stresses (Erpen et al. 2018), or to improve plant nutritional or flavour properties (Kannappa Reddy et al. 1993). Another option is metabolic engineering, which is utilised to either accelerate the production of certain metabolites found naturally in their tissues or to create whole new compounds (Satish et al. 2019; Jensen and Scharff 2019).

3.1 Genetically Modified Plants: Future Perspectives/Directions

- In the future, new vaccines, antibodies, and other therapeutic proteins will likely be produced in plants as factories.
- New biopharmaceuticals and 'plantibodies' may find their best expression via molecular farming.
- As technology continues to advance, it is envisaged that significant economic benefits will be gained.
- These efforts must include boosting yields; improving the scale-up of production; distributing transgenic plant material; and developing and validating production systems that successfully segregate pharmaceutical manufacture from human and animal feeds.

3.2 Modern Pharmacological Research of Artemisia

Since ancient times, *Artemisia* has been used to cure a variety of ailments. With recent discovery, *Artemisia* species have been referred to as 'natural combination medicines' due to their high concentration of bioactive chemicals.

3.2.1 Artemisinin from *Artemisia*

Metabolic engineering has been shown to be a successful technique for increasing the artemisinin content of *A. annua* during the last two decades. Previously, researchers used metabolic engineering strategies to increase artemisinin production, such as overexpression of genes involved in the artemisinin biosynthetic pathway (Banyai et al. 2010; Ma et al. 2015) and overexpression of transcription factors (TFs) known to promote artemisinin biosynthetic gene expression (Han et al. 2016). However, in recent studies, *A. annua* cultivars with low artemisinin content (0.02–0.4% dry weight) were used as transgenic recipients, or the increase in artemisinin content was not as effective when high-artemisinin cultivars (0.82–1.0% dry weight) were used as transgenic recipients. Meanwhile, *A. annua* has been intensively studied for its ability to cure a variety of diseases, including inflammatory and neoplastic illnesses, as well as viral, bacterial, and parasite-related infections, throughout the previous few decades (Efferth 2017). Furthermore, previous investigations focused only on upstream or downstream components of the artemisinin biosynthetic pathway, failing to successfully increase metabolic flow towards artemisinin synthesis. In addition, a synthetic biology method to artemisinin synthesis was described, in which the whole biochemical route for artemisinic acid, the precursor of artemisinin, was inserted into tobacco plants (Fuentes et al. 2016). However, it must be determined if this method enables efficient artemisinin synthesis in additional plant species. Additionally, its derivates, artesunate and artemether, have superior pharmacokinetic properties, which explains why they are widely utilised in different anti-malaria combo therapies worldwide. Given their good safety scoring in humans and their potential for wider availability at a relatively cheap cost, artemisinin-based medicines seem to be a viable choice for COVID-19 repurposing. (Zhou et al. 2021).

3.2.2 Sesquiterpenes from *Artemisia*

Sesquiterpenes, the major component of the Asteraceae family, include a very diversified chemical library generated by plants, that include frameworks to bioactive components (Saito et al. 2019). Eight unexplained sesquiterpenes, comprising three dimers, and ten known sesquiterpenes from the aerial parts of *A. sieversiana* have been found to exhibit anti-inflammatory activities. Compounds 4, 9, 12, 15, 16, and 17 displayed strong inhibitory activity against IL-1, IL-6, and TNF- in

LPS-induced RAW 264.7 cells (Nuermaimaiti et al. 2021). Likewise recent study reported that sesquiterpenoids and their dimers from *A. argyi* were shown to have strong NO inhibitory and anti-proliferative properties (Xue et al. 2019). Four novels highly oxidised sesquiterpenoids (1–4), as well as two recognised compounds, were isolated from *A. argyi* leaves (5 and 6) (Zhao et al. 2021).

3.2.3 Pros and Cons

- For plant-derived biopharmaceuticals to be safe and effective, they must fulfil the same requirements as those products generated from non-plant sources.
- Continuous monitoring of the environment is necessary to ensure that any errant compounds do not harm non-target creatures.
- In order to prevent the overexpression of potentially hazardous proteins in transgenic pollen, gene containment methods will continue to be developed.
- No doubt, there will be an ongoing discussion concerning the use of transgenic food plants, rather than non-food crops, as a source of novel medications.
- Antibodies, vaccines, various medicines, and even high-volume plasma proteins that under pipeline that are produced using recombinant plant DNA technology should pass through double checkpoint before it reaches the market.

4 Conclusion and Future Perspectives

Numerous molecules now employed in medicine are derived from plants, and the evidence indicates an increasing trend towards organic biologically active compounds. In comparison with chemically derived molecules, many compounds of natural origin are more biologically safe, have less adverse effects, and are often produced at a cheaper cost. However, owing to their limited adaption, the diversity of medicinal plant species in their natural habitats has been diminishing in the face of fast and unfavourable climate change and rising human environmental degradation. In contrast to this, and the ever-increasing need for plant-derived compounds, new and more efficient in vitro plant-growing techniques are being developed. The data analysis demonstrated that, under some conditions, high-throughput in vitro plant cultures may be used to manufacture a large number of natural secondary metabolites when paired with currently available precision approaches in molecular biology and genetic engineering. Modern green biotechnology, which allows manipulation of cellular processes at several levels, has the potential to be a viable alternative to traditional methods of producing biologically active molecules. The ability to synthesise various genetic constructs and insert them into the plant genome offers an efficient platform for the synthesis of a wide range of chemicals used in medicine, diagnostics, and industry. Intensive research is currently being performed on innovative biotechnological technologies and sustainable alternative strategies for producing high-value plant metabolites.

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Chapter 17
Hairy Root Cultures: A Novel Way to Mass Produce Plant Secondary Metabolites

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1 Introduction

Plants have the capability to synthesize a variety of chemicals and biologically active molecules via metabolic processes, making them impending sources for numerous vital medications. Both primary and secondary metabolisms are carried out by the plant cell. The secondary metabolism is triggered during the specific phases of plant's growth and development, as well as times when resources are few or when microbes are present (Yazaki et al. 2008). Plants produce a wide and diversified set of chemicals like alkaloids, flavonoids, anthocyanins, saponins anthraquinones, and terpenes as secondary metabolites (SMs) that play vital roles in the pharmacological, cosmetic, perfumery, and flavor industries (Chandran et al. 2020). These chemicals are found in trace concentrations and typically include chiral centers. As a result, they are tough to synthesize chemically and involve higher cost. Consequently, the most cost-effective way to get these crucial secondary metabolites has been to extract them from field-grown plants. Extraction of these useful biochemicals via

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M. K. Swamy, A. Kumar (eds.), *Phytochemical Genomics*,
https://doi.org/10.1007/978-981-19-5779-6_17

usual field-grown approaches could encompass extensive uprooting of the plants and, therefore, unavoidable devastation of their habitat and biodiversity. Truncated growing rates, constrained cultivation regions, environment reliance, plant diseases and pests, extreme labor requisite, tissue-/organ-specific metabolite production, inconsistency in impurities, complications in purification procedures, and financial costs involved in the selection and application of valuable materials have limited the extensive production of bioactive compounds using exclusive habitats and field-grown plants (Cho et al. 2003; Almagro et al. 2013; Halder et al. 2018). To address this issue, in vitro culture technology might be used to effectively propagate, conserve, and produce secondary metabolites from these plants on a massive scale. In this case, hairy root culture technology appears as an outstanding, easy, and proficient organ-centered tissue culture system alternative to acquiring wild or in vitro grown plants to create key biologically active metabolites in short period (Murthy et al. 2014; Mitra et al. 2020; Das et al. 2020). Hairy root cultures are created by taking use of soil-dwelling bacteria called *Agrobacterium rhizogenes*' unique natural capacity to develop hairy roots at infection sites. Hairy root are particularly beneficial for the generation of secondary metabolites, because numerous compounds are generated in roots, however not produced in callus or suspension cultures. Even in circumstances, when secondary metabolites amass solely in the plant foliage, hairy root cultures have been demonstrated to accrue the compounds (Zhou et al. 2011). The genetic stability has traditionally been one of hairy root cultures' major assets in biosynthetic supremacy together with hormone autotrophy, quicker growth potential, similar biosynthetic capacity, imitating that of the parental plants, and comparatively low-budget cultural requisites (Gutierrez-Valdes et al. 2020; Shi et al. 2021). The past three decades have used hairy roots as a biological matrix for diverse biotechnological activities, such as metabolic engineering, bioreactor designing, recombinant protein production, phytoremediation, molecular breeding for crop improvement, biotransformation-mediated derivatization studies, and rhizosphere physiology/plant–microbe interaction analysis. Furthermore, during repeated subculturing and plant regeneration, hairy root is frequently able to grow full sustainable plants and keep their genetic steadiness. Hairy root cultures have also been observed to yield a variety of bioactives that are not seen in the parental plant (Veerasham 2004). Furthermore, by scaling up from research laboratory shake culture to large-scale hairy root cultures in bioreactors, extensive synthesis of various secondary metabolites is achievable. Hairy root culture can also be utilized to identify intermediary and important enzymes linked in secondary metabolite production (Hu and Du 2006). The ease with which elicitors can be applied to hairy roots and metabolic reactions induced and allows for differential screening to identify genes implicated in secondary metabolite paths in plants. Recently, attempts have been made to conceptualize the development of hairy root-mediated transgenic plants and their field culture, giving this technology a "soil–laboratory–soil transition." Thus, the current chapter summarizes information on the successful production of a variety of useful secondary metabolites in hairy roots by means of a number of biotechnological tactics, its current trend, ideas for overcoming challenges, and

recent developments in future examination in this convincing field of plant biotechnology.

2 *Rhizobium Rhizogenes*: Mode of Infection and Transformation

The terminology "hairy root" first appeared in literature in 1900, when Stewart et al. (1900) introduced it to describe contaminated fruit crops. The phrase, "hairy root syndrome," was used by Hildebrandt (1934) to describe a set of disease symptoms that comprise the appearance of a tiny, hairy-like roots mass due to bacterial infection. Nevertheless, it was Riker et al. (1930), who identified, defined, and designated the causative entity as *A. rhizogenes*, and it was Ackermann (1977), who showed its use in plant transformation. The first in vitro culture investigations of this technology began in the early 1980s (Willmitzer et al. 1982). *A. rhizogenes*, the pathogenic soil bacterium, commonly identified as *Rhizobium rhizogenes* is currently a defined member of the genus *Agrobacterium* (the family, Rhizobiaceae) (Gelvin 2003; Veena and Taylor 2007) The usual host range of *A. rhizogenes* is confined to a small number of dicotyledonous plant species, although *A. rhizogenes* can develop hairy roots in several monocotyledonous and gymnosperm plant species under laboratory circumstances (Tepfer et al. 1989). When the bacteria infects a cell, it transfers its T-DNA from the Ri plasmid's TR and TL sections to the infected cell, where it integrates into the host cell's nuclear genome. The T-DNA (10–30 kb) segment of DNA is found on the Ri (root-inducing) plasmid (200 kb) in the bacterial cell. *A. rhizogenes* strains are classified into five categories based on opine production: agropine, mannopine, octopine, cucumopine, and nopaline (Zhou et al. 1998; Hirapure et al. 2019). Agropine strains are being the most preferred choice because of their superior root-inducing capabilities. Agropine strains have 2 substantially divided T-DNA regions, i.e., the TR-DNA and the TL-DNA on their Ri plasmid. The TR-DNA has the genes required for the production of auxin. The Tms1 and Tms2 loci are similar to the tms1 and tms2 of the Ti plasmid (Rawat et al. 2019). For the numerous stages of T-DNA transmission to the host cell, adequate synchronization of T-DNA genes (placed on bacterial pRi), multiple chromosomal genes (*chvA*, *chvB*), and vir (*virD1*, *virD2*, and *virE1*, *virE2*) genes are necessary (Chandra 2012). The genes (*rol* genes, i.e., *rol A*, *rol B*, and *rol C*) present in the bacteria's plasmid help in the transformation. The multiple genes included in T-DNA are encoded upon incorporation, resulting in the generation of auxin and cytokinins which induce the development of HR-like outgrowths from the wounded regions (Guillon et al. 2006; Gantait and Mukherjee 2021). T-DNA transfer is divided into seven stages, beginning with the stimulation of vir genes by sugar and phenolic compounds produced by damaged plant tissues and ending with the *rol* genes expression and T-DNA integration into the host genomic DNA (Hwang et al. 2017). All *rol* (*rolA, rolB*, and *rolC*) genes have a specific role and are involved in inducing and proliferating hairy

roots, according to earlier researches (Pavlova et al. 2014). All of the *rol* genes are involved in the typical development of hairy roots, as well as the generation and amassing of bioactive substances. Independently, *rolA* controls the formation of roots and their growth, *rolB* is responsible for root initiation and callus development, *rolC* is in charge of root growth, and *rolB* and *rolD* are accountable for suppressing the growth of callus. The *rolA*, *rolB*, and *rolC* oncogenes of *A. rhizogenes* are being known to be key stimulators of plant cell differentiation and growth. The discovery that the *rol* genes are impending secondary metabolism activators revealed a new purpose for these genes in plant–*Agrobacterium* interactions. In some circumstances, the activator impact of distinct *rol* genes is strong enough to overcome cultivated plant cells' incapability to create substantial quantities of plant metabolites (Bulgakov 2008). The best notable instance of the *rolB* transformation's efficiency was validated in *Vitis amurensis* cells, where in the gene resulted in increased resveratrol production (up to 100-fold). The amount of rolB mRNA transcripts was linked to the ability to synthesize resveratrol (Kiselev et al. 2007). Individual *rolA*, *rolB*, and *rolC* genes were shown to upsurge anthraquinone (AQ) synthesis in *Rubia cordifolia* calli, according to Shkryl et al. (2008). The enhanced transcription of the isochorismate synthase (ICS) gene, a crucial gene in AQ biosynthesis, was responsible for the stimulatory impact. When compared to the control, non-transformed calli, an *R. cordifolia* cultures expressing *rolB* at higher levels showed the strongest AQ-stimulating activity, with *rolB* ensuring a 15-fold rise in AQ accumulation. In comparison to control calli, the *rolA*- and *rolC*-expressing cultures yielded 2.8- and 4.3-fold greater quantities of AQs, correspondingly. The effect of *rolA, rolB*, and *rolC* on AQ synthesis on the other hand was not synergistic, since *rolA* and r*olC* appeared to dampen the inducing effects of *rolB* on AQ biosynthesis (Shkryl et al. 2008). In cultivated plant cells, the *rolA* gene appears to be a secondary metabolism stimulant. The strongest activator appears to be the *rolB* gene. In this regard, the *rolC* gene has received the most attention, and its use appears to be promising in terms of secondary metabolism activation. HRs can sometimes create substances that are not seen in non-transformed roots. For example, roots generated following Ri T-DNA-mediated transformation of *Scutellaria baicalensis* accrued glucoside conjugates of flavonoids rather than glucose conjugates found in non-transformed roots (Nishikawa et al. 1999). The transformed roots often show variable growth and SMs accumulation patterns because to the location ambiguity of T-DNA integration into the host cell genome and the off-time physiological state of the host cell. HRs have the unique virtue of genetic and biosynthetic stability, and these culture systems can create desired SMs across multiple generations without losing this property. The hairy roots grow quickly and yield secondary metabolites that are similar to or greater than those produced by usual roots. As a result, hairy roots are frequently utilized as alternate organs for the generation of secondary metabolites. Till date, in more than 100 therapeutically valued plants, hairy roots have been established (Dhiman et al. 2018). Figure 17.1 illustrates the stages of hairy root culturing and their numerous uses.

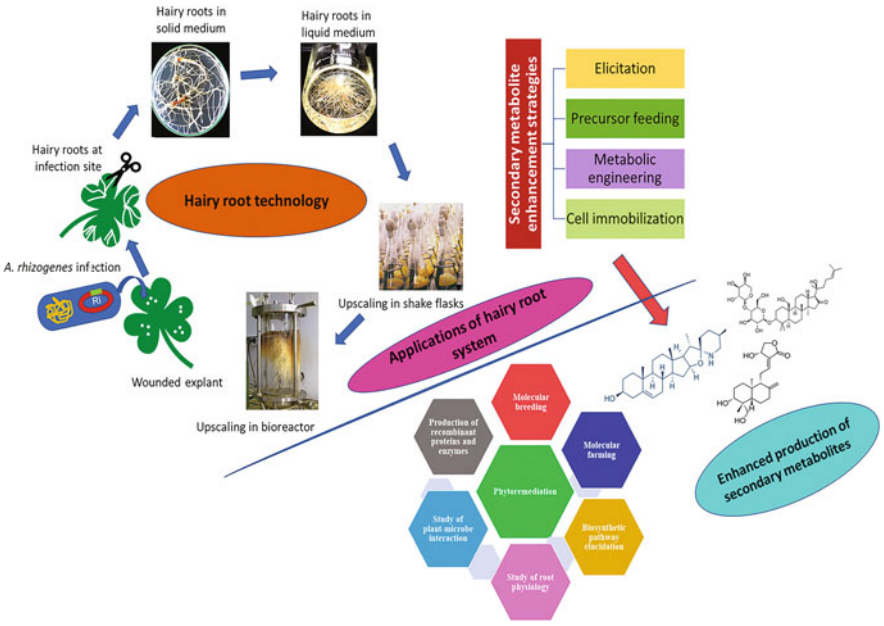

Fig. 17.1 Schematic diagram showing the various stages of hairy root culturing, secondary metabolite enhancement strategies, and multiple applications of hairy root system

3 Hairy Roots: Green Factories of Plant Secondary Metabolites

Hairy roots assist as a worthy way to overproduce commercially important phytocompounds on demand. Plant roots often accumulate commercially valuable secondary metabolites. These chemicals have complicated structures and are present in extremely small quantities, making extraction problematic. The high expense of organic synthesis, as well as the risk of plant extinction through direct root harvesting, made their collecting increasingly difficult. As a result, an alternative approach that can match the commercial need while also maintaining natural germplasm is required. Although biotechnological innovations have made cell suspension cultures, a viable option for producing secondary metabolites, this method has drawbacks like metabolites production in specialized cells at different developmental stages and cell genetic variability. Hairy root cultures alternatively yield a wide range of bioactive compounds that nearly resemble the production prospective of the integral root system. Other distinguishing characteristics, including as high genetic and biochemical firmness, rapid expanding time, and the potential to create novel chemicals, are often enough to justify the use of in vitro hairy root cultures for various applications. Some of the notable examples of phytocompounds isolated from hairy roots such as tanshinone from *Salvia miltiorrhiza*, camptothecin from *Ophiorrhiza alata,* taxol from *Taxus media, s*hikonin from *Arnebia* and

Lithospermum, indole alkaloids from *Catharanthus roseus,* azadirachtin from *Azadirachta indica,* betalain from *Beta vulgaris,* artemisinin from *Artemisia annua,* withanolides from *Withania somnifera,* ginsenoside from *Panax ginseng,* ajmaline and ajmalicine from *Rauvolfia micrantha,* reserpine from *Rauvolfia serpentina,* bacopasaponins from *Bacopa monnieri,* resveratrol from *Arachis hypogea,* scopolamine and hyoscyamine from *Hyoscyamus muticus,* gymnemic acid from *Gymnema sylvestre,* plumbagin from *Plumbago rosea,* andrographolide from *Andrographis panniculata,* and nicotine from *Nicotiana tabacum* are tabulated in recent reviews (Gantait and Mukherjee 2021; Shi et al. 2021). Hairy roots develop more quickly than adventitious roots or even regular plant cultures (Paek et al. 2009) and collect larger quantities of certain useful chemicals than adventitious roots and natively grown-up plant roots (Miao et al. 2017; Hao et al. 2020). For example, hairy roots of *S. miltiorrhiza* had a total tanshinone concentration equal to 15.4 mg/g DW (dry weight) compared to field-cultivated plant roots, which contained just 1.7–9.7 mg/g DW tanshinone (Kai et al. 2011a, b). Hairy root cultures, rather than adventitious roots or typical plant tissues, can create a variety of unique bioactive chemicals. Hairy root cultures are also beneficial for producing several secondary metabolites that are generated or amassed in aerial regions of plants as these compounds can occur in hairy root cultures in very small concentrations, if at all. For instance, vindoline is produced from tabersonine, which naturally occurs in the green sections of plants, and thus it is essential to use hairy roots in the form of improved green hairy roots (Abbasi et al. 2007). Berkov et al. (2003) observed the synthesis of a novel tropane alkaloid ester in *Datura stramonium* tetraploid hairy roots. Unique natural triterpene saponins and cadaverine, for example, are being identified in the hairy roots of *Brugmansia candida* and *Medicago truncatula*, possibly as a result of transformation or stresses, however not in roots and leaves of intact plants (Carrizo et al. 2001; Pollier et al. 2011). Thus, hairy root cultures may be utilized to isolate and synthesize novel chemicals with potential medicinal use. Hairy root cultures also have advantages over bacteria. For example, compared to microorganisms, such as *Saccharomyces cerevisiae* and *Escherichia coli,* they give an alternate platform, which is more alike to that of the natural host plant. Further, the expressed plant-derived proteins in this route properly fold in hairy roots when compared in microbes. Till date, hairy root cultures have been established in several hundreds of medicinal herbs to produce valuable secondary metabolites and some the recent reports are tabulated in Table 17.1, demonstrating that continuous progress is made in this area.

4 Commercial Scale-Up Process of Hairy Roots

There is growing interest in evaluating the feasibility of producing secondary metabolites from medicinal plants on an industrial scale using bioreactors of various dimensions and features (Park and Paek 2014; Georgiev and Weber 2014). Bioreactors have a number of advantages, including consistent temperature regulation

Table 17.1 Successful production of some of valuable secondary metabolites in hairy root cultures of different plant species

S. No	Plant species	*A. rhizogenes* strain used	Explant used	Culture media	Secondary metabolites	References
1	*Helicteres isora* L.	ATCC-15834, MTCC-534	*In vitro* leaf and nodes	MS liquid media	Diosgenin	Kumar et al. (2014)
2	*Rehmannia glutinosa* Libosch	A4	*In vitro* shoot tips and leaves	WPM liquid media	Iridoids, and phenylethanoid glycosides	Piątczak et al. (2015)
3	*Salvia sclarea*	ATCC15834	Axenic leaf sections	MS liquid media	Aethiopinone	Vaccaro et al. (2017)
4	*Calendula officinalis* L.	ATCC 15834 with and without pCAMBIA 1381Z vector	Cotyledons or hypocotyls	½ MS liquid media	Oleanolic acid	Długosz et al. (2013, 2018)
6	*Isatis tinctoria* L.	LBA9402	Petiole	½ MS liquid media	Alkaloids and flavonoids	Gai et al. (2019)
7	*Silene linicola*	A4	Cotyledon leaves	B5 liquid media	Ecdysteroids	Erst et al. (2019)
8	*Echinacea purpurea* L.	ATCC 43057	Seedling leaves	MS liquid media	Phenolics, flavonoids, and caffeic acid derivatives	Demirci et al. (2020)
9	*Echium plantagineum* L.	ATCC15834	Seedling leaves and stem	1/2B5 and M9 media	Shikonin	Fu et al. (2020)
10	*Corylus avellana* L.	K599, 15,834, and c58c1pRiA4	Seedling-derived leafstalks (lamina + petiole), lamina, petioles, and stems	¼ WPM, 1/2 MS, and ½ SH liquid media	Paclitaxel	Parizi et al. (2020)
11	*Salvia przewalskii* Maxim.	ATCC 15834	*In vitro* leaves	6,7-V media	Phenolic acid and tanshinone	Li et al. (2020a, 2020b)
12		LBA 9402	Leaves	MS liquid media	Steviol glycosides	Libik-Konieczny et al. (2020)

(continued)

Table 17.1 (continued)

S. No	Plant species	*A. rhizogenes* strain used	Explant used	Culture media	Secondary metabolites	References
	Stevia rebaudiana Bertoni					
13	*Turbinicarpus lophophoroides*	A4 agropine	Stem disks	MS and B5 liquid media	Feruloyl glucoside	Solis-Castañeda et al. (2020)
14	*Mentha spicata*	A13	Internode segments	½ MS liquid media	Phenolic acids	Yousefian et al. (2020)
15	*Pelargonium sidoides*	LBA-9402, C58C1, and *A. tumefaciens* C58C1(pRiA4)	Seedling leaves	MS liquid media	Coumarin and phenolic compounds	Yousefian et al. (2021)
16	*Apocynum venetum*	Ar.119	Stem	WPM liquid medium	Flavonoids	Zhang et al. (2021)
17	*Macleaya microcarpa*	ACCC 10060	Cotyledons, hypocotyls, leaves, petioles of seedlings	MS liquid media	Benzylisoquinoline alkaloid	Zheng et al. (2021)
18	*Papaver armeniacum* L.	C58C1, ATCC15834, GM and R1000	Seedling-derived hypocotyls, shoots, and roots	MS liquid media	Benzylisoquinoline alkaloids	Naeini et al. (2021)
19	*Sphaeralcea angustifolia*	K599/pTDT (cucumopine type), A4/pTDT or ATCC 15,834/pTDT (agropine type)	Seedling-derived leaves and nodes	MS liquid media	Scopoletin and sphaeralcic acid	Reyes-Pérez et al. (2021)
20	*Litchi chinensis*	MSU440 containing binary vector pCAMBIA1300-eGFP or pCAMBIA1300-LcMYB1-eGFP	Seedling-derived leaf disks and stem segments	MS solid media	Anthocyanins, proanthocyanins, and flavonols	Qin et al. (2021)
21	*Antirrhinum majus L*	AR1193 strain bearing plant expression binary vectors pBI35S:ROS1-35S:DEL, pBI35S:ROS1, pBI35S:DEL, and pBI121	Seedling-derived hypocotyl segments	MS solid media	Anthocyanins	Piao et al. (2021)
22		MTCC 532	Calli	MS solid media	Celastrol	Moola et al. (2021)

	Celastrus paniculatus Willd					
23	*Cynara cardunculus* L.	A4, LBA9402	In vitro-derived hypocotyls	SH liquid media	Hydroxycinnamic acid	Folgado et al. (2021)
24	*Arachis hypogaea* L.	K599	Seedling-derived leaf, petiole, and hypocotyls	½ MS liquid media	Prenylated stilbene compound	Pilaisangsuree et al. (2018); Chayjarung et al. (2021)
25	*Satureja sahendica* Bornm	ATCC 15834, A4, and LBA 9402	Seedling-derived node and internode	MS liquid media	Thymol	Bahmani et al. (2021)
26	*Salvia virgata* Jacq.	A4, ATCC15834, R1000, GM1534 and C58C1	Seedling-derived leaves	½ MS liquid media	Phenolic acids	Dowom et al. (2021)
27	*Calendula officinalis*	ATCC 15834 with and without pCAMBIA 1381Z vector	Cotyledons or hypocotyls	½ MS liquid media	Oleanolic acid and sterols	Alsoufi et al. (2021)
28	*Uraria picta*	ATCC-15834	Seedling-derived leaf, stem, and root	MS liquid media	Lipids, benzenoids, and organic acids	Acharya et al. (2021)

during operation, improved nutrient uptake, and the ability to handle huge amounts of culture (Uchendu et al. 2011). As a consequence of these facts, bioreactors have become an ideal system for modern industrialized plant tissue and cell cultures. The growth of hairy roots and metabolites secretion in bioreactors has arisen as an encouraging approach for commercial-scale setups in the early 1990s, with international firms eager to culture hairy root biomass to produce metabolites (Mehrotra et al. 2015; ROOTec bioactives Ltd., Switzerland; http://www.rootec.com; CBN Biotech, South Korea). Green2Chem in Belgium (http://www.green2chem.com/), and Root Lines Technology in France (http://www.rootlines-tech.com/) are presently the most popular commercially viable implementation of hairy root cultures technology for extensive pharmaceutical manufacture. By employing bioreactors of different forms and dimensions, the firms use transformed hairy root cultures to produce pharmaceutical plant secondary metabolites on a massive scale for use in the industrial synthesis of pharmaceuticals. During the late 1980s, investigations on the culturing of hairy roots were conducted utilizing the hairy roots of belladonna (*Atropa belladonna*) and bindweed (*Calystegia sepium*) in stirred tank reactors to create tropane alkaloids (Jung and Tepfer 1987). Hairy root culture can be done in submerged (liquid-phase), gas-phase, and a combination of liquid- and gas-phase (hybrid) bioreactors (Mishra and Ranjan 2008). The hairy roots of *Hyoscyamus niger* (black henbane) cultivated in a hybrid bubble column/spray bioreactor produced significant amount of alkaloids (Jaremicz et al. 2014). Using mist bioreactors, hairy root culture of the *Azadirachta indica* (neem tree) produced azadirachtin (13.3 g L^{-1} DW) (Srivastava and Srivastava 2012). A tenfold increase in biomass and production of caffeic acid derivatives was achieved in *Echinacea purpurea* hairy root cultures grown in balloon-type bubble bioreactors (a type of air lift bioreactor) of 5 L capacity (Jeong et al. 2009). Cardillo et al. (2010) have achieved enhanced biomass and accumulation of tropane alkaloids such as hyoscyamine, anisodamine, and scopolamine in hairy root cultures of *Brugmansia candida* grown in modified 1.5-L stirred tank bioreactor. The highest ginsenoside production was obtained in *Panax quinquefolium* hairy root cultures elicited with 250 μM L^{-1} methyl jasmonate nutrient sprinkle bioreactor (Kochan et al. 2018). Recently, bioreactor upscaling of transgenic *Atropa belladonna* hairy roots has resulted in 2.3-fold enhanced production of curcumin (Singh et al. 2021), while the flavonoid content of *Apocynum venetum* hairy roots cultured in bioreactors has exceeded the shake flask production by 43.97%. Likewise, the yield enhancement of several pharmaceutically important phytocompounds was successfully achieved in various bioreactor systems, some of which are tabulated (Table 17.2).

Table 17.2 Upscaling of hairy roots and secondary metabolite production of selected plant species in bioreactors

S. No	Plant species	Type of bioreactor	Medium	Secondary metabolites	References
1	*Cichorium intybus* L.	Acoustic mist bioreactor	MS medium	Esculin	Bais et al. (2002)
2	*Beta vulgaris*	Various airlift bioreactors of 5 l capacity (cone, balloon, bulb, drum, and column)	½ MS medium	Betacyanin	Shin et al. (2002)
3	*Ophiorrhiza pumila*	3-l capacity glass bioreactor	B5 medium with 2% sucrose	Camptothecin	Sudo et al. (2002)
4	*Astragalus membranaceus*	30-l airlift bioreactor	Modified MS medium	Astragaloside IV and polysaccharide	Du et al. (2003)
5	*Pueraria phaseoloides*	2.5-l airlift bioreactors	MS or 6,7-V medium	Puerarin	Kintzios et al. (2004)
6	*Beta vulgaris*	3-l capacity bubble column bioreactor	MS medium	Betalaine	Suresh et al. (2004)
7	*Panax ginseng*	20-L air bubble bioreactor	½ MS medium	Ginseng, acidic polysaccharide, and phenolic compounds	Jeong and Park (2005)
8	*Beta vulgaris*	3-L capacity bubble column bioreactor	MS medium	Betalaine	Savitha et al. (2006)
9	*Harpagophytum procumbens*	3-l bubble column bioreactor	MS medium	Harpagide and harpagoside	Ludwig-Müller et al. (2008)
10	*Salvia sclarea*	10-L sprinkle bioreactor	1/2 B5 medium	Ferruginol, salvipisone, aethiopinone, and 1-oxoaethiopinone)	Kuźma et al. (2009)
11	*Brugmansia candida*	Modified 1.5-L stirred tank	Gamborg B5/2 medium	Scopolamine, anisodamine, and hyoscyamine	Cardillo et al. (2010)
12	*Azadirachta indica*	3-l stirred tank bioreactor	Modified MS medium (MM2)	Azadirachtin	Srivastava and Srivastava (2012)
13	*Azadirachta indica*	3-L stirred tank reactor, 3-L bubble column reactor, and 4-L	Modified MS medium (MM2)	Azadirachtin	Srivastava and Srivastava

(continued)

Table 17.2 (continued)

S. No	Plant species	Type of bioreactor	Medium	Secondary metabolites	References
		nutrient spray bioreactor			(2012, 2013)
14	*Panax quinquefolium*	10-l capacity sprinkle bioreactor	B-5 medium	Ginsenoside	Kochan et al. (2012, 2014, 2016, 2018)
15	*Centaurium maritimum* (L.)	RITA® temporary immersion bioreactors	½ MS medium	Secoiridoid glycosides	Mišić et al. (2013)
16	*Hyoscyamus niger*	Bubble column bioreactor and a hybrid bubble column/spray bioreactor	MS medium	Scopolamine, hyoscyamine, anisodamine, and cuscohygrine	Jaremicz et al. (2014)
17	*Artemisia annua*	Modified 3-L stirred tank bioreactor	MS medium	Artemisinin	Patra and Srivastava (2014, 2015, 2016)
18	*Vinca minor*	5-l stirred tank bioreactor	¼ B5 medium	Vincamine and total alkaloids	Verma et al. (2014)
19	*Talinum paniculatum* Gaertn	1-L balloon-type bubble bioreactor	MS medium	Saponin	Manuhara et al. (2015)
20	*Catharanthus roseus*	3-l bubble column bioreactor	1/2 B5 medium	Ajmalicine	Thakore et al. (2017)
21	*Apocynum venetum*	3-L bubble bioreactor	woody plant liquid medium (WPM)	Flavonoids	Zhang et al. (2021)
22	*Atropa belladonna*	Modified stirred tank 10-L bioreactor	½ MS medium	Curcumin	Singh et al. (2021)
23	*Arachis hypogaea* L.	5-L capacity stirred tank bioreactor	½ MS medium	Trans-resveratrol, trans-arachidin-1, and trans-arachidin-3	Eungsuwan et al. (2021)

5 Recent Biotechnological Strategies to Enhance Plant Secondary Metabolites in Hairy Roots

5.1 Metabolic Engineering

Genetic manipulation of key genes involved in the production of substrates and/or precursors, intermediates, and end products has facilitated the enhanced production of secondary metabolites in hairy roots. For example, enhanced accumulation of tanshinones and phenolic acids were achieved in metabolically engineered *Salvia miltiorrhiza* hairy roots by overexpressing key genes coding for enzymes and transcription factors such as *GGPPS-DXS2, HMGR-DXR, HMGR-GGPP, MYB98, WRKY1, WRKY2, and HPPD* (Kai et al. 2011a, b; Shi et al. 2014, 2016; Hao et al. 2020). Various key genes involved in the biosynthesis of camptothecin such as *MYB1, G10H-STR, G10H,* and *SLS* were successfully targeted to achieve greater metabolite occurrence in hairy roots of *Ophiorrhiza pumila* (Cui et al. 2015; Rohani et al. 2016; Shi et al. 2020). Likewise, fourfold increased yield of valerenic acid was obtained in hairy roots of *Valeriana officinalis* by overexpressing the key enzyme valerena-4,7(11)-diene synthase (*VDS*; Ricigliano et al. 2016).

Metabolic engineering offers enormous potential for enhancing secondary metabolite accumulation by targeting transcription factors that coordinately regulate several biosynthetic pathway genes. Various transcriptional factors have recently been overexpressed in different medicinal plants to attain greater metabolite accumulation. CrORCA4 overexpression boosted tabersonine synthesis in *Catharantus roseus* hairy roots by more than 40-fold (Paul et al. 2017), while transgenic hairy roots overexpressing Ii049 yielded 425.60 g/g lariciresinol, an 8.3-fold increase over wild-type levels (Ma et al. 2017).

Recently, genome editing methods like CRISPR/Cas9 have been effectively employed in metabolic engineering of medicinal plants to fine-tune genome editing and characterize the enzymes and pathways linked to produce secondary metabolites. The use of CRISPR/Cas9 to knock out *SmMYB98* in *S. miltiorrhiza* resulted in a drop in phenolic acid and tanshinone levels, showing that *SmMYB98* plays a favorable role in phenolic acid and tanshinone production (Hao et al. 2020). For example, in *O. pumila* hairy roots, CRISPR/Cas9 was utilized to remove *OpG10H* and *OpSLS*, resulting in a 90% reduction in camptothecin levels (Shi et al. 2020).

Nowadays, omics-related approaches like genomics, transcriptomics, proteomics, and metabolomics are being utilized to explore the hairy roots of medicinally valued plants in order to find novel genes and metabolic processes. For instance, transcriptomic examination of diverse tissues, hairy roots, or elicitor-treated samples has identified several new genes linked to tanshinone biosynthesis and novel transcription factors that govern salvianolic acid and/or tanshinone biosynthesis (Zhou et al. 2017). Glutamate was abundant in hairy roots of *Verbascum nigrum*, however absent in mother plant tissues as revealed from the nuclear magnetic resonance-based metabolomics studies of transgenic roots (Georgiev et al. 2015). Furthermore, genes presumably involved in specialized metabolism in *O. pumila* and

S. miltiorrhiza were also discovered using a combination of metabolomic and transcriptome analysis (Udomsom et al. 2016). A detailed information on various recent biotech tactics used to increase secondary metabolites yield in hairy roots of different plant species was reviewed by Shi et al. (2021). Other yield enhancement approaches like cell immobilization and precursor feeding techniques were elaborated by Dhiman et al. (2018).

5.2 Elicitation

The biotic (bacteria, fungi, virus, insects, and herbivores) and abiotic (high/low temperature, draught, salinity, UV radiation) stresses gradually affect the plant growth and survival. The plant systems rapidly produce low molecular weight compounds which help to protect the plants by the activation of several biological pathways (Halder et al. 2019). Secondary metabolites are not only involved in defense mechanism but also take part in pollination, oviposition, seed dispersal, and symbiotic relations between other species. Due to the abundant biological properties, secondary metabolites have received the great interest from scientific community (Wang and Wu 2013). However, natural availability and concentrations of secondary metabolites are varying in each plant system. Usually, plants are having lower concentrations of secondary metabolites. Therefore, in order to fulfill the commercial and pharmaceutical demand, the secondary metabolite production rate was increased using biotechnological approaches.

Recently, many reports were focused more on elicitors to produce commercially important therapeutic compounds (Naik and Al-Khayri 2016). Elicitation is the most efficacious and extensively used biotechnological approaches for producing new secondary metabolites (Mishra et al. 2012; Halder et al. 2019). Elicitors activate the signal transduction pathway of several genes related to secondary metabolite secretion by change the transcription level of many regulatory genes. Various physicochemical parameters, types and concentrations of elicitor, time and light conditions, and the medium composition are the main factors that can influence the productive rate (Mishra et al. 2012; Halder et al. 2019). In elicitation, many biotic elicitors' composition is known such as alginate, chitin, elicitin, xanthan, pectin, glycoprotein, inactivated enzymes, glucans, and purified polysaccharides; however, various unknown compositions of biotic elicitors such as bacterial extract, fungal extract, and yeast homogenate are also used. So far, numerous abiotic elicitors such as chemical elicitors (cadmium chloride ($CdCl_2$), silver thiosulfate ($Ag_2S_2O_3$), cupric chloride ($CuCl_2$), silver nitrate ($AgNO_3$), copper sulfate ($CuSO_4$), nickel sulfate ($NiSO_4$), vanadyl sulfate ($VOSO_4$), and selenium), osmotic elicitors (polyvinyl pyrrolidone, sodium chloride, sorbitol, mannitol, cadmium chloride, and potassium chloride), gaseous elicitors (nitric oxide (NO) and ethylene), physical elicitors (drought, temperature, salinity, light, and UV radiation), signaling molecule elicitors (methyl jasmonate, jasmonic acid, acetyl salicylic acid, salicylic acid, and systemin), and polyamine elicitors (putrescine and spermidine) were used for secondary metabolite production (Table 17.3). Recently, enhanced production of many biologically

Table 17.3 Different types of biotic and abiotic elicitors used for secondary metabolite production in hairy root culture

Elicitors	Plant species	Metabolites	Reference
Bacterial elicitor			
Bacillus cereus	*Salvia miltiorrhiza*	Tanshinone	Wu et al. (2007)
Yeast elicitor			
Yeast extract	*Panax ginseng*	Ginseng saponin	Jeong et al. (2005)
Fungal elicitor			
Crude extracts of *Fusarium conglutinans* mycelia	*Tagetes patula*	Thiophenes	Mukundan and Hjortso (1990)
Signaling molecule elicitors			
Jasmonic acid (JA)	*Azadirachta indica*	Azadirachtin	Satdive et al. (2007)
JA	*Catharanthus roseus*	Indole alkaloid	Peebles et al. (2009)
Methyl jasmonate (MeJA)	*Plumbago indica*	Plumbagin	Gangopadhyay et al. (2011)
MeJA	*Centella asiatica*	Asiaticoside	Kim et al. (2007)
Salicylic acid (SA)	*Cichorium intybus*	Sonchuside A	Malarz et al. (2007)
SA	*Hyoscyamus muticus*	Lubimin and solavetivone	Morgan and Shanks (2000)
Acetylsalicylic acid (ASA)	*Tropaeolum majus*	Glucotropaeolin	Wielanek and Urbanek (2006)
ASA	*Panax ginseng*	Ginseng saponin	Jeong et al. (2005)
Abiotic elicitors			
Ag + ($Ag_2S_2O_3$)	*Salvia miltiorrhiza*	Tanshinone	Ge and Wu (2005)
$CuCl_2$	*Plumbago indica*	Plumbagin	Gangopadhyay et al. (2011)
Light 385–790 nm	*Artemisia annua*	Artemisinin	Wang et al. (2001)
Low temperature (19.5 °C)	*Catharanthus roseus*	Linolenic acid and Indole alkaloids	Toivonen et al. (1992)
High temperature (40 °C, 45 °C, 50 °C)	*Beta vulgaris*	*Pigment*	Thimmaraju et al. (2003)
Osmotic stress elicitors			
Sorbitol	*Salvia miltiorrhiza*	Tanshinones	Shi et al. (2007)
NaCl	*Datura stramonium*	Hyoscyamine	Khelifi et al. (2011)

important secondary metabolites was achieved in hairy roots via elicitation strategy (Table 17.4).

6 Multiple Applications of Hairy Roots

In addition to producing secondary metabolites, hairy root cultures are also used as a potential tool for various studies including fundamental research and environmental and commercial applications (Gantait and Mukherjee 2021). This technology covers a wide-ranging applications in diverse fields, such as conservation of threatened medicinal plants through tissue culture, production of secondary metabolites, novel recombinant proteins, phytoremediation, metabolic engineering, bioresource technology, and phytomining, and Table 17.5 clearly summarizes the potential applications of hairy root culture technology.

7 Constraints Encountered with Hairy Root Culturing

In spite of many profitable advantages, hairy root culture is not simple and having lot of troubles to raise the stable successful protocol in many plant species (Gantait and Mukherjee 2021). The efficiency of hairy root culture is decided by the important factors such as internal inhibitors, recalcitrant, contaminations, optimization of various surface sterilization protocol, selection of explants, culture conditions, incubation time, temperature, and light and dark conditions. There are major limiting factors in hairy root culture establishment such as chromosomal aberrations, genetic changes, gene suppressions, differential production of secondary metabolites, morphological alterations in regenerants, problems during phytoremediation, no stable gene expression, and silencing of gene expression at late phases of subculturing (Gutierrez-Valdes et al. 2020; Gantait and Mukherjee 2021) (Fig. 17.2).

8 Conclusion and Future Prospects

Differentiated hairy root cultures are more suited for biosynthesizing important compounds than undifferentiated cell suspension cultures, due to their genetic and biochemical reliability. Furthermore, as alternative biotechnological methodologies like genetic engineering, elicitation, and the use of metabolic traps are now being investigated, HRs' potential is always rising. Metabolic engineering has promise for increasing yields, but it necessitates an understanding of the control of metabolic pathways at the enzyme and gene levels, comprising features like transporting and compartmentation. Elicitation boosts the generation of secondary metabolites and aids in the creation of metabolic traps for product adsorption from culture medium.

Table 17.4 Recent documentation of elicitation of hairy root cultures of selected plant species for enhanced accumulation of valuable metabolites

Plant	Agrobacterium strains used	Elicitor molecule and its concentration	Effect	References
Eclipta prostrata	*R. rhizogenes* LBA 9796	100 μM of JA	5.2-fold increase in wedelolactone, a 1.6-fold increase in demethylwedelolactone, and a 2.47-fold increase in 3,5-di-O-cafeoylquinic acid	Maciel et al. (2021)
Arachis hypogaea		CHT + MeJA + CD	Highest amounts of *trans*-arachidin-1 and *trans*-arachidin-3, highest TPC, and antioxidant potential	Chayjarung et al. (2021)
Senna obtusifolia		MJ 100 μM	Increased accumulation of betulinic acid	Kowalczyk et al. (2021)
Salvia virgata	ATCC15834	MJ 22.4 PPM	Maximum accumulation of TPC, TFC, rosmarinic acid, and salvianolic acid A	Dowom et al. (2021)
Psammosilene tunicoides		200 mg L^{-1} chitosan	Quillaic acid, gypsogenin, and gypsogenin-3-O-β-D-glucuronopyranoside increased expression of genes involved in saponin metabolism	Qiu et al. (2021)
Atropa acuminata and *A. belladonna*	A4 and LBA9402	50 Mm methyl-β-cyclodextrin and 0.5 μM coronatine	Increased production of Hyoscyamine and scopolamine	Fattahi et al. (2021)
Psammosilene tunicoides		Salicylic acid 5 mg/L	Saponin contents increased by 2.5-fold	Su et al. (2021)
Ficus carica		*Piriformospora indica* culture filtrate 2%	Enhanced accumulation of gallic acid (80.5-fold), caffeic acid (26.2-fold), coumaric acid (4.5-fold), and cinnamic acid (60.1-fold), apigenin (27.6-fold), and rutin	Amani et al. (2021)
Pelargonium sidoides	LBA 9402	100 μM MJ	Gallic acid, vanillic acid, and umckalin	Yousefian et al. (2021)
Papaver armeniacum	*R. rhizogenes* strain C58C1	100 μM MJ	Highest contents of thebaine, codeine, and morphine by enhancing the expression of *SalAT*, *COR*, and *T6ODM* genes	Naeini et al. (2021)
		100 μM SA	Highest contents of papaverine and noscapine by upregulating *DBOX* and *BBE* genes	
Atropa belladonna		Yeast extract 1.5 mg/L	Highest content of scopolamine and atropine	

(continued)

Table 17.4 (continued)

Plant	Agrobacterium strains used	Elicitor molecule and its concentration	Effect	References
	A7 strain of *Agrobacterium rhizogenes*			Hedayati et al. (2021)
Centella asiatica		200 μM MJ	Triterpenoids	Baek et al. (2020)
Linum album		200 mg L^{-1} chitosan	Podophyllotoxin, 6-methoxy podophyllotoxin, catechin, and vitexin	Samari et al. (2020)
Salvia przewalskii		400 μM MeJA	Caffeic acid, rosmarinic acid, salvianolic acid B, cryptotanshinone, and tanshinone IIA.	Li et al. (2020a)
		50 μM SA	Tanshinone	
Mentha spicata		MJ	Rosmarinic acid content (55.44 μg g^{-1} dry weight, about 11.84-fold) after 6 h exposure to elicitor. Moreover, caffeic acid, chlorogenic acid, and cinnamic acid	Yousefian et al. (2020)
Hyoscyamus species		Silicon dioxide nanoparticles 100 mg L^{-1}	Hyoscyamine (140.15 μg g^{-1} FW) and scopolamine	Hedayati et al. (2020)
Celastrus paniculatus	MTCC532	10 μg mL^{-1} of silver nanoparticles	Celastrol content 2.25-fold	Moola et al. (2021)

Table 17.5 Multiple applications of hairy roots

Applications	Plants	References
Secondary metabolite production	Coniferin from *Linum flavum*	Lin et al. (2003)
Analysis of gene function	Glucocorticoid-inducible promoter expression studies in *Catharanthus roseus*	Hughes et al. (2002)
Transformation studies and transgenic technology	Using Ri plasmid of *A. rhizogenes* as a vector gene transfer in tobacco	Comai et al. (1985)
Protein encoding from related and unrelated taxa	Induction of *Pisum sativum* lectin gene into *Trifolium repens*	Diaz et al. (1995)
Metabolic engineering	Solanoside glycoside produced from *Solanum khasianum* hairy roots	Putalun et al. (2003)
Biotransformation	Hairy roots of *Coleus forskohlii* were biotransformed the ethanol and methanol substrates into β-D-ribo-hex-3-ulopyranosides and β-D-glucopyranosides, respectively.	Li et al. (2003)
Phytoremediation	Hairy roots of *Brassica napus* were cleansing up the 2,4-dichlorophenol pesticides	Agostini et al. (2003)
Production of novel compounds or proteins	Novel flavonoid glucoside conjugates were found in hairy roots of *Scutellaria baicalensis* rather than normal glucose conjugates	Nishikawa and Ishimaru (1997)
Structural conversion in the metabolite	The constitutive expression of H6H gene in *Atropa belladonna* showed the conversion of hyoscyamine to scopolamine in the root tissues.	Hashimoto et al. (1993)
Plant regeneration	Plants were regenerated from hairy roots of *Catharanthus roseus*	Choi et al. (2004)
Promoting root induction in vegetative propagation	Many recalcitrant plants apple and *Pinus spp.* were induced for root production using hairy root culture	Gantait and Mukherjee (2021)
Study of the host–pathogen interaction	Effects of fungal cultures (*Glomus mosseae* and *Gigaspora margarita*) on hairy roots of *Convolvulus sepium*	Mugnier and Mosse (1987)
Study the interaction of rhizosphere and nematodes	Development of nematode resistant in sugar beet against beet cyst nematode (*Heterodera schachtii*) by transforming Hs1pro1 gene in susceptible hairy roots of sugar beet	Cai et al. (1997)
Recombinant protein production	Human interferon alpha-2b production from *Daucus carota* hairy roots	Luchakivskaia et al. (2012)
Specialized metabolites production	Tropane alkaloids production from *Hyoscyamus reticulatus* hairy roots	Khezerluo et al. (2018)
Phytomining	Accumulation of Ni metal by *Alyssum bertolonii*	Boominathan et al. (2004)

Apart from secondary metabolite generation, the hairy root system has been used in metabolic pathway elucidation and investigations involving microorganism–root interactions. Advances in omics-related approaches and newly emerging genome editing techniques, including CRISPR/Cas9, may help in identifying the target compound's biosynthesis pathways, main stages, and regulatory mechanisms.

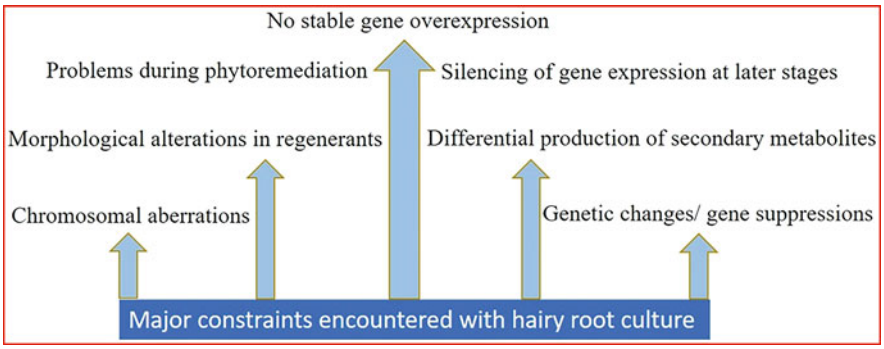

Fig. 17.2 Major limiting factors in hairy root culture

Hairy roots provided with appropriate concentrations of nutrients and oxygen, modifying operational factors as the culture progresses, and improving elicitor types, product recovery, and assortment of hairy root lines with powerful enzyme pools are still being explored. Thus, HRs will become a potent and sustainable phytochemical production system as breakthroughs in plant transcriptomics, proteomics, and metabolomics are combined with in silico modeling of metabolic fluxes and genetic engineering.

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Chapter 18
Secondary Metabolite Production from Roots/Rhizomes: Prospects and Challenges in Developing Differentiated Cultures Using the Plant's Hidden Half

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1 Introduction

Medicinal plants constitute a valuable resource for bioprospecting secondary metabolites (SMs) or natural products that had received attention over centuries due to their diverse biological activities. Worldwide market for plant-based drugs is growing every year with approximately 70% of drugs approved by US Food and Drug Administration in the past 25 years being based on natural products. These include artemisinin from *Artemisia annua*, paclitaxel from *Taxus brevifolia*, vinblastine and vincristine from *Catharanthus roseus,* and solamargines from *Solanum* species (Newman and Cragg 2020). SMs hold economic value considering their application as drugs, pigments and dyes, flavoring and fragrance agents, food additives, and also botanical pesticides. For many of these natural products, chemical synthesis has been developed; however, for many, the intrinsic structural complexity of the metabolite (s) remains a challenge such that biological sources are still relied for their production. Reliance on plants from wild as raw materials for extraction is often associated with environmental issues of genetic diversity loss and habitat destruction due to large-scale harvesting of medicinal plants for such high-value, low-volume metabolites (Canter et al. 2005). Such overharvesting for high-value compounds has pushed the status of many medicinal plants to threatened or endangered species. These issues have been addressed by developing plant *in vitro* strategies like cell and tissue cultures for controlled production of various secondary metabolites (SMs).

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M. K. Swamy, A. Kumar (eds.), *Phytochemical Genomics*,
https://doi.org/10.1007/978-981-19-5779-6_18

For example, callus and cell culture systems have been optimized for the production of various SMs (Christoph and Zotchev 2021) like isoquercetin, rutin, and rosmarinic acid from *Ocimum basilicum* (Açıkgöz 2020), artemisinin from *Artemisia annua* (Zebarjadi et al. 2018), ginsenosides from *Panax quinquefolius* (Biswas et al. 2018), paclitaxel from *Corylus avellana* (Salehi et al. 2019), and withanolides from *Withania somnifera* (Ahlawat et al. 2017). However, for many of the metabolites, the concentration of active principle in cell/suspension cultures is often found to be very low compared to those obtained from the field-grown plants possibly due to inherent biochemical or genetic instability of such culture systems over several passages (Gaosheng and Jingming 2012). Being undifferentiated, callus and cell cultures display limited ability to produce certain SMs which can be surpassed by developing differentiated cultures like root, shoot, and/or embryo cultures (Verpoorte et al. 2002).

Underground plant organs like roots, tubers, corms, and rhizomes hold enormous metabolic potential with their biosynthetic capabilities as diverse as any other plant part. Root biochemical diversity is well evidenced from their medicinal uses recorded in the traditional pharmacopeias (Bais et al. 2001). There are several metabolites like nicotine which are biosynthesized in roots and then transported to apoplast for accumulation in aboveground tissues (Zenkner et al. 2019). Other metabolites found exclusively in roots and underground tissues include thiarubrine in marigold roots, emetine in *Cephaelis ipecacuanha* and *C. acuminata* (Garcia et al. 2005), rotenone from *Derris* spp. and *Lonchocarpus* spp. (Zhang et al. 2020), forskolin from *Coleus forskohlii* (Singh and Suryanarayana 2019), and shikonin from (*Lithospermum erythrorhizon*) (Sharma et al. 2013a, b). For many of the secondary metabolites, production is feasible only in differentiated culture systems like root culture (Sharma et al. 2013a, b). Due to this reason, root cultures have received much research attention for production of root-specific SMs considering the (1) high proliferation rate; (2) metabolite production limited to roots; (3) enhanced potential for SM production; and (4) genetic stability of cultures (Srivastava and Srivastava 2007; Sharma et al. 2013a, b; Silja and Satheeshkumar 2015; Babich et al. 2020). Therefore, unlike other *in vitro* cultures, root cultures that include hairy root (HR) and adventitious root (AR) cultures are differentiated tissue systems that constitute prospective strategies for enhancing SM production. Development of root cultures thus constitutes an attractive alternative to cell/callus cultures with potential for commercial scale-up, particularly for plants that are known to produce metabolites in the underground tissues (Bais et al. 2001).

2 Hairy Root Cultures: Advancements and Prospects

Hairy roots are differentiated cultures with the roots arising profusely from wounded sites of explants following infection with the symbiotic bacterium, *Agrobacterium rhizogenes* (renamed as *Rhizobium rhizogenes*) (Stewart et al. 1900) (Fig. 18.1). During 1930s and 1960s, profuse root formation in horticultural plants was

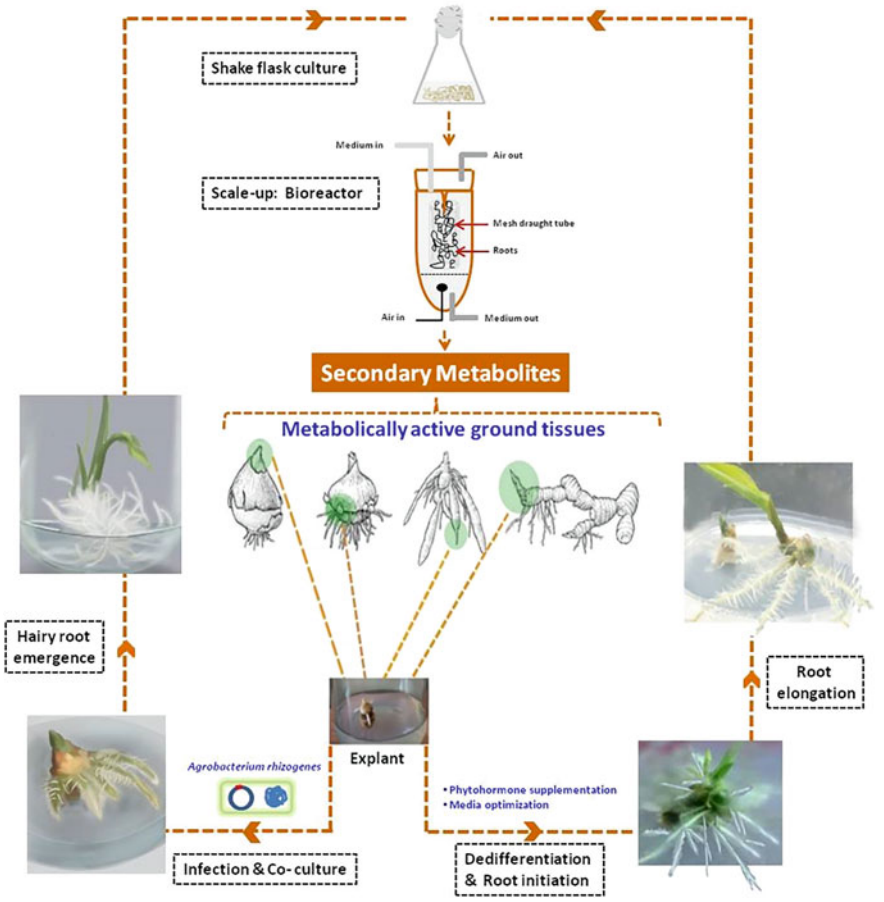

Fig. 18.1 Diagrammatic representation of the workflow of generating differentiated root cultures, i.e., hairy root (HR) and adventitious root (AR) cultures from explants of belowground tissues from medicinal plants for production of bioactive secondary metabolites (SM)

recognized to be resulting consequent to microbial invasion (Riker et al. 1930; Gutierrez-Valdes et al. 2020). Later studies conducted from 1970s to the 1980s identified the etiological agent as *A. rhizogenes*, a gram-negative soil bacterium (Ackermann 1977; Tepfer 1984). The bacterium was identified to induce formation of neoplastic and plagiotropic roots referred as hairy roots (HRs) (Sevon et al. 2002) (Fig. 18.2a). This ability of *A. rhizogenes* is attributed to the transfer of specific DNA fragments (T-DNA) from its extrachromosomal replicon, that is, root-inducing (Ri) plasmid to the plant cells (Nilsson and Olsson 2006; Babich et al. 2020). Transfer of T-DNA which is bordered by direct repeats (~25 nucleotides) is initiated from right border and proceeds to the left border with T-DNA integration often incomplete and truncated at the left part. The T-DNA which encodes for opine biosynthetic genes can occur either as single copies or as tandem or inverted repeats

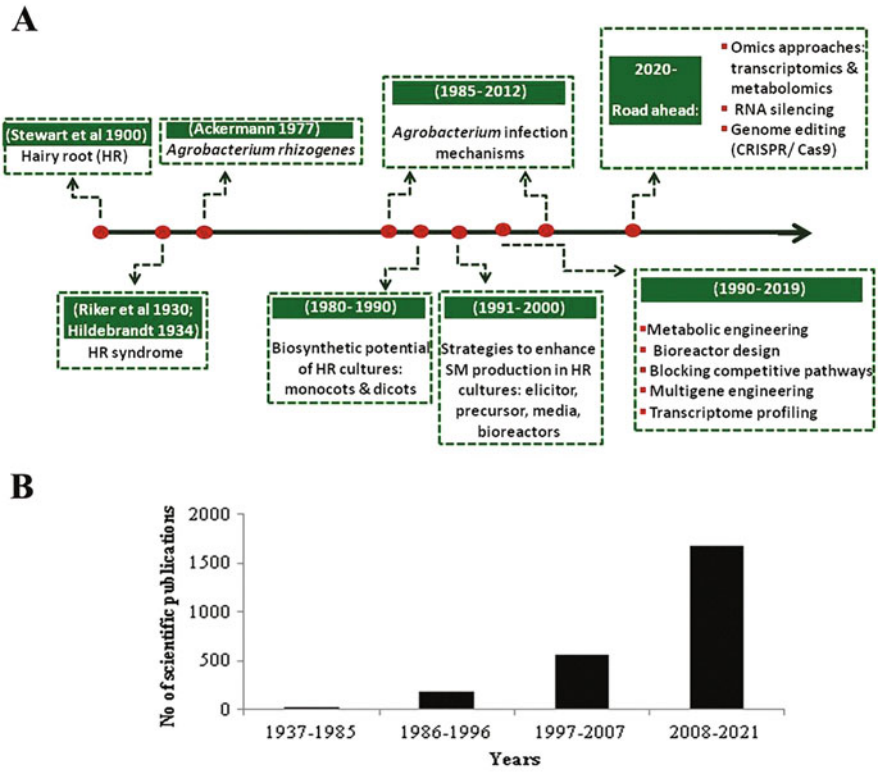

Fig. 18.2 Chronology of research undertaken in HR culture establishment. (**a**) Timeline portrayal of research undertaken till date and future prospects in HR culture technology. (**b**) Increase in number of research publications reporting HR culture establishment over the past four decades. Data have been generated by searching PubMed database using the keyword "hairy root"

(Bulgakov 2008). Opines are conjugation products of primary metabolites such as amino acids, α-keto acids, and sugars and cannot be metabolized by plants. Following uptake and integration of T-DNA in host cells, expression of T-DNA genes leads to abnormal root growth and production of specific opines which are utilized by the bacterium for its growth (Nilsson and Olsson 2006; Bulgakov 2008; Gutierrez-Valdes et al. 2020).

Till date, optimization and development of HR cultures have been heralded as a powerful biotechnological tool for SM production on commercial scale (Babich et al. 2020). The process holds several advantages over classical *in vitro* approaches like callus and suspension cultures (Verpoorte et al. 2002; Chandran et al. 2020) which can be summarized as:

- **Genetic and Phenotypic Stability**: Due to chromosomal stability of HRs that are cultivated in the absence of growth regulators and hence do not display somaclonal variations (Baíza et al. 1999; Häkkinen et al. 2016).

- **Fast Growth in Hormone-Free Environment**: Display profuse lateral branching due to transfer of *rol* genes from *A. rhizogenes* to explant that alters auxin metabolism and response (Nilsson and Olsson 2006).
- **Non-Geotropic Branching**: HR phenotype characterized by lack of geotropism and high lateral branching compared to normal roots (David et al. 1984; Gutierrez-Valdes et al. 2020) due to *rol* gene integration (Bulgakov 2008).
- **Production of Plant Biomass for Continuous Production of Desirable Metabolites**: Production is independent of variations in seasonal, climatic, and/or weather conditions which ensure a stable, continuous production system (Srivastava and Srivastava 2007).
- **High Biomass and SM Production**: Effective *A. rhizogenes* strains guarantee overproduction of target SM compared to its production in the normal plant under field-grown conditions (Srivastava and Srivastava 2007).
- **Contamination-Free Production**: The organized HR cultures are free of phytopathogenic contamination/infestation and thus are free of pesticides and herbicides. They are also free of organic and inorganic pollutants to which the plants are constantly exposed to under field conditions (Gutierrez-Valdes et al. 2020). This ensures production of plant biomass not only in large quantity and but also with high ecological purity.

In view of the abovementioned advantageous features of HR cultures (Mehrotra et al. 2015), there is increased interest among academic research groups, biotechnology companies, and pharmaceutical industries for optimization and development of HR cultures for various medicinal plants with pharmacological properties (Canter et al. 2005; Chandran et al. 2020; Gutierrez-Valdes et al. 2020; Christoph and Zotchev 2021). This is evident from the publications related to HR culture establishment available from 1990s to 2021 in the public database, PubMed as shown in Fig. 18.2b.

Prior to optimizing HR culture development, several parameters need to be considered such as (1) the targeted plant species (growth capacity; ability to be transformed and metabolite composition); (2) targeted metabolite (toxicity and molecular weight); and (3) stability of HR cultures overtime with respect to production capacity by the HR cultures (Häkkinen et al. 2016; Gutierrez-Valdes et al. 2020). The most important determining factor is selection of plant species that should display the ability to produce SMs in HR cultures in significant quantity and be amenable to scale-up (Sevon et al. 2002).

2.1 Factors Influencing HR Formation

The process of HR culture establishment starts with infection and co-cultivation of a wounded explant with *A. rhizogenes* strain followed by transfer to hormone free, antibiotic containing media to eliminate residual microbe and incubation till emergence of HR. For several medicinal plants, browning of explant tissue during

Table 18.1 Representative *A. rhizogenes* strains classified based on the opines produced (Babich et al. 2020)

Agropine	Mannopine	Cucumopine	Mikimopine
ATCC15834	LMG63	K599	MAFF30-1724 MAF6602-102 MAFF210266
LBA9402	LMG150	NCPPB2588	
HRI	NCIB8196	NCPPB2659	
NCPPB1855 LMG152	TR7	NCPPB2657	A13
R1601	TR101		A5
A4 MTCC 532 Arqua1	TR105 C58C1 ATCC11325 NCPPB2991 ATCC25818 NCPPB2626 LMG149		A6

Agrobacterium-mediated transformation process due to presence of high polyphenol content has been reported as major bottleneck in transformation (Rana et al. 2016). These problems can be overcome by selecting appropriate virulent *Agrobacterium* strain and also by manipulating culture media composition by adding antioxidants (Sandal et al. 2007). The various factors critical in influencing HR formation are as follows:

Agrobacterium Strains: Choice of bacterial strain is one of the main factors influencing successful HR induction (Sharifi et al. 2014). Many strains of *A. rhizogenes* have been used for optimizing HR production (Table 18.1). Selection of an effective *Agrobacterium* strain for SM production from transformed root cultures significantly depends on the plant species and must be determined empirically. Correlations have been inferred for the differences in virulence, morphology and growth rate of HR cultures to the variety of Ri (root-inducing) plasmids harbored within each bacterial strain (Park and Facchini 2000). Among the T-DNA genes in Ri plasmid, the *rol* oncogenes cause striking phenotypical and biochemical alterations in the transformed HR cultures (Thwe et al. 2016). Studies have shown the *rol* genes as potential activators of secondary metabolism in transformed cells of plants belonging to Solanaceae, Araliaceae, Rubiaceae, Vitaceae, and Rosaceae families (Bulgakov 2008). Majority of the scientific literature has reported ATCC15834 and A4 as the effective strain for HR induction.

Explants Source: Source of explants has a significant effect on transformation frequency with various explants reported to be used for HR formation like protoplast, leaf, cotyledons, hypocotyls, shoot tips, stem, stalk, storage root, and tubers (Panda et al. 2017). General induction of HR is by infecting sterile explants with *A. rhizogenes* strains. Compared to dicotyledonous plants, monocotyledonous plants which are not natural host of *Agrobacterium* species are not easily transformed with *A. rhizogenes* (Sood et al. 2011). However, with the understanding of plant

physiology and advent of molecular techniques, several monocots have been transformed by certain wild-type *A. rhizogenes* like A13 (Akutsu et al. 2004).

Infection Time: It is the duration of infection being identified as a factor influencing HR formation, various time period ranging from 10 to 40 min have been tried out in different plant taxa (Panda et al. 2017). For explants from some plants like peach, the optimal time for cotransformation has been identified as 30 min (Xu et al. 2020), while for some like *Withania somnifera* L., it is reported as 10–20 min (Saravanakumar et al. 2012).

Culture Conditions: Medium components, temperature, and pH are known to greatly affect transformation efficiency. For explants from different genotypes, the most popular medium of choice is MS (Murashige and Skoog) medium (Murashige and Skoog 1962) or modified MS-based medium. Reducing salt strength in inoculation and coculture medium has been reported by many to enhance T-DNA delivery.

Media: Conventional media used include MS basal medium, Gamborg (B5), and white media for explant preparation and subsequent induction of HR in infected explants. MS has been identified as the most suitable media for HR induction in *Psoralea corylifolia* L (Shinde et al. 2010), *Macleaya cordata* (Willd.) R.Br (Huang et al. 2018), *Althaea officinalis* L (Tavassoli and Afshar 2018), *Isatis tinctoria* L (Gai et al. 2015), *Withania somnifera* L (Saravanakumar et al. 2012), *Scutellaria baicalensis* Georgi (Lee et al. 2013a), *Vitis vinifera* L (Hosseini et al. 2017), *Cucumis anguria* L (Sahayarayan et al. 2020a, b), and *Solanum laciniatum* Ait (Okršlar et al. 2002). Some other studies have identified Gamborg's B5 medium as optimal for HR induction in plants like *Echinacea* spp., namely *E. purpurea* (L.) Moench, *E. pallida* (Nutt.) Nutt. and *E. angustifolia* (DC.) Hell) (Romero et al. 2009), *Gentiana scabra* Bunge (Huang et al. 2014), *Valeriana officinalis* L (Parizi et al. 2014), *Hyptis suaveolens* (L) Poit (Bazaldúa et al. 2014), *Salvia sclarea* L. (Kuzma et al. 2008), and *Solanum aculeatissimum* Jacq. (Ikenaga et al. 1995).

Sugar Concentration: Sugar concentration in the culture medium has been reported to induce *vir* genes synergistically with acetosyringone (Chandran and Potty 2011). This results in enhanced HR induction and also promotes rapid growth of HR. Various concentration of sucrose ranging from 2 to 5% have been tried to induce HR formation in various plant species (Sivanandhan et al. 2012a, b; Verma et al. 2015). Optimal sucrose concentration has been observed to be dependent on the selected plant with 2% determined as optimal for soybean (Cheng et al. 2021), while 3% reported to be optimal for plants like *Pueraria phaseoloides* (Roxb.) Benth. (Liang et al. 2004), *Macleaya cordata* (Willd.) R.Br. (Huang et al. 2018), *Solanum aculeatissimum* Jacq. (Ikenaga et al. 1995), *Isatis tinctoria* L. (Gai et al. 2015), and *Aconitum heterophyllum* Wall (Giri et al. 1997). HR cultures of *Panax quinquefolium* L exhibited maximum growth rate at 2–3% of sucrose in media (Kochan et al. 2013). Higher concentration of sucrose (4%) was reported to enhance withaferin A and withanone production in *Withania somnifera* L (Dunal) (Sivanandhan et al. 2012a, b), while for *Talinum paniculatum* Gaertn, sucrose concentration was optimized as 6% for maximum biomass production and 5% for high SM production (Yosephine et al. 2015).

Elicitors: In addition to nutrient media, elicitation by biotic and abiotic agents (Naik and Al-Khayri 2016) is an efficient way of enhancing SM production in HR cultures. Elicitors generally enhance SM production by stimulating defense responses of the plant (Halder et al. 2019; Isah 2019). Types of elicitor, dosage, and exposure duration are major factors determining SM production following treatment of HR cultures with elicitors. Some of the elicitors commonly applied to HR cultures are summarized in Table 18.2. Besides enhancing SM production, the application of elicitors very often stimulates efflux of intracellular products thus easing product recovery and purification of desired metabolite. This makes elicitation a commercially viable strategy to enhance production of low-volume, high-value metabolites.

2.2 Enhanced Secondary Metabolite Production through HR Cultures

Production of various SMs of high-value has been optimized in HR cultures developed from various taxa, such as HR cultures developed for production of rosmarinic acid (Grzegorczyk et al. 2006), artemisinin (Cai et al. 1995), baicalin (Sung-Jin 2006), aconitine (Giri et al. 1997), anthraquinone (Guo et al. 1998), tropane alkaloids (Jouhikainen et al. 1999), and nicotine (Zhao et al. 2013) (Table 18.3). Higher level of SM accumulation over shorter time period compared to normal root is the main advantage of developing HR cultures. Examples include the enhancement in tanshinone content in transgenic *S. miltiorrhiza* hairy roots (15.4 mg/g dry weight) compared to field-grown root (1.7–9.7 mg/g dry weight) (Kai et al. 2011; Hao et al. 2020). Despite several experimental systems revealing HR cultures as excellent models for ensuring stable and enhanced SM production (Baíza et al. 1999; Häkkinen et al. 2016), the technique faces challenges for several medicinal plants in terms of significant increase in SM levels and stability of cultures. This necessitates the need to undertake experiments in medicinal plants particularly those wherein SMs are biosynthesized and accumulate in belowground tissues (Kai et al. 2011; Kim et al. 2015; Zhao et al. 2016). In this direction, the various biotechnological strategies proposed like use of elicitors, precursor addition, and blocking competitive pathways (Xiao et al. 2011) using metabolite pathway inhibitors (Aswati et al. 2020) and multigene engineering (Schweizer et al. 2018; Kai et al. 2011) need to be explored (Sharma et al. 2013a, b). For several of the medicinal plants wherein the entire biosynthetic pathway is yet to be characterized (Guo et al. 2013; Qiu et al. 2020), omics-based approaches particularly transcriptomics and metabolomics will facilitate in regulatory gene identification (Zhou et al. 2017). The prospects of using recent technologies like genome editing using CRISPR/Cas9 (Cheng et al. 2021) and RNA silencing will yield valuable insights into gene (s) regulating SM biosynthetic pathways (Shi et al. 2021).

Table 18.2 Elicitors used in the hairy root cultures of different plants

Plant	Elicitors	Concentration	Metabolite elicited	Reference
Ammi majus L	Benzo (1,2,3)-thiadiazole-7-carbothionic acid S-methyl ester (BION®) Autoclaved lysate of cell suspension of bacteria—*Enterobacter sakazakii*	2.5 mg 15 mL/L	– Umbelliferone Bergapten	Staniszewska et al. (2003)
Astragalus membranaceus Moench	Methyl jasmonate	283 μM	Isoflavonoid	Gai et al. (2016)
Brugmansia candida Pers	Salicylic acid (SA)	0.01, 0.10, and 1.00 mM	Tropane alkaloids	Pitta-Alvarez et al. (2000)
	Yeast extract	0.8 mg/mL	(Scopolamine and hyoscyamine)	
	$CaCl_2$	100 mM		
	$AgNO_3$	1.0 mM		
	$CdCl_2$	1.0 and 2.0 mM		
Arachis hypogaea L	Cyclodextrin + methyl jasmonate	100 μM + 9 g/L	Resveratrol, piceatannol, arachidin-1, and arachidin-3	Yang et al. (2015)
Cichorium intybus L	*Phytophthora parasitica* var. nicotiana filtrate	1% v/v	Esculin/ esculetin	Bais et al. (2000)
Echinacea purpurea (L) Moench	Gibberellic acid	0.025 μM	Caffeic acid derivatives	Abbasi et al. (2012)
Fagopyrum tataricum (L) Drejer	UV-B	30 min. (light intensity1.26 μW/ cm^2 on sample surface)	Rutin, quercetin	Huang et al. (2016)
Panax ginseng CA Mey	Methyl jasmonate	22.4 mg/L	Ginsenoside	Palazón et al. (2003a, b)
Papaver orientale L	Methyl jasmonate	100 μM	Morphine	Hashemi and Naghavi (2016)
Plumbago indica L	Jasmonic acid + chitosan	80 μM + 200 mg/ L	Plumbagin	Gangopadhyay et al. (2011)
Psoralea corylifolia L	Jasmonate	1 and 10 μM	Daidzin	Zaheer et al. (2016)
	Acetyl salicylic acid	10 and 25 μM		
Salvia castanea Diels	Methyl jasmonate	200 μM	Tanshinone	Li et al. (2016)
	Ag+	15 μM		

(continued)

Table 18.2 (continued)

Plant	Elicitors	Concentration	Metabolite elicited	Reference
Scopolia parviflora (Dunn) Nakai	Bacteria spp.	2 mL per 15 mL hairy root culture	Scopolamine	Jung et al. (2003)
Rauwolfia serpentina (L) Benth ex Kurz	NaCl	100 mM	Ajmalicine ajmaline	Srivastava et al. (2016)
	Mannan	100 mg/L		
Solanum khasianum CB Clarke	Cellulase	100 µg/mL	α-solanine Solasodine	Srivastava et al. (2016)
	NaCl	100 mM and 200 mM NaCl		
Brugmansia candida Pers	Hemicellulase	50 mM	Scopolamine and hyoscyamine	Pitta and Pitta-Alvarez (2000)
	$CaCl_2$	0.5 U/mg		
Hyoscyamus reticulatus L	Iron oxide nanoparticles	900 mg/L	Scopolamine and hyoscyamine	Moharrami et al. (2017)
	FeNPs	450 mg/L		
Catharanthus roseus (L) G. Don	*Penicillium* spp. homogenate	–	Catharanthine and ajmaline	Sim et al. (1994)
Nicotiana tabacum L	Yeast extract, *Botrytis fabae* extract	–	Sesquiterpene phytoalexins	Wibberley et al. (1994)
Datura metel L	*B. cereus* cultures	13.3 mL	Scopolamine	Shakeran et al. (2017)
	S. aureus cultures	100 mL		
Gentiana dinarica Beck	*Chitosan*	50 mg/L	Xanthone compounds	Dijana et al. (2017)
Withania somnifera (L) Dunal	*Salicylic acid*	150 µM	Withanolide A, withanone	Sivanandhan et al. (2012a)

3 Adventitious Roots: Induction and Prospects

Adventitious roots (ARs) are differentiated *in vitro* cultures which develop in response to wound (De Klerk et al. 1999; Silja and Satheeshkumar 2015) from explants that are non-root in origin such as leaves, stem nodes, and internodes (Gonin et al. 2019). AR emerges either directly by organogenesis from cambium cells or indirectly from callus tissues (Silja and Satheeshkumar 2015; Lakehal and Bellini 2019). Emergence of AR is influenced by numerous endogenous factors like hormone fluctuations and/or exogenous factors like environmental stress including mechanical damages and mineral deficiency/nutrient deprivation (Sorin et al. 2005; Steffens and Rasmussen 2016; Li 2021). AR cultures are simpler to establish as they do not involve genetic modification like HR culture establishment (Gaosheng and Jingming 2012; Gonin et al. 2019). AR induction techniques have been successfully

Table 18.3 Secondary metabolites produced through hairy root cultures

Plant	*Agrobacterium rhizogenes* strain	Secondary metabolite	Reference
Isatis tinctoria L	LBA9402	Flavonoids	Gai et al. (2015)
Gentiana scabra Bunge	ATCC15834	Iridoids and secoiridoids	Huang et al. (2014)
Withania somnifera (L) Dunal	R1000	Withaferin A	Saravanakumar et al. (2012)
Rhaponticum carthamoides (Willd.) Iljin	A4	Caffeic acid derivatives	Skala et al. (2015)
Panax ginseng CA Mey	A4	Ginsenoside	Palazón et al. (2003a, b)
Echinacea purpurea (L) Moench	ATCC 43057	Caffeic acid derivatives	Abbasi et al. (2012)
Cichorium intybus L	LMG 150	Coumarin	Bais et al. (2000)
Psoralea corylifolia L	LBA 9402	Phytoestrogens	Shinde et al. (2010)
Vitis vinifera subsp. sylvestris	ArA4	Resveratrol	Hosseini et al. (2017)
Artemisia annua L	R1601	Artemisinin	Cai et al. (1995)
Genista tinctoria L	ATCC 15834	Isoliquiritigenin	Łuczkiewicz and Kokotkiewicz 2005
Salvia sclarea L	LBA 9402	Diterpenoid	Kuzma et al. (2008)
Solanum aculeatissimum Jacq	ATCC15834	Steroidal saponin	Ikenaga et al. (1995)
Talinum paniculatum Gaertn	LB510	Saponin	Yosephine et al. (2015)
Salvia officinalis L	ATCC 15834	Rosmarinic acid	Grzegorczyk et al. (2006)
Cassia obtusifolia L	LBA9402	Anthraquinone	Guo et al. (1998)
Rauvolfia micrantha Hook	ATCC15834	Ajmalicine, ajmaline	Sudha et al. (2003)

optimized for several plant species toward production of high-value SMs of pharmaceutical, nutraceutical, and industrial importance (Murthy et al. 2008). AR cultures are advantageous for commercial production of SMs that are root-specific in terms of biosynthesis and/or accumulation (Paek et al. 2009a, b; Baque et al. 2012a, c). Rapid growth (Hahn et al. 2003; Silja and Satheeshkumar 2015), stability, and active SM biosynthesis make AR cultures a promising strategy for enhanced biomass and SM production (Carvalho and Curtis 1998). The enhanced research interest in AR culture development is evidenced from the increase in number of research publications till current year in the PubMed database (Fig. 18.3).

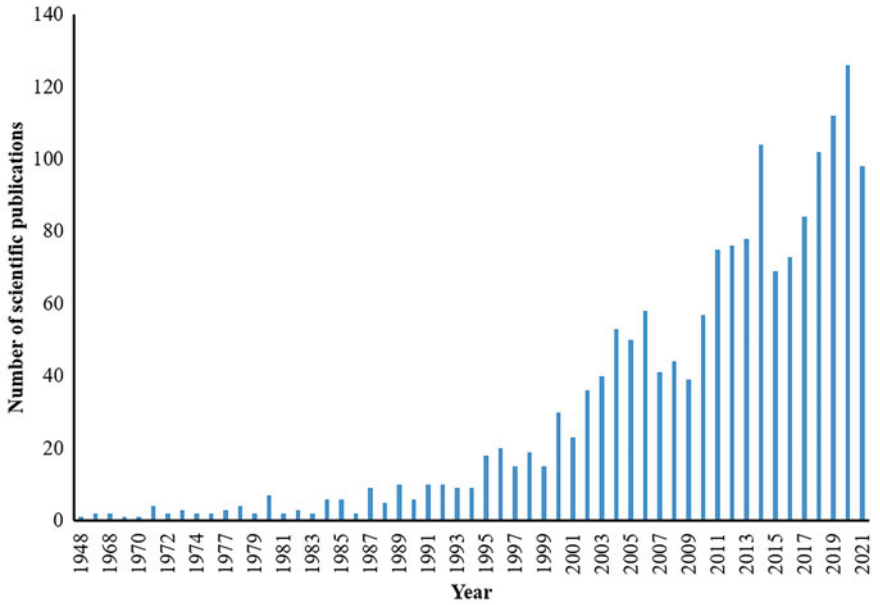

Fig. 18.3 Rise in scientific publications over the past seven decades, as identified in PubMed database using the keyword "adventitious root"

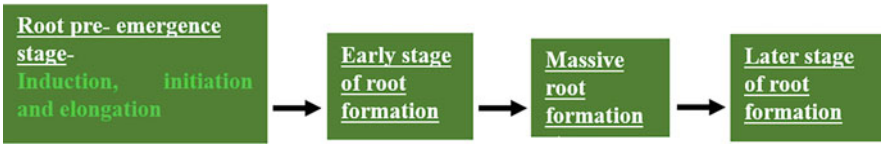

Fig. 18.4 Summary of stages involved in adventitious root formation

3.1 Factors Influencing AR Formation

Organogenesis leading to AR formation can be summarized in four steps (Figs. 18.1 and 18.4) (Jasik and De Klerk 1997; Rahmat and Kang 2019). First is the root preemergence stage which includes induction (dedifferentiation) and comprises of formation of root initials, development of an organized root primordia and elongation or emergence of root primordial. This is followed by second step which comprises of early phase root formation followed by third step involving massive root growth and final fourth step of root development and proliferation (Zhang et al. 2017; Rahmat and Kang 2019). These stages are influenced by changes in endogenous concentrations of hormones like auxin which stimulates AR induction and cytokinin that triggers differentiation of root tissue and ethylene resulting in root elongation (Haissig 1974; Jarvis and Yasmin 1987; Nag et al. 2001; Pop et al. 2011; Druege et al. 2016).

Different culture conditions including media, growth hormones, type and concentration of carbon source, culture conditions and pH need to be optimized for different plant species to generate *in vitro* AR. Plant growth hormones significantly affect AR induction with enhanced AR formation observed in presence of auxin, abscisic acid (ABA), jasmonic acid (JA), and polyamines (Haissig 1974; Hansen 1976; Nag et al. 2001; Steffens et al. 2006; Lischweski et al. 2015; Druege et al. 2016) while inhibited by ethylene (Nordström and Eliasson 1984; Wang and Pan 2006). Gibberellin and cytokinin have been reported to inhibit initiation of root primordia at high concentrations (Hansen 1976; Higuchi et al. 2004; Mao et al. 2019).

Explant Source: Explants used for AR induction can be leaf (Praveen et al. 2009; Lee et al. 2011; Sharma et al. 2013a, b), stem (Jasik and De Klerk 1997; Srikanth et al. 2016), or roots (Paolillo Jr and Zobel 2002). AR has been successfully established from callus developed from root explant (Murthy and Paek 2016) or cell suspension culture, obtained from friable callus (Raju et al. 2015). Adventitious shoots and roots regenerated in aeroponic system from explants have also been used for rapid induction of AR, in less than a week, from stem explants of six *Brassica* spp. cultivar varieties, without use of any plant hormones (Srikanth et al. 2016).

Culture Conditions: Different culture conditions such as media strength, pH, addition of growth hormones and their concentration, carbon source, and elicitors have been optimized for SM production in different medicinal plant species through *in vitro* generation of AR (Wawrosch and Zotchev 2021). A summary of the optimized culture conditions for various reported AR induction processes is given in Table 18.4.

Media: Compared to B5 and SH media, MS media at different strength is most commonly used for the induction and proliferation of AR cultures. Optimal conditions for AR induction have been reported in half MS media for *Curcuma amada* (Raju et al. 2015), *Withania somnifera* (Praveen and Murthy 2010) and *Psoralea corylifolia* (Baskaran and Jayabalan 2009). Full strength MS media was reported to be suitable for AR induction in *Andrographis paniculata* (Sharma et al. 2013a, b).

Sugar Concentration: Concentration of sucrose as carbon source is another crucial media parameter that regulates flux of SM biosynthetic pathways (Herbers et al. 1996; Bhardwaj et al. 2016). Considering the fact that the absorption and metabolism of carbon is species dependent (Ho et al. 2020), studies have been carried out using sucrose at concentration ranging from 1 to 5% to optimize AR induction in various plants as summarized in Table 18.5. Higher sucrose concentration triggers osmotic stress resulting in accumulation of SM (Ho et al. 2020). This has been reported in AR of *E. angustifolia* (Wu et al. 2007), *H. perforatum* (Cui et al. 2010) and *Polygonum multiflorum* (Ho et al. 2021).

pH: Medium pH is a critical factor to be optimized for AR induction and SM production. Medium pH is reported to be altered consequent to various factors like differential uptake of ammonium (NH^{4+}) and nitrate (NO^{3-}) (Skirvin et al. 1986), autoclaving of media (Skirvin et al. 1986), and interaction of AR with media.

Role of Phytohormones: Phytohormones are known to coordinate and guide each step of AR formation from the primary event of cell reprogramming till

Table 18.4 Explants used and optimized culture conditions for induction of AR in different plants for the production of secondary metabolites

Plant species	Secondary metabolites	Explant used for AR induction	Optimized culture condition	References
Andrographis paniculata (Burm.f) Wall	Andrographolide	Leaf	MS + 2.7 μM NAA + 30 g/L sucrose	Praveen et al. (2009)
			MS + 1.0 mg/L NAA	Sharma et al. (2013a, b)
Echinacea purpurea (L) Moench	Caffeic acid derivatives, alkylamides, polyacetylenes, and polysaccharides	Root	MS having ammonium and nitrate ratio is 5: 25 mM+ 9.8 m M IBA +50 g/L sucrose.	Paek et al. (2009a, b)
Artemisia amygdalina Decne	Phenolic compounds and essential oil. Terpenes	Leaf	MS + 1.0 mg/L NAA+ 4% sucrose	Taj et al. (2019)
Panax ginseng CA Mey	Ginsenosides	Root	MS + 30 g/L sucrose +24.6 μM IBA	Murthy et al. (2016)
Psoralea corylifolia L	Psoralen	Hypocotyl explant	½ MS liquid media +3 μM IBA.	Baskaran and Jayabalan (2009)
Podophyllum hexandrum Royle	Podophyllotoxin	Root explants derived from *in vitro* seedlings.	MS solid medium + IBA (1.5 mg/L)	Rajesh et al. (2012)
Curcuma amada Roxb	Isosorbide and n-hexadecanoic acid	Friable callus-derived cell suspension culture	1/2 MS liquid media +0.3 mg/L IBA + 3% of sucrose	Raju et al. (2015)
Boerhavia diffusa L	Punarnavine	Leaf	MS + NAA (1.0 mg/L).	Jenifer et al. (2012)
Hypericum perforatum L	Hypericin	Root	MS + 1 mg/L; IBA + 30 g/L sucrose; blue light as elicitor	Najafabadi et al. (2019)
Plumbago zeylanica L	Plumbagin	Leaf	MS + 1.0 mg/L IBA + 0.5 mg/L NAA	Sivanesan and Jeong (2009)
Rumex crispus L	Flavonoids	Leaf	MS + 5 μM NAA + 0.5 μM Kn	Mahdieh et al. (2015)
Aloe vera L	Aloe-emodin and aloin	Leaf	MS + 0.5 mg/L NAA + 0.2 mg/L 6-benzylaminopurine	Lee et al. (2013b)

(continued)

Table 18.4 (continued)

Plant species	Secondary metabolites	Explant used for AR induction	Optimized culture condition	References
Withania somnifera (L) Dunal	Withanolide A	Leaf	½ MS semisolid medium (0.8% agar) + 0.5 mg/L IBA + 30 g/L sucrose	Praveen and Murthy (2010)
Gynura procumbens (Lour) Merr	Flavonoids	Leaf	MS + 5 mg/L IBA + 3% sucrose, temporary immersion bioreactor	Kusuma et al. (2017)

Table 18.5 Optimized sucrose concentration for AR induction in various plant species

Plant species	Optimized sucrose concentration	Reference
Decalepis salicifolia Venter	2% (for maximal biomass) 5% (for 2-hydroxy-4-methoxybenzaldehyde induction)	Rodrigues et al. (2021)
Podophyllum hexandrum Royle	2% (for maximal biomass) 6% (for podophyllotoxin induction)	Rajesh et al. (2014)
Gymnema sylvestre R.Br.	3%	Lee et al. (2006)
Panax ginseng CA Mey		Kim et al. (2005)
Pseudostellaria heterophylla (Miq) Pax	4%	Yin et al. (2013)
Allamanda cathartica L		Khanam et al. (2018)
Echinacea angustifolia (DC) Hell	5%	Wu et al. (2007)
Eurycoma longifolia Jack		Hussein et al. (2012)
Panax notoginseng (Burkill) F.H. Chen		Zhao et al. (2020)

emergence and outgrowth (Lakehal and Bellini 2019). Auxin is the major growth-promoting phytohormone and central regulator controlling the AR initiation in plants along with an array of other phytohormones through a complex crosstalk (Li 2021). Auxins including 2,4-dichlorophenoxy-acetic acid (2,4-D), indole-3-butyric acid (IBA), indole-3-acetic acid (IAA), and α-naphthalene acetic acid (NAA) are the most widely used root stimulators in culture practice (Jarvis and Yasmin 1987; De Klerk et al. 1999; Pop et al. 2011). IBA at varying concentration (3–24.6 μM) is the most commonly used auxin for induction of SM in AR cultures of medicinal plants like *Curcuma amada* (Raju et al. 2015), *Psoralea corylifolia* (Baskaran and Jayabalan 2009), *Panax ginseng* (Murthy et al. 2016) and *Centella asiatica* (Ling et al. 2009a). In *Malus hupehensis,* IAA (100 mg/mL), NAA (300 mg/mL) and green growth regulators (GGR) (300 mg/mL) shortened the rooting time by 25 ± 47.4% and increased the rooting percentage in cuttings by 0.9 ± 1.3 times (Zhang et al. 2017).

Table 18.6 Some of the elicitors reported to enhance secondary metabolites (SM) production in adventitious root (AR) cultures of different medicinal plants

Plant	Elicitor used	Concentration	Metabolite	References
Panax ginseng CA Mey	Methyl Jasmonate	100 µM	Ginsenosides	Kim et al. (2004)
	Organic germanium	60 mg/L	Ginsenosides	Yu et al. (2005)
Panax quinquefolius L	*A. panax* and *C. destructans* fungi extracts	4 mg/L and 20 mg/L	Ginsenosides	Yu et al. (2016)
Polygonum multiflorum Thunb.	Methyl Jasmonate	50 µM	Phenolic compounds	Ho et al. (2018)
Hypericum perforatum L	UV-B (60 min), low temperature (4 °C for a period of 72 h)	–	Hypericin	Tavakoli et al. (2020)
Morinda citrifolia L	Chitosan	0.2 mg/mL	Anthraquinones, phenolics, and flavonoids	Baque et al. (2012a, b)
Aloe vera L	Salicylic acid	1000–2000 µM	Aloe-emodin and chrysophanol	Lee et al. (2011, 2013b)
Oldenlandia umbellate L	Pectin	50 mg/L	Anthraquinones	Krishnan and Siril (2018)
Perovskia abrotanoides Kar.	Yeast extract and $AgNO_3$	100 and 200 mg/L, 25 µM, respectively	Tanshinones	Zaker et al. (2015)
Withania somnifera (L) Dunal	Chitosan	100 mg/L	Withanolides	Sivanandhan et al. (2012c)
Plumbago rosea L	Jasmonic acid	50 µM	Plumbagin	Silja and Satheeshkumar (2015)

Elicitation: Various biotic or abiotic elicitors, when applied in small quantity, are known to modulate the plant defense mechanisms by enhancing SM production in plant *in vitro* cultures (Ramirez-Estrada et al. 2016; Naik and Al-Khayri 2016; Isah 2019). Abiotic elicitors used include both physical (light, UV, osmotic stress, salinity, drought and thermal stress) and chemical (methyl jasmonates, salicylic acid, $CuSO_4$, $AgNO_3$, sorbitol, phenyl acetic acid, caffeic acid, oxalic acid and ethephon) (Naik and Al-Khayri 2016; Rahmat and Kang 2019). Examples of various elicitors used for enhanced SM production from AR cultures are summarized in Table 18.6.

3.2 Enhanced Secondary Metabolite Production Through AR Cultures

The immense possibilities offered by AR cultures for SM production includes option to scale-up, enhance production by optimizing medium composition and/or elicitation (Murthy et al. 2016). Compared to hairy root cultures, AR is not genetically modified (Fig. 18.1) offering these cultures the advantage of being a natural strategy for commercial production of SMs (Murthy et al. 2016).

4 Applications of AR Culture

SMs obtained from wild or field-grown medicinal plants are heterogeneous and are known to fluctuate, both in quality and quantity (Canter et al. 2005; Baque et al. 2012b; Chandran et al. 2020; Christoph and Zotchev 2021). Various biotic and abiotic factors significantly affect the quantity of SMs in field-grown medicinal plants (Beppu et al. 2004). The quality also gets affected by factors like presence of soil pollutants that can be harmful to human health. In this context, AR cultures constitute a worthwhile strategy to enhance production of pharmaceutically and nutraceutically important root-specific bioactive SMs, in bulk and of uniform quality (Gaosheng and Jingming 2012; Murthy et al. 2016). Strategies to scale-up *in vitro* AR cultures by incorporating bioreactor technologies (Gerth et al. 2007; Baque et al. 2012b; Rahmat and Kang 2019) could facilitate commercial production of such high-value SMs. Establishment of AR cultures on commercial scale also ensures round-the-year production that can be modulated to enhance SM production by media manipulation, elicitor and precursor addition. Like HR cultures, the technique also limits the destructive harvesting of field plants for roots that affects the biodiversity and threatens natural plant population driving them toward extinction. Other attractive attributes of AR culture for long-term, large-scale SM production is with respect to culture stability in terms of root morphology, biomass and metabolite production (Le et al. 2019). Besides culture stability, AR cultures also display better biosynthetic ability compared to suspension cultures (Rahmat and Kang 2019). In view of these advantageous features, AR cultures have been established for commercial production of SMs like saikosaponin from *Bupleurum falcatum* and ginsenosides from *Panax ginseng* (Rodrigues et al. 2021). Other examples of commercially established AR cultures are tabulated in Table 18.7.

Table 18.7 Examples of commercially significant secondary metabolites reported to be produced in AR cultures

Plant species	Metabolites	Importance	Reference
Panax ginseng CA Mey	Ginsenosides	Prevention of cardiovascular diseases, improvement in blood circulation, neuroprotective	Kim et al. (2017)
Hypericum perforatum L	Hypericin	Depression treatment and photodynamic therapy	Najafabadi et al. (2019)
Withania somnifera (L) Dunal	Withanolide A	Neuroprotective	Praveen and Murthy (2010)
Morinda citrifolia L	Anthraquinones, phenolics, and flavonoids	Anticancer, antioxidant, antibacterial, antiviral, hepatoprotective, antiallergic	Baque et al. (2012a)
Podophyllum hexandrum Royle	Podophyllotoxin	Anticancer	Rajesh et al. (2012)
Perovskia abrotanoides Kar	Tanshinone	Anticancer, anti-inflammatory, antioxidant	Zaker et al. (2015)
Scopolia Parviflora (Dunn) Nakai	Scopolamine	Anticholinergic	Jung et al. (2003)
Eurycoma longifolia Jack	Quassinoid	Anticancer, antimalaria, to treat stomach and intestinal problems	Hussein et al. (2012)
Orthosiphon Stamineus Benth.	Rosmarinic acid	Antimicrobial, immunomodulatory, antidiabetic, antiallergic, anti-inflammatory	Ling et al. (2009b)
Silybum marianum (L) Gaertn.	Silymarin	Cirrhosis	Riasat et al. (2015)
Withania somnifera (L) Dunal	Withanolide	Asthma, parasitic disease, glaucoma, headache, hepatopathy	Thilip et al. (2015)

5 Conclusion and Future Perspectives

Plant roots constitute one of the main raw materials in about 60% of ethnomedicinal formulations (Rahmat and Kang 2019). For many of the SMs of pharmaceutical relevance, the complex chemical structure makes the chemical synthesis routes unfeasible. Under such circumstances, establishment of differentiated root culture is of particular relevance to ensure stable SM production without relying on large-scale plant harvesting for extraction that is associated with negative environmental impacts. These observations necessitate the need to undertake research on medicinal plants of ethnopharmacological relevance, toward prospecting and developing appropriate production systems for the many root-based SMs. Genetic stability and biochemical stability of HR and AR cultures as well as efficient metabolite

productivity amenable to scale up make these differential *in vitro* systems advantageous over callus and cell suspension cultures. For some medicinal plants, HR techniques have been successful in enhancing SM production, but for many others, AR has been found to be relatively more efficient (Rahmat and Kang 2019). Both HR and AR techniques are of particular significance for enhancing production of SMs, biosynthesis of which is localized in roots/rhizomes/tubers. Considering the species-specific factors significantly influencing HR and/or AR culture induction and establishment, the information being reviewed will be of immense help to researchers engaged in optimization of root culture conditions. The information can be used to optimize and develop appropriate commercial production systems for more medicinal plants towards enhancing availability of root-based bioactive SMs. While traditional methods that include integrative application and optimization of elicitors, precursors and culture media offer excellent opportunity for large-scale production of bioactive SMs, the newer technologies of transcriptomics and metabolomics, genome editing, metabolic pathway engineering, and gene silencing will help to unravel the regulatory gene(s), thereby providing detailed understanding to the SM biosynthetic pathway regulation. In these respects, HR and AR cultures offer tremendous scope as models for undertaking research in understanding SM biosynthesis and regulation.

Acknowledgements Authors acknowledge the facilities extended at CUK for undertaking research in the reviewed area. The grant received from UGC (University Grants Commission, Govt. of India) for Junior Research Fellowship is acknowledged by Harsha K (No.20/1/2015(ii)EU-V; Sr No. 2121530466) and Harshitha K (No. 20/12/2015(ii)EU-V; Sr. No. 2121530467). Shilpa T acknowledges the Junior Research Fellowship (09/1108(0030)/2018-EMR-I) received from CSIR (Council for Scientific and Industrial Research).

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Chapter 19
Elicitation: An Efficient Strategy for Enriched Production of Plant Secondary Metabolites

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1 Introduction

Secondary metabolites (SMs) are the chemical compounds responsible for activating plant defense mechanism. They do not have direct roles in the central life processes of plants, but provide protection against pathogens and environmental stresses. They also involve in creating ecological connections between other species and act as herbivore deterrents, pollination attractants, and mitigators of oxidative stress. They possess paramount commercial value, because of their potential use as pharmaceutical drugs as they have been identified as antibiotic, antifungal, antiviral, antioxidant, anticancer, anti-inflammatory agents, etc. Further, they have been recognized as food additives, flavoring agents, sources for innumerable industrial products, etc. (Kabera et al. 2014).

Plants secrete SMs via different metabolic pathways, which are instigated from the principal metabolite paths. Some of the vital metabolite biosynthetic pathways in most of the plants are conserved. A frequent methylation, glycosylation,

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M. K. Swamy, A. Kumar (eds.), *Phytochemical Genomics*,
https://doi.org/10.1007/978-981-19-5779-6_19

phosphorylation, acylation, hydroxylation, oxidation, prenylation, and a few chemical alterations cause wide-ranging alterations in the elementary structures of metabolites. On the basis of biosynthesis pathways, SMs may be classified into three major categories: (1) phenolic compounds (the shikimate pathway-mediated biosynthesis); (2) terpenes (the mevalonic pathway-mediated synthesis); and (3) nitrogen-containing compounds (the tricarboxylic acid cycle-mediated synthesis) (Jan et al. 2021). Phenolics comprise the largest group of metabolites ranging from simple structures with one or more phenol groups to complex polymeric substances. Some phenolic compounds are treasured for their medicinal properties with high market value and demand. The subtypes of this major secondary metabolite are simple phenols, flavonoids, tannins, lignans and lignins and coumarins. Important candidates include caffeic acid, ferulic acid, hydroxy cinnamic acid, chlorogenic acid, gallic acid, myricetin, apigenin, and luteolin genistein.

Terpenes constitute a large class of hydrocarbonated metabolites having isoprene units. Being lipid soluble, these chemical constituents possess varied structures and act as essential elements of various hormones, sterols, and plant pigments. Their chief roles include pollinator attractants, herbivore deterrents, and defensive toxins. On the basis of the occurrence of isoprene unit numbers, they are categorized as monoterpenes, diterpenes, triterpenes, sesquiterpenes, and polyterpenes. All types of compounds of terpenes exhibit significant pharmacological activities, such as antioxidant, antimicrobial, antiaggregating, anti-inflammatory, antispasmodic, antihistaminic, cardioprotective, and anesthetic properties. Distinguished representations of this group of metabolites are citral, thymol, paclitaxel, forskolin, salvinorin, rubber, and so on. Nitrogen-containing compounds are structurally very diverse and termed as alkaloids, which can be categorized as aromatics, acridones, ephedras, carbolines, imidazoles, ergots, bisindoles, indoles, indolizidines, oxindoles, quinolines, manzamines, quinozolines, phenylethylamines, phenylisoquinolines, piperidines, pyrrolidines, purines, pyridines, pyrroloindoles, pyrrolizidines, and simple tetra hydroisoquinolines. They exhibit a wide-ranging pharmacological activities, including local anesthesia, analgesia, respiratory stimulation, cardiac stimulation, vasoconstriction, antineoplastic, hypertensive, hypotensive, antimicrobial, and allelopathic activities. Monocrotaline, tomatidine, senecionine, vincristine, vinblastine, pyrrolizidine, and mimosine are some examples of commercially important alkaloids (Hussein and Anssary 2018).

Secondary metabolites are biosynthesized at lower levels from usual precursors at the specific physiological and developmental stages of plants. The large-scale industrial production of phytochemicals involves lots of challenges such as availability, overexploitation, truncated yield, seasonal variations, tissue or organ-specificity, problems in purification. Moreover, the chemical synthesis approach is not viable economically, due to their chemocomplexity and stereospecificity. At this juncture, plant cell cultures have been progressed as encouraging alternatives to produce plant metabolites in large quantities. They can also be induced in a synchronized mode in order to achieve enhanced accumulation of metabolites. Tissue culture-based plant biotechnological techniques like cell suspension cultures, callus cultures, micropropagation, and adventitious root cultures have been advanced for

the commercial production of plant metabolites. In addition, numerous approaches, including screening and selection of high-yielding lines, culture media optimization, physical parameters, elicitation, precursor feeding, hairy root culturing, biotransformation, plant cell immobilization, and metabolic engineering, have been merged to fabricate high valuable phytochemicals in huge masses. This review is intended to focus on one of the most efficient strategies for improved synthesis of secondary metabolites, i.e., elicitation. Various attributes of elicitation and the effect of different types of elicitors with special reference to nanoelicitors have been conscripted in this review.

2 Mechanisms of Elicitation and Types of Elicitors

Elicitation is one of the ways to enhance the production of SMs by adding appropriate quantities of elicitors, which are the chemical compounds of biotic and/or abiotic origin (Radman et al. 2003). Elicitors stimulate stress reactions in plant cells, leading to enhanced biosynthesis and accumulation of SMs. Some of the parameters, including types of elicitors and their concentrations, period of exposure, culture types, cell lines, media compositions, presence or lack of plant growth regulators, stages of the culturing at the time of elicitor addition, determine the efficacy of the elicitation approaches on both biomass and yield of SMs (Naik and Al-Khayri 2016). As signals, elicitors initiate the signal transduction cascades after attaching to the elicitor-specific receptor sites occurring on plant cell membrane and eventually alter the expression levels of several regulatory genes and transcription factors involved in the secondary metabolic pathways. Overall, these reactions result in the enhanced biosynthesis and yield of SMs. The binding of elicitors to receptors on cell membranes lead to increased influx of Ca^{2+} ions, prompt modifications in protein phosphorylation patterns, instigation of protein kinases and G-proteins, acidification of cytoplasm, enhanced accumulation of ROS, and all of which have a cumulative effect on the transcriptional activation of defense genes (Namdeo 2007). In recent years, various transcription factors associated with regulation of genes involved in defense mechanism, stress tolerance, and biosynthetic pathways of phytochemicals have been identified. In fact, they are DNA binding proteins, which bind to the promoter regions of specific genes, and modify the expression rate, especially the initiation process of transcription by RNA polymerase (Jan et al. 2021). WRKY, MYB, bHLH, bZIP, AP2/ERF, and NAC are few examples of transcription factors highlighted in ongoing research works, and they have been found to be activated by means of diverse agents of elicitation. On the basis of origin, elicitors are broadly sorted into two groups—biotic and abiotic elicitors. Here, we deliberate about the subtypes of biotic and abiotic elicitors and their effects on improved synthesis of valuable compounds and nanoelicitors.

3 Effect of Biotic Elicitors on Secondary Metabolite Production

Biotic elicitors are derived from the living organisms, and their roles are linked to the receptor sites and function by triggering or deactivating a few or more enzymes or ion channels (Patel and Krishnamoorthy 2013). They comprise molecules from bacteria, fungi, herbivores, plant cell wall fragments, and exudates released due to pathogenic or herbivores attacks in plants (Namdeo 2007). Examples include polysaccharides, glycoproteins, inactivated enzymes, pectin, purified chitosan, alginate, chitin, xanthan, elicitin, yeast extract, and fungal homogenate. These compounds are found out to induce resistance feedback in both whole and cultures from plant cells. They are categorized into four groups as shown in Fig. 19.1.

3.1 Carbohydrates and Proteins

Carbohydrate molecules and proteins from living organisms are being used efficiently for enhanced production of phytochemicals. They elucidate their role of ion channels in cellular membranes of plants for the signal relocation prompted due to external stimuli. Lectins, glycoproteins, pectolyase, cryptogein, and oligandrin are examples of proteins used as elicitors. These proteins were successful in eliciting

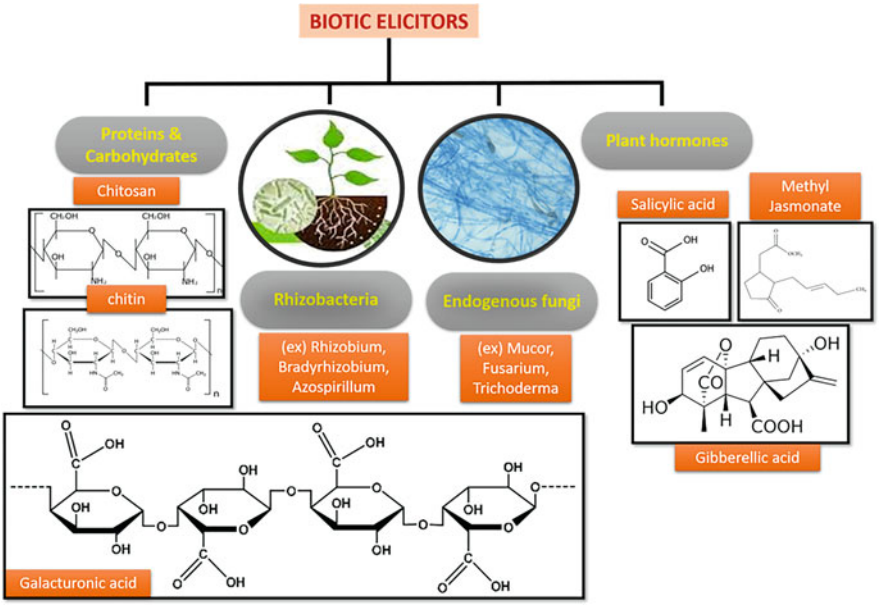

Fig. 19.1 Different agents of biotic elicitors

SMs production (Thakur et al. 2019). Studies have witnessed the use of carbohydrates like oligogalacturonic acid, agaropectin, chitosan, chitin, and yeast extract or yeast polysaccharide as effective elicitors of plant SMs. Oligogalacturonic acid induced the synthesis of phytoalexins in *Glycine max* cotyledons and *Panax ginseng* cell suspension cultures (Hu et al. 2003). The effect of chitosan on improved accumulation of metabolites has been confirmed in a number of plants like *Ruta graveolens*, *Vitis vinifera* (Xu et al. 2016), *Barringtonia racemosa* (Osman et al. 2018), and *Hypericum perforatum* (Badiali et al. 2018). Yeast extract has also been proved as a beneficial elicitor molecule in several reports (Jan et al. 2021).

3.2 Rhizobacteria

Rhizobacteria are a group of bacteria colonizing rhizosphere portion of plants and are present in specialized structures known as nodules of root cells. They are well known to enhance plant growth and SMs production through various mechanisms (Viveros et al. 2010). Rhizospheric microbes are considered as the greatest biotic elicitors as they induce the biosynthesis of SMs in plants through induced systemic resistance mechanism. They also stimulate the key enzymes that are involved in SMs synthetic pathways and related to plants' defensive responses (Isah 2019). Examples of rhizobacteria used as elicitors are as follows: *Pseudomonas putida, P. fluorescens, Bacillus subtilis, B. coagulans, Azospirillum brasilense, Glomus aggregatum, Trichoderma harzianum, T. viride, Azotobacter chroococcum,* etc. Among these, *P. putida* and *P. fluorescens* have been testified to augment the accumulation of metabolites in many plants, namely *Stevia rebaudiana* (Vafadar et al. 2014), *Hyoscyamus niger* (Ghorbanpour et al. 2013), *Hypericum perforatum* (Manero et al. 2012), *Glycine max* (Solano et al. 2010), *Catharanthus roseus* (Jaleel et al. 2008), *Origanum majorana* (Banchio et al. 2010), and *Pisum sativum* (Bahadur et al. 2007).

3.3 Fungal Cells

One of the best approaches for inducing biosynthetic pathways in plant cells is by using the preparations of pathogenic or nonpathogenic fungi as eliciting factors. Whole cell extracts, cell filtrates of different fungal cells, and elicitor molecules produced by fungi have been described to encourage the synthesis of phytochemicals for more than two decades (Chamkhi et al. 2021). Asiaticoside content in *Centella asiatica* was boosted by the usage of autoclaved fractions of fungus, *Piriformospora indica* (Jisha et al. 2018). *Corylus avellana* cell suspension cultures treated with two endogenous fungi, *Chaetomium globosum* and *Paraconiothyrium brasiliense,* were exhibited to accumulate highest amounts of paclitaxel, an anticancer compound. About 291.5 μg L^{-1} of paclitaxel was achieved by the use of 10%

(v/v) of this fungal elicitor during late log phase of cell culture cycle, and it nearly 4.1 times higher compared to the quantity attained in the control cultures (Salehi et al. 2020). Several fungal species such as *Glomus mosseae*, *Trichoderma harzianum, Azotobacter* sp., *Azospirillum* sp., *Trichoderma viride, Claviceps purpurea, Aspergillus niger, Protomyces gravidus, Mucor hiemalis, Fusarium moniliforme, Fusarium oxysporum, Botrytis cinerea, Trichoderma atroviride, Pythium aphanidermatum, Phytophthora megasperma, Alternaria carthami, Penicillium chrysogenum, Phytophthora megasperma, Colletotrichum lindemuthianum, Coriolus versicolor, Ganoderma lucidum, Hyoscyamus muticus, and Rhizoctonia solani* have been studied to enhance the active molecules synthesized in a variety of plants (Thakur et al. 2019). In addition, recent reports on secondary metabolite enhancement through biotic elicitors have been detailed in Table 19.1.

3.4 Hormones

Plant growth regulators/hormones are chemical messengers that function together with the explicit tissues and cause various physiological reactions in plants. They have major roles in organogenesis, flowering, micropropagation, and secondary metabolite production. They have been classified as auxins, cytokinins, and gibberellins. Some plant growth regulators have also been utilized as elicitors as they have the capability to stimulate the genetic expression of genes coding for enzymes involved in several metabolic pathways. Very frequently utilized plant growth regulators as elicitors include jasmonic acid, methyl jasmonate (MeJA), and salicylic acid (SA) (Fig. 19.1).

MeJA is the ester of jasmonic acid and is derived from the catabolism of linolenic acid and modulates many physiological routes in plants like development of roots, senescence, and defensive responses by acting as secondary messengers. MeJA is an ever-present signaling molecule and assists plant responses to varied stresses, such as wounds, insect, and pathogen attacks (Wang et al. 2014). MeJA prompts an extensive transcriptional reprogramming and leads to activate the whole metabolic pathways. Thus, MeJA triggers the biosynthesis of several kinds of SMs that are helpful for plants to acclimatize, essentially under ecological challenges (Han et al. 2006). Numerous studies have witnessed the efficacy of MeJA in improving the production of SMs (Thakur et al. 2019; Jan et al. 2021). Recently, Hashemi et al. (2021) indicated the highest accumulation of chelidonine and sanguinarine after 72 and 24 h of MeJA elicitation, respectively. Their research investigation also exposed the upregulation of cheilanthifoline synthase and tetrahydroprotoberberine *N*-methyltransferase genes. In grape cells, MeJA along with calcium enhanced E-ε-viniferin and E-resveratrol levels by 140% and 180%, correspondingly (Martins et al. 2021). The total flavonoid and phenolic contents and increased expressions of lycopene beta cyclase and phenylalanine ammonia-lyase genes were reported in *Lactuca sativa* upon elicitation with 90 μM MeJA (Escamilla et al. 2020). A few more recent studies are summarized in Table 19.2.

Table 19.1 Influence of biotic elicitors on increased accumulation of plant-based bioactive compounds

Plant	Biotic elicitor used	Enhanced secondary metabolites	Reference
Salvia miltiorrhiza	Endophytic fungus *Mucor fragilis*	Salvianolic acid B, rosmarinic acid, stearic acid, and oleic acid and enhanced expression of SmAACT, SmGGPPS, and SmPAL genes	Xu et al. (2021)
Dracocephalum kotschyi	Chitosan	Rosmarinic acid, quercetin, and apigenin	Kahromi and Khara (2021)
Ocimum basilicum and *Melissa officinalis*	Chitosan lactate	Rosmarinic acid, anthocyanins, and TPC	Nowak et al. (2021b)
Azadirachta indica	Chitosan	Azadirachtin, mevalonic acid, and squalene	Farjaminezhad and Garoosi (2021)
Carthamus tinctorius	Yeast extract	Flavonoids, phenylpropanoids, alkaloids, fatty acids, and aromatic glycosides	Liu et al. (2021)
Morus alba	Yeast extract and MeJA	Mulberroside A, oxyresveratrol, and resveratrol	Inyai et al. (2021)
Valeriana jatamansi	Yeast extract and MeJA	Valerenic acid and hydroxy valerenic acid	Partap et al. (2020)
Linum sp.	MeJA and coronatine	Podophyllotoxin, 6-methoxypodophyllotoxin, and 6 methoxy podo phyllotoxin-7-*O*-β-glucoside	Alfieri et al. (2021)
Allium jesdianum	MeJA and putrescine	Increased TPC, TFC, and anthocyanin	Yazdanian et al. (2021)
Arachis hypogaea	Chitosan, MeJA, and cyclodextrin	*Trans*-arachidin-1 and *trans*-arachidin-3, and enhanced antioxidant activity	Chayjarung et al. (2021)
Hordeum vulgare	Marine protein hydrolysates and chitosan oligosaccharide	Phenolics and antioxidative enzymes	Ramakrishna et al. (2019)
Blackberry	*Pseudomonas fluorescens*	Catechin, epicatechin, anthocyanin, quercetin, and kaempferol derivative	Rivilla et al. (2021)
Salvia officinalis	*Pseudomonas fluorescens*	Cis-thujene, camphor, and 1,8-cineol	Ghorbanpour et al. (2016)
Vitis vinifera	*Trichoderma viride*	Trans-resveratrol, δ- and ε-viniferins	Sak et al. (2021)
Corylus avellana	*Camarosporomyces flavigenus*	Paclitaxel	Salehi et al. (2020)
Salacia chinensis	MeJA	Increased TPC, TFC, antioxidant activity, and increased accumulation of mangiferin	Chavan et al. (2021)

(continued)

Table 19.1 (continued)

Plant	Biotic elicitor used	Enhanced secondary metabolites	Reference
Panax ginseng	MeJA	Production of four known flavanone derivatives, one new curcubinoyl derivative, jasmogin A, and six new curcubinoyl-flavanone conjugates, and jasmoflagins A-F for the first time	Liu et al. (2021)
Salvia virgata	MeJA	Increased TPC, TFC, rosmarinic acid, and salvianolic acid accumulation	Dowom et al. (2021)
Trigonella foenum-graecum	MeJA and SA	Trigonelline	Beygi et al. (2021)
Salvia miltiorrhiza	SA	Dihydrotanshinone, cryptotanshinone, tanshinone I and tanshinone IIA, and total tanshinone level	Szymczyk et al. (2021)
Rubia tinctorum	SA	Total AQ, alizarin, and purpurin	Demirci et al. (2021)
Musa acuminata	SA	TPC, TFC, total saponins, and diosgenin	Jirakiattikul et al. (2021)

MeJA methyl jasmonate, *SA* salicylic acid, *TPC* total phenolic content, *TFC* total flavonoid content

SA is a well-known inducer of plant systematic acquired resistance in plant–pathogen interaction. During pathogenic attacks, it rapidly amasses and passes to other plant parts for inducing wide-ranging defensive responses (Zhao et al. 2005). Also, it is one the most extensively investigated stress-signaling molecules. It stimulates resistance in plants against pathogenic attack and other environmental stress factors (Kang et al. 2004). In addition, it has been found to influence the germination of seeds, seedling establishment, cell growth, and biotic and osmotic stress responses (Vicente and Plasencia 2011). Though SA is not a ubiquitous inducer for defense-related metabolite production in plants, it encourages gene expressions that are linked to the synthesis of several classes of plant SMs (Schenk et al. 2000; Taguchi et al. 2001). For example, the treatment with 100 μM SA has shown to increase the secretion of phenol (6.5%), 5-hydroxymethyl furfural (6.3%), and (Z)-9-octadecenamide (8.8%) in *Piper cumanense* cell suspensions cultures (Sanchez et al. 2020). Enhanced accumulation of guanosine (2.5-fold), inosine (2.1 fold), and ephedrine (3.1-fold) were detected in the presence of 100 μM SA for about 15 days (Duan et al. 2019a, b). SA has also elicited the accumulation of total phenols, flavonoids, flavonols, and particular metabolites, such as chlorogenic acids and rosmarinic acid in the shoot culture of *Knautia sarajevensis* (Karalija et al. 2019). A review by Ali et al. (2019) has detailed the impact of SA on the improved synthesis of phytochemicals in various plant species. Few more recent reports are listed in Table 19.2.

Table 19.2 Effect of nanoelicitation on improved production of plant secondary metabolites

Plant	Nano particles	Concentration	Effect	References
Isatis constricta	Ag	2 mg/L	Enhanced production of indigo by 1.15-fold and tryptanthrin by 1.71-fold	Karakas (2020)
Stevia rebaudiana	ZnO and CuO	2 mg/L ZnO and 20 mg/L CuO	Improved accumulation of rebaudioside A and stevioside and enhanced total phenolic content, total flavonoid content, and DPPH activity	Ahmad et al. (2020)
Arabidopsis thaliana	Ag	5 ppm	Increased accumulation of 47 compounds including camalexin and anthocyanins	Kruszka et al. (2020)
Camelina sativa	ZnO	80 mg/L	Increased total phenols, flavonoids, carotenoids, and anthocyanins	Hezaveh et al. (2020)
Silybum marianum	ZnO	500 ppm	Enhancement in total phenolics, total flavonoids, antibacterial and anticancer activities	Saeed et al. (2021)
Hyoscyamus species	SiO_2	100 mg/L	Highest amount of hyoscyamine, scopolamine, and increased expression of pmt. and h6h genes	Hedayati et al. (2020)
Celery	Se	5 mg/L	Improved production of total phenols, flavonoids, phytohormones, amino acids, and vitamin-C	Li et al. (2020)
Nigella arvensis	Al_2O_3, NiO, TiO_2	50 and 1000 mg/L of NiO	Highest quercetin with 50 mg/L NiO and highest glaucine and kaempferol with 1000 mg/L NiO	Modarresi et al. (2020)
Thymus daenensis	Carbon nanotubes	250 μg/mL	Increased TPC, TFC, and antioxidant activities	Samadi et al. (2020)
Fagonia indica	Iron-doped ZnO	62.5 μg/mL	Increased TPC, TFC, antioxidant potential and accumulation of epigallocatechin gallate	Khan et al. (2021a, b)
Dracocephalum kotschyi	FeO	75 mg/L	Enhanced production of rosmarinic acid, xanthomicrol, cirsimaritin, and isokaempferide and increased expression of pal and ras genes	Nourozi et al. (2019a)
Dracocephalum kotschyi	SiO_2	100 mg/L	Enhanced production of rosmarinic acid, xanthomicrol, cirsimaritin, and isokaempferide and increased expression of pal and ras genes	Nourozi et al. (2019)
Raphanus sativus	MgO	20 mg/L	Increased TPC, TFC antioxidant potential, and Pb phytoaccumulation	Hussain et al. (2019)

(continued)

Table 19.2 (continued)

Plant	Nano particles	Concentration	Effect	References
Linum usitatissimum	ZnO	100 mg/L	Increased TPC, TFC, and accumulation of dehydrodiconiferyl alcohol glucoside and guaiacylglycerol-β-coniferyl alcohol ether glucoside	Abbasi et al. (2019)
Gymnema sylvestre	CuO	3 mg/L	Increased TPC, TFC, and accumulation of gymnemic acid II	Chung et al. (2019)
Prunella vulgaris	Ag and Au	30 µg/L of AgNPs +90 µg/L AuNPs	Increased TPC, TFC, and antioxidant activities	Fazal et al. (2019)
Thymus sp Zataria multiflora	ZnO	150 mg/L	Improved production of thymol and carvacrol	Mosavat et al. (2019)

TPC total phenolic content, *TFC* total flavonoid content

4 Influence of Abiotic Elicitors on Secondary Metabolite Production

Abiotic elicitation refers to inciting the secretion of plant SMs by using substances of nonbiological origin which may be physical or chemical (Thakur et al. 2019). Abiotic elicitors are broadly classified into physical and chemical elicitors as detailed in Fig. 19.2. Heavy metals and inorganic salts fall under chemical elicitors, and light, temperature, water, and sound waves come under physical elicitors (Veersham 2004). Upon the exposure to these abiotic stress factors, dehydration occurred in plant cells, which led to the osmotic pressure generation and elimination of water from the cytoplasm to vacuoles. This is followed by the production of biologically active metabolites (Verma and Shukla 2015).

4.1 Physical Elicitors

The production of plant SMs was found to increase upon exposure to physical forms of elicitors, such as temperature, light, and sound waves. Temperature influences metabolic activities of plants and studies confirmed that both heat stress or cold stress tend to enhance the secretion of SMs in plants. In addition to this, plants also biosynthesize some cryoprotectants, such as sugar alcohols (ribitol, sorbitol, inositol, etc.), soluble sugars (stachyose, raffinose, trehalose, saccharose, etc.), and nitrogenous compounds like proline, betaine, glycine, and protective antifreeze proteins for overcoming temperature stresses (Griffith and Yaish 2004). The shoot cultures of *Physalis peruviana*, when exposed to 45 °C for up to 5 h, have caused a significant

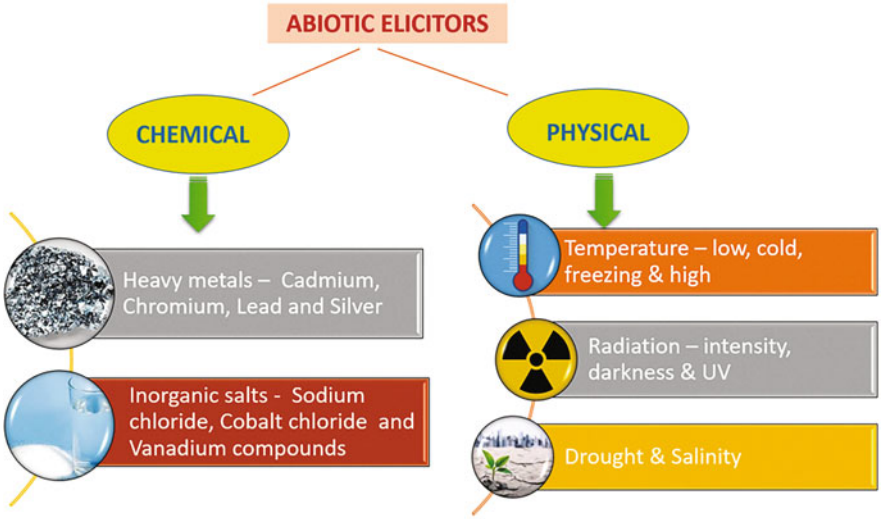

Fig. 19.2 Categories of abiotic elicitors

alterations in withanolide accretion, and about 12.5-fold increase in their yield was noticed (Şahin et al. 2020). The increased levels of phenolic compounds secretion were observed in *Robinia pseudoacacia* seedlings when exposed to higher temperatures (Zhao et al. 2016).

Light, a physical factor, can also affect the SMs secretion in several plant species, especially the UV-B light. Different aspects of light radiation such as light intensity, photoperiod, different monochromatic lights, and UV exposure played a significant part in increased secretion of a number of SMs in different plant species. The most prevalent and multidimensional abiotic elicitor of plant kingdom is drought stress, which typically leads to ecological, morphological, biochemical, physiological, and molecular variations in plant species (Isah 2019). In addition, plants adapt to dry circumstances via the accumulation of SMs like phenolic compounds, terpenes, and flavonoids. Recently, Abbasi et al. (2021) reported maximum TPC, TFC, antioxidant activity, kaempferol, apigenin, myricetin, and isorhamnetin in the callus cultures of *Fagonia indica* grown under photoperiod (16 L/8D h) after exposure to UV-C radiation. In 2020, Khursid et al. investigated growth and developmental aspects of *Eclipta alba* under multispectral lights and reported that red light significantly enhanced total phenolics (57.8 mg/g), total flavonoids (11.1 mg/g), coumarin (1.26 mg/g), eclalbatin (5.00 mg/g), wedelolactone (32.54 mg/g), β-amyrin (0.38 mg/g), dimethyl wedelolactone (23.67 mg/g), and luteolin (0.39 mg/g) in callus cultures of *E. alba*. Enhanced total flavonoid content, total phenolic content, antioxidant potential, and increased levels of silymarin, silybins (A and B), silydianin, isolsilychristin, and silychristin were observed in *Silybum marianum* callus cultures grown under constant light (Shah et al. 2019).

A detailed investigation was performed to find out the effect of different light emitting diodes on the callus cultures of *Ocimum basilicum*. The results have shown that callus grown under blue lights increased maximum total phenolic content as compared to control. Further, high-performance liquid chromatography (HPLC) analyses revealed 2.46 times and 2.25 times increased concentrations of rosmarinic acid and eugenol in callus grown under blue light, respectively. Further, highest amounts of chicoric acid were noted in callus grown under the uninterrupted white light, and the highest amount of peonidin and cyanidin were found in callus culture grown under red light (Nadeem et al. 2018). Shoot cultures of three *Aronia* sp. exposed to different monochromatic lights, such as red, far-red, blue lights, under darkness, UV-A irradiation, and under white light (control), were analyzed for important metabolites. About five times increased production of chlorogenic acid, neochlorogenic, rosmarinic acid, protocatechuic acid, cynaroside, quercitrin, hyperoside, and rutoside was noticed under blue light (Szopa et al. 2018). Kapoor et al. (2018) also reported the maximum production of TPC, TFC, and salidroside under blue light in the callus cultures of *Rhodiola imbricata.*

The asiaticoside, madecassoside, and total centellosides contents were augmented with the exposure of NaCl (12.5 and 25.0 mM) in *Centella asiatica* (Pipatsitee et al. 2021). NaCl (17 mM) treated callus of *Capsicum annum* showed the improved yield of capsaicin and dihydrocapsaicin (Gammoudi et al. 2019). In addition to this, drought, sound waves, and gaseous toxins were also reported to be efficient abiotic elicitors (Thakur et al. 2019; Jan et al. 2021). But no recent (after 2010) reports are available using these physical factors. Recent reports are focusing on using different monochromatic lights, photoperiods, and UV-B/C radiation exposure as eliciting agents for increased production of phytochemicals.

4.2 Chemical Elicitors

These refer to various chemical substances including metal ions, heavy metals, metal oxides, and salts, which act as elicitors of plant SMs. Metals, such as Ni, Fe, Ag, and Co and metal ions such as La^{3+}, Eu^{3+}, Ag^{+}, and Cd^{2+} have been reported to have significant role on enhanced production of phytochemicals (Verpoorte et al. 2002). Heavy metals influence the change in the plant's metabolic activities, and upset the formation of sugars, proteins, photosynthetic pigments, nonprotein thiols, and biologically active metabolites by fluctuating the features of secondary metabolism.

In *Melissa officinalis*, a hike in the quantity of total phenolics, soluble flavonols, anthocyanins, and phenolic acids was demonstrated upon treatment with 100 mM NaCl (Nowak et al. 2021a, b) and enhanced production of hydroxycinnamic acid derivatives was noticed on using chloride salts of Cd and Co (Urdova et al. 2015). The application of MeJA (50 μM) and Ag^{+} (15 μM) significantly improved the secretion of caffeic acid, rosmarinic acid, salvianolic acid B, and salvianolic acid A in 2 *Salvia* species of Iran, *S. officinalis* and *S. verticillate* (Pesaraklu et al. 2021). Vanadium compounds, such as $NHVO_3$ and $VOSO_4$ resulted in increased

accumulation of genistin, genistein, biochanin A, daidzein, and formononetin in *Trifolium pratense* (Kubes et al. 2019) and genistin in *Genista tinctoria* (Skalicky et al. 2019). In soybean, improved production of glyceollin I was reported on using $AgNO_3$ as elicitor (Farrell et al. 2017).

4.3 Nanoelicitation

Materials having the sizes that range between 1 and 100 nm are outlined as nanoparticles (NPs). They possess distinctive chemical and physical properties on the basis of their nanoscale which varies from the bulk materials. They have been reported to be used in various branches of medicine, electronics, biotechnology, food and agriculture as drugs, optical devices, sensors, antibiotics, and diverse materials with a variety of applications (Montejo et al. 2021). Many recent investigations have witnessed the usage of NPs as novel abiotic elicitors, and they act as inducers of enhanced production of phytochemicals. Some studies have confirmed the ability of NPs to stimulate gene expressions that are linked with the SMs biosynthesis (Khan et al. 2021b). NPs bind with the plasma membrane receptors and exchange ions (Cl^-, K^+ efflux, and Ca^{2+} influx) into the cytoplasm which modulates various signal transduction pathways, thus leading to the SMs production. NPs promote the release of reactive oxygen species and secondary signaling messengers that lead to transcriptional regulation in plant secondary metabolism.

Based on the source, NPs are classified into carbon-based, metal-based, metal oxides, metal salts, quantum dots, and nano-sized polymers. Figure 19.3 displays the different types of NPs with examples under each category. Among these, metal-based NPs and metal oxides are effectively utilized as elicitors in the recent years.

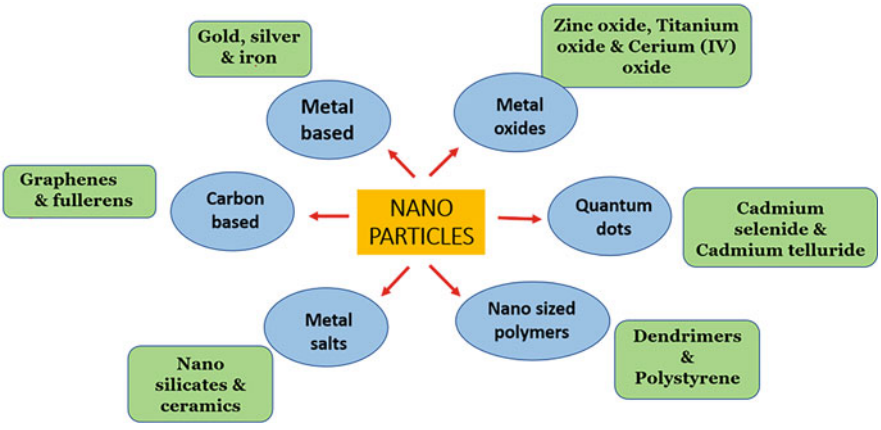

Fig. 19.3 Classification of nanoparticles

Some of the studies conducted in 2021 on NPs based elicitation are summarized below.

The influence of ZnONPs (zinc oxide nanoparticles) on growth of callus and silymarin biosynthesis in *Silybum marianum* under different light environments was demonstrated. Maximum callus weight (2294 mg/L FW) was obtained by adding 0.15 mg/L ZnONPs in the culture medium. Further, the metabolite profiling revealed maximum total phenolic content of 37 mg/g DW, total flavonoid content of 8.9 mg/g DW, superoxide dismutase activity (4.1 nM/min/mg FW), 2,2-diphenyl-1-picrylhydrazyl antioxidant activity (91.5%), and the highest silymarin content of 14.6 mg/g DW in the callus cultures grown in media added with 0.15 mg/L ZnO NPs (Shehzad et al. 2021).

The potential of CSNPs (chitosan nanoparticles) in mitigating salt stress was described in *Catharanthus roseus*. To create a salt stress condition, plants were exposed to NaCl (150 mM), and 1% CSNPs were smeared as a foliar spray. The observations clearly indicated that CSNPs arrested chlorophyll levels, influenced activities of ascorbate peroxidase, glutathione reductase, catalase, and promoted the accumulation of alkaloid levels. In addition, the expression levels of geissoschizine synthase, mitogen-activated protein kinases, and octadecanoid-derivative responsive AP2-domain genes were considerably raised in CSNPs sprayed plants under salt stress. Thus, the application of nanoparticles can be used as elicitors to augment the production of alkaloid and provide an enhanced protection against salinity stress (Hassan et al. 2021).

The role of ZnONPs on plants' growth, antioxidative responses, and accumulation of lead (Pb) in *Persicaria hydropiper* was studied. The oxidative stresses were ameliorated in ZnONP-treated seedlings via increased secretion of free proline, flavonoids, phenolics, stimulation of antioxidative enzymes, and improved Pb amassing (Hussain et al. 2021). The effects of ZnONPs on the oxidative stresses and antioxidative reactions in *Linum usitatissimum* seedlings and their in vitro cultures were studied. The supplementation of ZnONPs (500 mg/L) to seedlings showed the superior antioxidative properties, increased total flavonoid content, and total phenolic content. Further, superoxide dismutase and peroxidase activities were also noticed to be the highest when compared to control treatments. Further, increased secretion of lariciresinol diglucoside, secoisolariciresinol diglucoside, dehydro diconiferyl alcohol glucoside, and guaiacyl glycerol-β-coniferyl alcohol was observed. In the 25 mg/L ZnONP-treated callus cultures, the highest antioxidant and other activities with improved rooting influence were noted (Zaeem et al. 2020).

Improved accumulation of SMs and antioxidant properties was reported in the callus of *Artemisia annua* by the supplementation of varied levels of CuO, ZnO, and CoO NPs. The shoot-derived callus treated with CuO NPs (0.1 mg/L) resulted in the highest phenolic content (60 µg). The addition of ZnO NPs (0.1 mg/L) resulted in higher antioxidant activity in root-derived callus cultures. Therefore, the study confirmed the positive effect of NPs on the induction of SMs in *A. annua* (Fatima et al. 2021). The influence of iron nanoparticles (FeNPs) on plant growth features, free radicle scavenging properties, and secretion of steviol glycosides in *Stevia rebaudiana* cultures was shown by Khan et al. (2020). FeNPs at reduced

concentrations (45 μg/L) influenced the morphological growth parameters, positively. The high levels of FeNPs (135 μg/L) increased the total phenolic content levels (3.2 mg/g DW), total flavonoid content (1.6 mg/g DW), and antioxidant activity (73%).

Still, a number of studies have been reported witnessing the magnificent utilization of nanoparticles as elicitors and some of them are summarized in Table 19.2 with the details of type of nanoparticles used, their concentration, and their specified effect on plant secondary metabolism.

5 Concluding Remarks

Aforementioned studies and reports corroborated the efficacy of many physical, chemical, hormonal factors, bacterial and fungal cells, and nanoparticles as reliable eliciting agents of a wide range of beneficial plant-based compounds. Nanoelicitation has enthralled the attention nowadays as many latest research articles have recently appeared. These elicitors have the advantage of triggering the accumulation of secondary metabolites in large quantities under in vitro conditions devoid of environmental and climatic conditions. Focussed and in-depth investigations examining the biosynthetic pathways of various metabolites, mode of action and environmental impacts of elicitors, are need of the hour for large scale commercial production of bioactive compounds through elicitation.

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Part III
Linking Phytochemical Genomics to Gene Editing Tools

Chapter 20
A Short Review on Genes Regulating Biosynthesis of Major Secondary Metabolites

Arun Kumar Kashyap, Sumit Kumar Dubey, Sujit Shah, and Ajay Kumar

1 Introduction

Plants synthesize thousands of different secondary metabolites, such as flavonoids, alkaloids, and terpenoids. Secondary metabolites play important roles in plants besides multiple in pharmaceutical and nutraceutical industry. The metabolites protect plants from different environmental stresses including abiotic and biotic stresses (Chezem and Clay 2016). Secondary metabolites produced are very helpful in survival of the plants. Secondary metabolites are helpful in fighting against bacteria, fungi, and insects; the metabolites also serve as the factor responsible for establishing symbiotic relation with other organisms (Demain and Fang 2000). Plant secondary metabolites are the source of many products including drugs, flavors, insecticides, and dyes. Due to the vast application of secondary metabolites, the field has been extensively explored and received wider attention in the recent times. Therefore, the plant-derived bioactive secondary metabolites have been a major area of research.

Secondary metabolites are derived from different metabolic pathways in the plants involving array of different enzymes. The metabolic pathways are affected by the expression of different genes. Gene regulation of the plant secondary

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M. K. Swamy, A. Kumar (eds.), *Phytochemical Genomics*,
https://doi.org/10.1007/978-981-19-5779-6_20

metabolites is mediated by several genes including transcription factors (TFs). Numerous TF families have been identified for their roles in pathways involved in secondary metabolite synthesis. TFs are known as key players to initiate transcription. TFs are actively engaged in the biosynthesis pathway regulation and accumulation of bioactive metabolites that are synthesized in plants (Patra et al. 2013). TFs direct integration of various cellular signals, binding to corresponding promoter regions (cis-elements), stimulate or suppress the genes expression of the enzymes, make network with other TFs, promote the formation of active complex and so on (Yang et al. 2012). The activators and repressors regulate the expression of specific sets of gene for relevant metabolic pathway in response to environmental signals including phytohormones and abiotic factors. The regulation of metabolic pathways is also triggered by posttranscriptional and posttranslational mechanisms. The clear elucidation of the gene regulatory networks is helpful to understand the central phenomenon that regulates the synthesis and accumulation of bioactive secondary metabolites of interest. The knowledge of the genes particularly the candidate genes that regulate the biosynthesis of the medicinally and pharmaceutically important metabolites is crucial for the enhancement of their production. Besides this, the identification of the genes paves the way towards their utilization in the creation of metabolically superior medicinal plants using metabolic engineering, genome modification and gene editing. Genes of several major secondary metabolites have been identified in the past. These genes have also been used for the genetic engineering and metabolic engineering of the medicinal plants. There has been successful attempts in creating the better varieties of medicinal plants. This chapter particularly focuses on the regulation of the plant-derived bioactive secondary metabolites (Table 20.1).

2 Genetic Regulation of Secondary Metabolite Biosynthesis

Plants produce many organic molecules which are broadly classified into two classes viz. primary and secondary metabolites. Primary metabolites are common in plants and help in growth, reproduction, and development whereas secondary metabolites are important molecules helpful for various defense responses and can serve as source of many valuable products with wide applications (Patra et al. 2013). Production of the secondary metabolities is not only regulated by the genes but also by the environmental conditions (Erb and Kliebenstein 2020). The quantity and quality of the secondary metabolites is also altered under the influence of biotic factors such as symbionts, endophytes, and plant pathogens. The response to different environment condition results in efficient signaling network which helps in expression of different genes, resulting in the alteration of biosynthesis and accumulation of secondary metabolite. The synthesis of metabolite is not just the result of the expression of gene, but also it is regulated by the posttranscriptional and posttranslational mechanisms (Patra et al. 2013). Figure 20.1 shows a generalised scheme of the regulation of plant secondary metabolite production and their role in plant defenses.

Table 20.1 List of gene/transcription factor/protein/enzyme associated with secondary metabolite

Secondary metabolite	Gene/transcription factor/protein/enzyme	Reference
Glucosinolates	HIGH INDOLIC GLUCOSINOLATE 1 (HIG1, MYB51 TF belonging to R2R3-MYB family), ALTERED TRYPTOPHAN REGULATION1; HAG2/MYB76 and HAG3/MYB29	Gigolashvili et al. (2007a); Malitsky et al. (2008); Gigolashvili et al. (2008)
Anthocyanin	*FtUFGT proteins; FtUFGT6, FtUFGT7, FtUFGT8, FtUFGT9, FtUFGT15, FtUFGT40* and *FtUFGT41*	Yao et al. (2019)
Artemisinin	*Aa-EGL3* and *Aa-TTG1*	Liu et al. (2009)
	3-hydroxyl-3-methyglutaryl CoA reductase (*HMGR*) and *farnesyl diphosphate synthase* (*FPPS*)	Wen and Yu (2011)
	CYP71AV1	Teoh et al. (2006)
	AaWRKY1 *AaERF1* and *AaERF2* *Artemisinic aldehyde delta-11(13) reductase* (*DBR-2* genes)	Shen et al. (2016)
	Aa-ECS, Aa-CPS, Aa-GAS, Aa-BFS, Aa-ADS, and *Aa-SQS*. *Aa-ABCG6* and *Aa-ABCG7*. *Aa-ALDH1* and *Aa-CYP71AV1*	Salehi et al. (2018)
Phenolic compounds	*TaPAL1, TaPAL2, TaC3H1, TaC3H2, TaC4H, Ta4CL1, Ta4CL2, TaCOMT1*, and *TaCOMT2*	Ma et al. (2016)
	R1-MYB, R2-MYB, R2R3-MYB	Mohanty et al. (2016)
	AtMYB4 gene *C4H*	Jin et al. (2000)
	MYB box-like, GARE-like, and pyrimidine box-like zinc-finger proteins (ZNFs) including *ZCT1, ZCT2*, and *ZCT3* *ERF* TF	Mohanty et al. (2016)
	WRKY TFs	Mohanty et al. (2016)
	MYB-bHLH-WD40 complex-*MYB PAP1 or PAP2*, *bHLH EGL3, GL3 or TT8* and *AtTTG1*	Nesi et al. (2000); Borevitz et al. (2000); Zhang et al. (2003); Zimmermann et al. (2004); Teng et al. (2005); Gonzalez et al. (2008)
	VvMYBPA2, VvMYBA1, and VvMYBA2	Kobayashi et al. (2005)

(continued)

Table 20.1 (continued)

Secondary metabolite	Gene/transcription factor/protein/ enzyme	Reference
Anthocyanins	MYB-bHLH-WD40-5 MYB *ZmC1 or ZmPL1*, *bHLH ZmR or ZmB and ZmPAC1* (both deoxyflavonoid and anthocyanin) *MYB ZmP1* (deoxyflavonoid) MYB-bHLH-WD40 complex (anthocyanin)	Goff et al. (1990); Roth et al. (1991); Tuerck and Fromm (1994); Selinger and Chandler (1999); Walker et al. (1999); Carey et al. (2004)
	VvMYBPA2, *VvMYBA1* and *VvMYBA2*	Kobayashi et al. (2005)
Flavonol	*AtMYB11, AtMYB12*, and *AtMYB111*	Zimmermann et al. (2004); Mehrtens et al. (2005); Stracke et al. (2007)
Suberin and cutin	*bHLH PsGBP1—CHS* gene	Qian et al. (2007)
	AtMYB41	Kosma et al. (2014)
Terpenoids	*CYP450* and *CRG* (*CRG329* and *CRG432*) *ORCA* and *AP2* domain *CYP450*	Ouwerkerk and Memelink 1999; Ouwerkerk et al. 1999a, b
	Tryptophan decarboxylase (*TDC*)	Ouwerkerk et al. (1999b)
	TPS14-like1, TPS14-like 2, and *TPS14-like*	Chen et al. (2018)

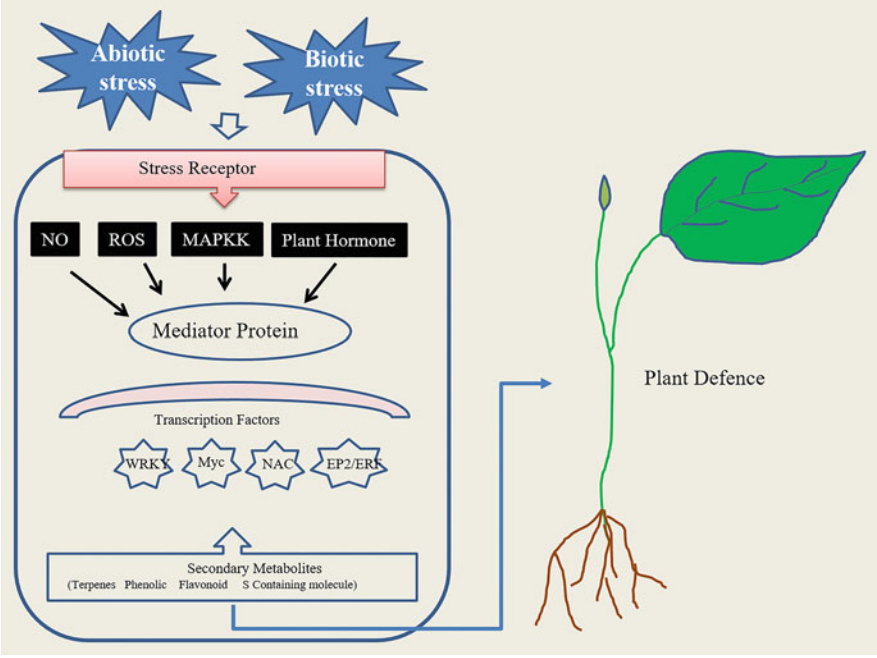

Fig. 20.1 A generalised schme of the regulation of plant secondary metabolites production

2.1 Genetic Regulation of Biosynthesis of Phenolic Compounds

Phenolic compounds are abundantly found in plants and are important due to their antioxidant properties (Dai and Mumper 2010). Phenolics are further divided into simple phenolic compounds (such as phenol, catechol, resorcinol, and phloroglucinol) and polyphenols (such as flavonoids, phenolic acids, tannins, lignans, and coumarins) depending upon the number of OH groups (Kougan et al. 2013; Luna-Guevara et al. 2018). There is a huge structural diversity of phenolic compound in the plants and their synthesis in plants generally talkes place through shikimic acid and the acetate malonate pathways (Kougan et al. 2013). Shikimic acid pathway leads to the production of array of phenolics such as benzoic acid and gallic acid, whereas phenylpropanoid pathway leads to the production of phenylpropanoid compounds such as cinnamic acid, caffeic acid and ferulic acid (Herrmann 1995; Herrmann and Weaver 1999; Kougan et al. 2013). Lignin, coumarins, lignans and flavonoids are some of the examples of the products of phenylpropanoid pathway (Fraser and Chapple 2011). Phenylpropanoid biosynthetic pathway in Arabidopsis plays a major role in monolignols and suberin (phenolic part) production and act as the host for UV-protecting sinapate esters (sinapic acid and sinapoyl malate derivatives) and Iron (Fe-III)-chelating coumarins (Ruegger et al. 1999; Koes et al. 2005; Schmid et al. 2014; Fourcroy et al. 2014). Kosma et al. (2014) revealed that *AtMYB41* acts as regulator for suberin and cutin production. This study further demonstrated that TF *MYB41* overexpression investigated in both *Arabidopsis thaliana* and *Nicotiana benthamiana* and results showed the increased production of suberin, total lignin content, and ferulate-conjugated fatty acids. Zamioudis et al. (2014) reported the that another TF *MYB72* in Arabidopsis modulates iron deficiency responses in its roots and triggers the production of phenolic compounds in iron deficiency. Shelton et al. (2012) have demonstrated the role of MYB TFs in the biosynthesis of isoflavonoids in *Lotus japonica*. This study found that, of the several MYB TFs, *LjMYB14* is crucial in the phenylpropanoid and isoflavonoid pathways. Two MYB TFs viz. *VvMYB14* and *VvMYB15* are reported to control the biosynthesis of stilbene (phenolic compound) in *Vitis vinifera* through the activation of an important gene stilbene synthases (STSs) (Höll et al. 2013). Xu et al. (2014) found that *EjMYB1* activates promoters of both *Arabidopsis* and *Eriobotrya japonica* lignin biosynthesis genes. This study further found that EjMYB1 acts as a repressor of lignin biosynthesis. Transformation of of 3 Arabdipsis MYB TFs viz. *AtMYB55, AtMYB61*, and *AtMYB63* in *Oryza sativa* resulted in enhanced production of lignin in the transgenic plants (Koshiba et al. 2017). Ma et al. (2016) found differential expression of various phenolic acid biosynthesis pathway genes such as *TaPAL1, TaPAL2, TaC3H1, TaC3H2, TaC4H, Ta4CL1, Ta4CL2, TaCOMT1*, and *TaCOMT2* in white, purple and red wheat varieties with varying phenolic content. Flavonoids belong to a polyphenolic class of secondary metabolites synthesized during cellular metabolism and comprises of nearly 9000 different structures (Martens and Mithöfer 2005).

Mostly all vascular plants synthesise certain groups of flavonoids (Williams and Grayer 2004). Flavonoids are further classified into different groups such as anthocyanidins, chalcones, flavonols, flavanones, flavan-3-ols, flavanonols, flavones, and isoflavonoids based on the chemical structure, oxidation, and unsaturation of the linking chain (Panche et al. 2016). Like other phenolic compounds, flavoinds biosynthesis is also controlled through several transcription factors. Kayani et al. (2021) found *AaYABBY5* regulates flavonoid biosynthesis in *Artemisia annua* through the actiation of the promoters of several genes scuh as *AaPAL, AaCHI, AaCHS*, and *AaUFGT*. A detailed overview of the transcriptional regulation of the flavonoid biosynthesis through *MYB, bHLH* and *WD40* TFs is provided by Hichri et al. (2011). This review extensively provides details about the roles of various TFs in the regulation of flavonoid biosynthesis in different species of flowering plants such as *Zea mays, A. thaliana, Vitis vinifera, Antirrhinum majus, Petunia hybrida*, and *Perilla frutescens*. Ramsay and Glover (2005) have also provided a review of the diverse roles of *bHLH*, *MY*B and *WD4*0 in the biosynthesis of plant secondary metaboites and epidermal cell diversity. The glycosylation is an important and often an end step in the biosynthesis of secondary metabolites including flavonoid biosynthesis. This glycosylation is an important step which is brought about by *Glycosyltransferases* (*GT*s) (Vogt and Jones 2000; Wang 2009). Several GTs have been identified from different flowering plants with their roles in the glycosylation of the different secondary metabolites including flavonoids (Vogt and Jones 2000; Wang 2009; Gachon et al. 2005). Similarly, Yao et al. (2019) have identified several GTs with their roles in the flavonoid biosynthesis in another medicinally important plant, *Fagopyrum tataricum*.

Lakshmanan et al. (2015) and Mohanty et al. (2016) demonstrated the role of several TFs such as *bHLH, bZIP, MYB,WRKY, ZnF* (*ZCT1, ZCT2* and *ZCT3*) and *ERF* in the regulation of phenolic compounds accumulation under the influence of blue light in rice.

Involvement of *TRANSPARENT TESTA8* (*TT8*), *TRANSPARENT TESTA GLABRA1* (*TTG1*) and *TT2* genes in the biosynthesis of flavonoids in Arabidopsis was demonstrated (Nesi et al. 2000). This study showed that *TT8, TTG1* and *TT2* genes control the flavonoid biosynthesis through the regulation of important flavonoid biosynthesis genes viz. *DIHYDROFLAVONOL 4-REDUCTASE* (*DFR*) and *BANYULS* (*BAN*). Walker et al. (1999) showed that *TTG1* gene encodes for a *WD40* Repeat Protein. Baudry et al. (2004) showed that *TT2, TT8*, and *TTG1* regulate the *BAN* gene expression through the formation of a ternary complex. Zimmermann et al. (2004) demonstrated the interaction of MYB with B-like BHLH in controlling flavonoid biosynthesis through TTG1. Several other studies also suggest the roles of MYB TFs in the regulation of phenolic compounds such as flavonoids (Zimmermann et al. 2004; Mehrtens et al. 2005; Stracke et al. 2007).

The role of various other TFs including JAZ has also been implicated in several processes including stress tolerance and anthocyanin regulation in plants (Seo et al. 2011; Song et al. 2011). Through interaction with several other components, TF *MYB21* has role in the accumulation of anthocyanin besides defense and

anther development (Song et al. 2011). Studies have also shown the role of JA in the regulation of anthocyanin production in plants (Song et al. 2011; Qi et al. 2011). JAZ proteins basically interacts with bHLH (*TT8, GL3*, and *EGL3*) and R2R3 MYB transcription factors (*MYB75* and *GL1*) to form a WD-repeat/bHLH/MYB transcriptional complex and represses the anthocyanin accumulation (Qi et al. 2011). However, in presence of JA, biosynthesis of the anthocyanin takes place through the degradation of JAZ proteins and release of the bHLH and MYB components from the WD-repeat/bHLH/MYB complexes (Qi et al. 2011). In summary, *JAZ* is a negative regulator of the JA mediated anthocyanin accumulation in plants. Another study shows the negative role of *AtMYBL2* in anthocyanin biosynthesis (Matsui et al. 2008). *C1, B* and *R* genes are known to regulate anthocyanin biosynthesis in maize (Goff et al. 1990). Besides *C1, B* and *R* genes, P (Myb-related transcriptional regulator) is also known to regulate the biosynthesis of anthocyanins in maize (Tuerck and Fromm 1994; Grotewold et al. 1998). Ectopic expression of the *C1/R* and *P* genes whowed accumulation of anthocyanins in maize suggesting that P is also an important gene for the anthocyanin biosynthesis (Grotewold et al. 1998). This study further showed that, the anthocyanin biosynthesis is regulated through the activation of biosynthetic genes viz. *c2* (*chalcone synthase flavanone/dihydroflavonol reductase*). Selinger and Chandler (1999) have identified one important gene *pale aleurone color1* (*pac1*) with role in anthocyanin biosynthesis. Mutation in this gene results in the reduction of anthocyanin concentration in maize. Spelt et al. (2000) have found that *Anthocyanin1* (*AN1*) is responsible for the anthocyanin biosynthesis in Petunia through the activation of *dfrA* gene that codes for *dihydroflavonol 4-reductase* enzyme (Carey et al. 2004). This pac1 gene of maize is similar in its function to TTG1 in Arabidpsis and AN1 in Petunia and apart from anthocyanin, it is also involved in several other functions in maize. Teng et al. (2005) showed that *MYB75/PAP1* gene controls the biosynthesis of anthocyanin under the induction of sucrose. The same study demonstrated that *MYB75/PAP1* encodes for Suc-induced anthocyanin accumulation (*SIAA1*) gene that in turn regulated the anthocyanin accumulation in a sucrose concentration dependent manner.

Besides *AN1*(*BHLH*) and *AN2* (*R2R3 MYB* gene), PH4 gene regulates the biosynthesis of anthocyanin in Petunia (Quattrocchio et al. 2006). Koes et al. (2005) have reviewed the transcriptional regulation of the anthocyanins in plants. This review provides details about the regulatory genes of flavonoids biosynthesis in Maize, Petunia and Arabidopsis. The role of *VvMYBA1* gene in the regulation of anthocyanin biosynthesis in grapevine was investigated (Kobayashi et al. 2005). Another study showed the role of one more *VvMYBA2* in anthycyanin regulation in grapewine besides *VvMYBA1* (Walker et al. 2007).

Jin et al. (2000) showed that *AtMYB4* negatively regulates the biosynthesis of sinapate esters (hydroxycinnamic acid derivatives) in Arabidopsis. This is achieved through its interaction with other targets and its expression is reduced under the exposure of UV-B light that allows the expression of *cinnamate 4-hydroxylase* which in turn is responsible for the production of sinapate esters. Similar to *AtMYB4*, Fornalé et al. (2014) demonstrated that *MYB7* also downregulates the biosynthesis of flavonoids in Arabidopsis through the downregulation of flavonoid

biosynthesis genes such as *DFR* and *UGT*. Preston et al. (2004) found that mutation in the gene AtMYB4 results in the alteration of the expression of the phenylpropanoid biosynthesis genes in Arabidopsis. Some of the genes showed upregulation (*COMT*), some downregulation (*DFR* and *ANS*), whereas some genes were unaffected (*PAL2* and *C4H*). This study suggest that *MYB4* might have role in the biosynthesis of some of the secondary metabolites.

2.2 Genetic Regulation of Terpenoids

Terpenoids or terpenes are a diverse group of secondary metabolites mainly found in plants and they are divided into mono, di, tri, tetra, and sesquiterpenes on the basis of a number of isoprene units (Cox-Georgian et al. 2019). The biosynthesis of terpenoids takes place through cytosolic (mevalonic acid, MVA) and plastidic (methylerythritol phosphate, MEP) pathways (Jin et al. 2021). Several genes from the cytosolic pathways such as *acetoacetyl-CoA thiolase* (*AACT*), *3-hydroxy-3-methylglutaryl- coenzyme* (*HMGS*, a synthase), *3-hydroxy-3-methylglutaryl coenzym*e (*HMGR*, a reductase), *mevalonate kinase* (*MVK*), *5-phosphatemevalonate kinase* (*PMK*), *mevalonate-5-phosphate decarboxylase* (*MDC*) and plastidic pathway such as *1-deoxy-d-xylulose-5-phosphate synthase* (*DXS*), *1-deoxy-d-xylulose 5-phosphate reductase* (*DXR*), *2-C-methyl-d-erythritol-4-phosphate cytidyltransferase* (*MCT*), *cytidyl(4-diphospho)-2-C-methyl-d-erythritol kinase* (*CMK*), *2-C-methyl-d-erythritol-2,4-cyclodiphohatesynthase* (*MDS*), *1-hydroxyl-2-methyl-2-(E)-butenyl-diphosphate synthase* (*HDS*), *4-hydroxy-3-methylbut-2-enyldiphosphate reductase* (*HDR*), *isopentenyl pyro-phosphate isomerase* (*IDI*) play crucial roles in the steps towards terpenoid biosynthesis (Jin et al. 2021). Further biosynthesis of the various types of terpenoids takes place through carious other enzymes including terpene synthases. Several authors have reviewed the various roles of biosynthetic genes in different plants. The regulation of these genes is important and it is brought about by several transcription factors. Several studies have proved the roles of diverse TFs, and their mechanisms of regulation of terpenoid biosynthesis. Chen et al. (2018) performed transcriptome analyses of *Cinnamomum camphora* and discovered several genes involved in the terpenoid synthesis. The monoterpene synthase genes, namely *TPS14-like*1, *TPS14-like2*, and *TPS14-like3*, have been shown to stimulate borneol-type terpenes, and their expressions were verified by qRT-PCR. Michael et al. (2020) revealed that the elongated *HYPOCOTYL5* (*HY5*) TF has major role in the light-mediated terpene biosynthesis through the regulation of terpene synthase gene (*AtTPS03*) in Arabidopsis thaliana. This study further found that this gene is crucial for light mediated terpene biosynthesis.

2.2.1 Artemisinin

Artemisinin a sesquiterpene lactone that is synthesized in the glandular trichomes of sweet wormwood (*A. annua*) and widely is utilized as an antimalarial drug (Krishna et al. 2008; Ferreira et al. 1996; Xiao et al. 2016). Among the various species of Artemisia, *A. annua* synthesises higher amounts of artemisinin. Wen and Yu (2011) provides a detailed review on the various genes and TFs that are important for artemisinin biosynthesis. *ENHANCER OF GLABRA3* (*EGL3*) and *TRANSPARENT TESTA GLABRA1* (*TTG1*) are the transcription factors that are important for glandular trichomes formation and biosynthesis of artemisinin (Liu et al. 2009). Wang et al. (2011) found that co-overexpression of the *HMGR* and *FPS* genes increases the artemisinin production in *A. annua* by 1.8 fold. Teoh et al. (2006) have shown that *CYP71AV1* (a cytochrome P450) is also an important gene in the biosynthesis of artemisinin. Teoh et al. (2006) investigated the identification of genes that are involved in the artemisinin biosynthesis pathway using expressed sequence tag (EST). To do so, glandular trichomes of *A. annua* were used to extract mRNA. The *CYP71AV1* cDNA clone encoding *cytochrome P450* was revealed by their expression in Saccharomyces cerevisiae culture, and the result exhibited that the biosynthesis of the antimalarial drug artemisinin was closely associated with *CYP71AV1*. Shen et al. (2016) has provided an important review on the transcriptional regulation of the artemisinin biosynthesis and its accumulation. This review also further discusses the role of various hormones such as JA, ABA in the induction of the TFs such as *ERF1/2, ORA, WRKY1, bHLH1* and *MYC 2*. These TFs then modulate the expression of artemisnin biosynthesis genes such as *amorpha-4,11-diene synthase* (*ADS*), *cytochrome P450 monooxygenase* (*CYP71AV1*), and *aldehyde D11(13) reductase* (*DBR2*) (Shen et al. 2016). Salehi et al. (2018) performed expression analysis of artemisinin biosynthesis and transporter genes in 5 species of Artemisia. This study found that *A. deserti* (S4) had a very high expression of *ALDH1* and *CYP71AV1* and low expression of *DBR2*.

2.2.2 Taxol

Taxol also known as paclitaxel is a well-known diterpenoid bioactive metabolite that was first discovered in the Taxus genus. This metabolite is secreted by the plant to protect itself against herbivores and pathogens. It is synthesised from geranylgeranyl diphosphate through the plastidial methyl erythritol phosphate pathway and involves several steps and enzymes as reviewed by Croteau et al. (2006). The role of JA signalling in the biosynthesis of taxol is well understood now. Wu and Ge (2004) found that production of the reactive oxygen species (ROS) and JA signalling is important for the elicitation of taxol biosynthesis in T. chinensis. This study found increased ROS production and JA biosynthesis under ultrasound exposure. The scavenging of the ROS resulted in the reduction of taxol as well as JA accumulation suggesting the important role of redox molecules in trigerring taxol biosynthesis.

Another study led by Sun et al. (2013) also suggested the role of MeJA signalling in the elicitation of taxol using transcriptome sequencing. Wang and Wu (2005) have demonstrated the role of NO in the biosynthesis of taxol. This study found that Taxus cells produce NO and ROS under the influence of Methyl Jasmonate (MeJA). Application of NO inhibitors led to the reduction of taxol accumulation suggesting that NO is an important part of signalling in the production of taxol. Changxing et al. (2020) provides a futuristic review on the use of biotechnology in the enhancement of high value plant secondary metabolites including taxol. Sun et al. (2013) used a plant cell culture approach to demonstrate that methyl jasmonate elicited paclitaxel synthesis (Sun et al. 2013). Comparative transcriptome profiling between *T. wallichiana* var. *mairei* and its cultivar Jinxishan showed that genes such as *taxadienol acetyltransferase* (*TAT*), *taxadiene 5-alpha hydroxylase* (*T5H*), *5-alpha-taxadienol-10-beta-hydroxylase* (*T10OH*), and *2-debenzoyl-7,13-diacetylbaccatin III-2-O-benzoyl-transferase* (*DBBT*) showed higher expression in the Jinxishan, which has higher taxol content (Wang et al. 2019). This study further found the differential exresion patterns of ERF, bHLH, MYB, and WRKY TFs between the WT and its cultivar, Jinxishan (Wang et al. 2019). Cui et al. (2019) demonstrated the role of jasmonic acid signalling in taxol biosynthesis, and found that two *EI ubiquitin ligase* genes (*COI1-1* and *COI1-2*), 7 *MYC bHLH* TFs (*MYC2, MYC3, MYC4, JAM1, JAM2, EGL3, TT8*) and 12 *JAZ* genes containing the ZIM domain and *MED25*, could have role in taxol biosynthesis in *T. media*.

Taxol is a well-known bioactive chemical that was first discovered in the Taxus genus. This chemical is secreted by the plant to protect itself against herbivores and diseases. Based on gene expression and regulation, the bioactive compound's level in plants can grow threefold (Croteau et al. 2006). There must be balance in the plant defense system for secondary metabolite production and plant growth and development (Erb and Kliebenstein 2020). Taxol production and plant growth hormone (jasmonic acid) biosynthesis are finely tuned processes (Wu and Ge 2004; Wang and Wu 2005; Wang 2009). The methyl jasmonate (MJ)-mediated taxol production pathway is incompletely understood. However, methyl jasmonate-induced Taxus cell suspension cultures have revealed the participation of specific transcription factors overexpression throughout the taxol production processes (Changxing et al. 2020).

Sun et al. (2013) used a plant cell culture approach to demonstrate that methyl jasmonate elicited paclitaxel synthesis (Sun et al. 2013). In comparison to untreated plant cells, they were able to ramp up paclitaxel synthesis with methyl jasmonate treatment for 7 days. They also revealed fresh information on taxol production in plant cell culture suspension mediated by the methyl jasmonate signaling network. They showed the participation of complex genes tightly controlled pathways in this respect, with 29 known genes engaged in terpenoid backbone biosynthesis and 18 genes involved in paclitaxel manufacture in the presence of methyl jasmonate. It was also hypothesized that miRNA would have a role in gene expression in methyl jasmonate-mediated taxol production.

The transcription factors involved in the production of taxol have recently been discovered. Taxadiene 5-alpha hydroxylase (T5H), 5-alpha-taxadienol-10-beta-

hydroxylase (T10OH), and 2-debenzoyl-7,13-diacetylbaccatin III-2-O-benzoyl-transferase are the enzymes involved (DBBT). These transcription factors are involved in the acetylation and hydroxylation processes that occur during taxol production (Wang et al. 2019). Similarly, Cui et al. (2019) demonstrated that the jasmonic acid signaling pathway for taxol biosynthesis, data obtained by RACE PCR at 5′ and 3′, 22 genes, was identified which is involved in jasmonic acid signaling. These genes are two EI ubiquitin ligase genes, COI1-1 and COI1-2; 7 MYC bHLH (basic/helix-loop-helix) type transcription factor (MYC2, MYC3, MYC4, JAM1, JAM2, EGL3, TT8); 12 JAZ genes containing the ZIM domain; and MED25, one of the components of the transcriptional complex. Among these transcription factor MYC2, MYC3 and MYC4 are responsible for the activation of taxol biosynthesis genes.

2.3 Gene Regulation of Nitrogen-Containing Alkaloids

Plants produce an array of N-containing compounds which are known as alkaloids are found in nearly 20 % of plant species and they have huge medicinal roles besides acting as protective agents for the plants themselves (Ain et al. 2016; Srivastava and Srivastava 2013). Various classes of alkaloids produced in plants include pyridine alkaloids, isoquinoline alkaloids, piperidine alkaloids, vinca alkaloid, indole alkaloids, aporphine alkaloids, pyrroloindole alkaloids, and lycopodium alkaloids (Hussain et al. 2018). Herbert (1999) have reviewed the biosynthesis of a number of alkaloids from plants. The roles of tryptophan decarboxylases in various plant species is reviewed in detail by Facchini et al. (2000). *Catharanthus roseus* produces a number of terpenoid indole alkaloids (TIAs) which have high medicinal potential including anticancer properties, Rischer et al. (2006) performad integrated metabolite-transcript analysis of the C. rosues and indicated that several transcripts such as CRG432, CRL13, CRG329 and CRG432 could be important for the biosynthesis of various metabolites (Ouwerkerk and Memelink 1999; Ouwerkerk et al. 1999a, b).

2.4 Regulation of Sufur-Containing Metabolites (Glucosinolates)

Glucosinolates (GSs) are sulphur-containing secondary metabolites produced by the members of the Brassicaceae family such as mustard, cabbage, and cauliflower. Plants produce various types of glucosinolates such as aliphatic (AGs), indole (IGs), and benzenic glucosinolates (BGs) (Chhajed et al. 2020). The biosynthesis of these glucosinolates involves several genes and TFs. Celenza et al. (2005) have demonstrated the role of *ATR1* gene in the biosynthesis of indole glucosinolates. Another

study found the involvement of *HIGH INDOLIC GLUCOSINOLATE* 1 (*HIG1, MYB51* TF belonging to R2R3-MYB family) in the biosynthetic regulation of indole glucosinolates. The study found that *HIG1/MYB51* leads to the activation of several genes that are involved in the biosynthetic indolic glucosinolates (Gigolashvili et al. 2007a). This study further found the higher accumulation of the indole glucosinolates in the *HIG/MYB51* overexpressed lines with increased defese against the herbivores. *ALTERED TRYPTOPHAN REGULATION1* [*ATR*1]-like and *MYB28-like* clade factors are known to regulate AGs and IGs respectively, Malitsky et al. (2008) showed the important roles of the MYB28-like and ATR1-like clade factors in the mediation of GSs. The role of *MYB34, MYB51*, and *MYB122* TFs was also demonstrated in the biosynthesis of IGs (Frerigmann and Gigolashvili 2014). This study showed that triple mutatnts of these three TFs do not produce IGs showing that these three MYB TFs play central role in the biosynthesis of IGs. Further it was found that, all these three TFs are important for the IG biosynthesis in shoots and roots, MYB34 is involved mainly in the root IG biosynthesis. These result suggest the organ specific spatial regulation of the GS biosynthesis and its regulation. *HIGH ALIPHATIC GLUCOSINOLATE 1*, (*HAG1; A MYB28* TF) was found to be involved in the regulation of biosynthesis of methionine-derived AGs through the regulation of everal GS biosynthesis genes (Gigolashvili et al. 2007b). This study also found the antiherbivore response of the plants with gain-of-function mutation. In another study, Gigolashvili et al. (2008) found the positive role of *HAG2/MYB76* and *HAG3/MYB29* in the regulation of AGs. Sønderby et al. (2007) found that MYB29 and MYB76 are required for the short-chained AGs whereas whereas *MYB28* plays role in both short- and long-chained AGs. Hirai et al. (2007) demonstrated the role of MYB28 and MYB 29 in the regulation of biosynthesis of AGs. Other studies have also demonstrated the roles of *bHLH* TFs in directing the synthesis of GSs. Schweizer et al. (2013) found the role of three *bHLH* TFs (*MYC2, MYC3, and MYC4*) in the biosynthesis of GSs. This study further showed that these TFs can also bind to the promoters of *MYB* TFs as well as GS biosynthetic genes. Major et al. (2017) have demonstrated the role of *JAZ* TFs (*JAZ1/3/4/9/10*) interactions with *MYC* TFs in controlling the GSs production in plants. The role of *JAZ* and *MYC* TF interactions in imparting herbivory resistance has been studied by Fernández-Calvo et al. (2011) and Niu et al. (2011).

2.5 Gene Regulation of Other Bioactive Compounds

Li et al. (2017) assessed E-geraniol (volatile mono-terpene derived from Citrus fruit) for their antifungal properties and reported that terpene synthase 16 (*CitTPS16*) stimulates E-geraniol synthesis in vitro, and *CitTPS16* overexpression in *Citrus sinensis* stimulates E-geraniol accumulation in vivo. Further, they analyzed the sets of gene involved in AP2/ERF transcription factor gene families (*CitERF71* and *CitTPS16*), and the result showed similar pattern of expression. Many studies have recently investigated the roles of TFs in the regulation of plant secondary

metabolites. For example, MYB TFs also regulate the biosynthesis of capsaicinoids in *Capsicum spp.* (Arce-Rodríguez and Ochoa-Alejo 2017; Sun et al. 2020). Podophyllotoxin is regulated by bZIP, MYB, WRKY, and bHLH TFs (Kumar et al. 2017). Several authors have reviewed the transcriptional regulation of plant secondary metabolites (Afrin et al. 2015; Jan et al. 2021; Patra et al. 2013; Yang et al. 2012; Vom Endt et al. 2002). All these studies suggest diverse roles of several TFs in the regulation of the biosynthetic genes of many secondary metabolites. Moreover, several hormones and hormonal factors also affect the quantity of the secondary metabolites in plants. Therefore, to get clear picture of plant secondary metabolites biosynthesis, we must integrate the environmental data with genomics, and metabolomics.

3 Conclusions

Plants produce various molecules for the survival which are broadly categorized into primary and secondary metabolites. The secondary metabolites help to combat the biotic and abiotic stress of plants. In the present chapter, a brief of the regulation of synthesis of different secondary metabolites by gene regulation at different level has been provided. Generally, the secondary metabolites are produced at the basal level in the plants; however, under stress condition, the plants get stimuli by different signals and the biosynthesis of secondary metabolites gets enhanced and accumulated in the plants. In this chapter, it is described that the specific secondary molecule is synthesized in response to specific condition and helps plants to combat environmental stress conditions. Moreover, the secondary metabolites have huge applications in various areas, including food, medicine and cosmetics.

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Chapter 21
Metabolic Engineering for High-Value Bioactive Compounds from Medicinal Plants

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1 Introduction

Plants are very important to humans and the ecological balance of the planet. Plants are the fundamental staple food for human and animal consumption, which make them an essential part of all living beings (Usman et al. 2014). They are remarkable for their enormous chemical products, notably secondary metabolites, which are advantageous biologically for humans (Buyel 2018). Medicinal plants have considerable therapeutic effects, and are utilized in the traditional medicinal products of many diseases. Additional metabolites besides primary metabolites called as secondary metabolites consist of a well-defined collection of high-value bioactive chemicals which demonstrate enormous functions in plants ranging from plant defense mechanism against pathogens, improved tolerance to biotic and abiotic

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M. K. Swamy, A. Kumar (eds.), *Phytochemical Genomics*,
https://doi.org/10.1007/978-981-19-5779-6_21

stresses, as attractants of insects and animals for fertilization, as pollinators, conferring resistance to several pathogens, and as a storehouse of medicinally important compounds (Wink 2008; Böttger et al. 2018). The nonnutrient, bioactive, and physiologically active compounds supplied by plants include terpenoids, steroids, coumarins, tannins, flavonoids, saponins, phenols, and glycosides. These are renowned for their health benefits (Tavakoli et al. 2021). The versatile therapeutic and curative benefits of herbal plants are principally determined by their phytochemical elements. Numerous bioactive metabolite secretions have been reclaimed as antibiotics, antitumor agents, antiviral substitutes, enzyme enhancers and inhibitors, immune boosters, modulators, protectors, rejuvenators, and growth promoters in plants. These have broadened the pyramid of healthy nutrition in the form of supplements and nutraceuticals, enhanced agricultural productivity naturally as biodegradable pesticides, insecticides, and ecofriendly industrial additives. The critical evaluation of bioactive compounds with their specific content in a vast range of germplasm and the restoration of indispensable genes and gene clusters from agriculturally enriched species becomes very important for the creation of a "gene pool." It becomes significant for crop improvement and product quality to understand the accumulation of bioactive compounds in various plant parts and the genes regulating particular biosynthetic pathways. The integrated application of pertinent analytical technologies for screening natural products, improved bioassay models, studies of molecular targets of the bioactive molecules and in vitro networking resulted in the isolation of a number of important herbal anticancer drugs (Newman and Cragg 2016). Secondary plant metabolites have many distinctive elements such as simple and complex aromatic rings, chiral centers, heteroatoms, and their specific amalgamation making them the focus for discovery of pharmaceuticals and synthetic medicines (Ajayi et al. 2019). The in vitro productivity of plants could also be increased by molecular techniques and studies of nutritional improvement (Cloutier et al. 2009). Metabolic engineering has become a potent method to transform new genes into plant cells, extend current routes or introduce novel chemicals to improve the intended metabolism (Kinney 2006, Cloutier et al. 2009,). Plant breeding and genetic engineering can improve the numerous characteristics of plants (Molinar 2012). Secondary metabolites are implicated in numerous divergent steps of plant development and transmit a comprehensive range of characters that imparts to their existence (Birchfield and McIntosh 2020). Metabolic engineering broadly allows manipulation of the overall bioprocess making it distinguishable from elementary genetic engineering. Engineering metabolites in plants pertains to altering inherent and endogenous pathways of plants specifically producing secondary metabolites to instigate one or more enzymatic reactions for the generation of new compounds or to enhance production of useful compounds or mediate the degradation of unproductive compounds (Kumar 2015). In fact it is quite a budding science in plants as the progress in plant metabolic engineering has speeded up only just the past 25 years. With the advancement of proficiency in substrate–product relationships in plant biosynthetic pathways as a consequence of use of radiolabeled experiments after 1975, attempts have now been made to use this understanding in engineering metabolism of plants. However, with the development

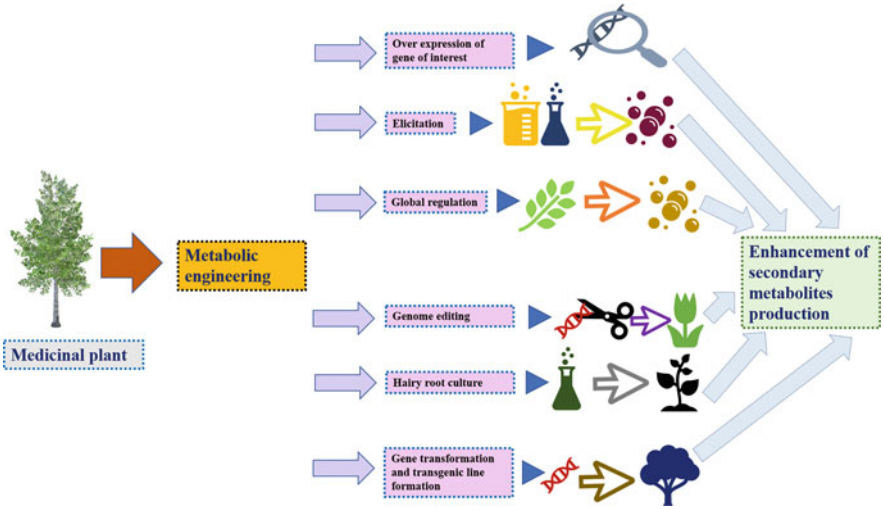

Fig. 21.1 Various approaches that can be used for metabolic engineering of high-value compounds

of fundamental molecular biological technologies, biochemical genetics, bioanalytical techniques like chromatography, electrophoresis, mass spectrometry, and nuclear magnetic resonance and genetic engineering techniques such as molecular cloning, plant transformation, promoter analysis, and protein targeting, plant metabolic engineering studies have proved to be a revolutionary factor in crop improvement (DellaPenna 2001). Bioanalytical techniques and genetic manipulations for synthetic biology for improving genomes protrude as a particularly significant set of enabling advanced technologies for the issues that come during the process (Fig. 21.1). Several methods like zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats CRISPR/Cas9 can be used to perform advanced plant genetic engineering (Mahfouz et al. 2014; Ain et al. 2015; Andersson et al. 2018; Castel et al. 2019). The first step is identification of the limiting factor that can be a gene or subsequently an enzyme regulating the expression of that gene and enzyme kinetics. Therefore, synchronized expression, multi-omics, and amalgamated network analysis are used to explain these phases, demonstrating the association between genes, proteins, and metabolites (Wong 2019). Plants have been used intensively and comprehensively as most suitable food, shelter, fiber, and pharmaceutical sources for generations. As many PNP-based pharmaceuticals get their origin from distinct plant metabolism and provide a composite framework for manufacture that is both efficient and cost-effective, plant natural products (PNPs) play fundamental role in improvement of human life (Courdavault et al. 2021). However, due to climate change and cultivation, natural plant habitats are increasingly losing out. Plant biotechnology provides a sustainable bioproduction technique for secondary metabolites employing in vitro approaches. The unique structural characteristics of sequential plant-derived metabolites such as their multitarget

spectrum and the resemblance in metabolite sequence have led to the development of several plant-based drugs encompassing about a majority of all medicinal products authorized by the leading food and health regulating agencies globally (Marchev et al. 2020). However, unless sustainable, large-scale pharmaceutical production is established, obtaining these in vitro plant metabolites will continue to be challenging. The difficulty is due to the uniqueness and complexity of plant cellular metabolism, accurate selection of bioreactor systems, and bioprocess optimization of secondary plant metabolite pathways (Marchev et al. 2020). In this chapter, we have briefly described various plant secondary metabolites, mechanisms, and use of different metabolic engineering and genome editing systems with special focus on medicinal plants, benefits and implementation of engineered nucleases as well as the regulatory aspects using engineered nuclease technologies.

2 Tools of Metabolic Engineering

Engineering in plants at the biosynthetic level of metabolism profoundly engages manipulating intrinsic pathways for increasing the content of a particular desirable compound holding special medicinal properties, or chemicals of economic importance, or diverging enzymatic reactions by mediating the degradation of compounds. In the following section, we survey design tools available for successful metabolic engineering with practical examples and prospect possible strategies for delivery of high-throughput biomolecules. Transcription factors (TFs), RNA interference (RNAi), and enzyme precursors have been described as follows as metabolic engineering tools. Table 21.1 provides a list of some of the examples of metabolic engineering in medicinal plants.

2.1 Transcription Factors

Transcription factors (TFs) which tend to manage multifold biosynthetic pathway steps have developed as an outstanding tool towards modifying composite plant metabolic processes, as compared to most structural genes in prokaryotes. There have been studies which describe the major findings of experiments in which transcription factors controlling plant metabolic pathways were selectively targeted and their prospective as potent tools for metabolic engineering was well established (Broun 2004). Transcription factors play a significant role in directing the development of metabolic biogenesis which may constitute salient resources for regulating the development of secondary plant metabolites. Various types of transcription factors have been shown to be employed in specific terpenoids exhibiting pharmaceutical standards (VomEndt et al. 2002; Xu et al. 2016).

APETALA 2/ethylene-responsive element binding factor (AP2/ERF) collection is a diverse set of distinctive factors that contain four primary subfamilies: the AP2,

Table 21.1 Some examples of metabolic engineering in medicinal plants

Plant	Gene	Approach used	Metabolite	Results	Reference
Lavandula latifolia	Linalool synthase	Overexpression	Linalool	Transgenic *Lavandula latifolia* plants overexpressed linalool synthase gene encoding LIS enzyme, responsible for catalyzing the synthesis of linalool	Mendoza-Poudereux et al. (2014)
Nicotiana tabacum	Geraniol synthase	Overexpression	Geraniol	Using hairy root culture method Geraniol synthase gene was introduced and expressed in *N. tabacum*	Ritala et al. (2014)
Artemisia annua	Amorpha-4,11-diene synthase gene (ADS), amorpha-4,11-diene 12-monooxygenase gene (CYP71AV1), cytochrome P450 reductase gene (CPR), and aldehyde dehydrogenase 1 gene (ALDH1)	Overexpression	Artemisinin	Using *Agrobacterium*-mediated transformation technology, transgenic *A. annua* plants were produced by overexpressing four mentioned transgenic artemisinin biosynthetic pathway genes	Shi et al. (2017)
Salvia miltiorrhiza	PAL	Overexpression	Tanshinones	100 μM MeJA influence the biosynthesis of biosynthesis of phenolic acids and tanshinones in hairy roots of *S. miltiorrhiza*	Xing et al. (2018)
Catharanthus roseus	bHLH iridoid synthesis 1 (BIS1)	Global regulation	Loganic acid and Secologanin	Endogenous phytohormone production and activation of terpenoid biosynthesis pathway. BIS 1 stimulates monoterpenoid indole alkaloid (MIA) pathway production in *Catharanthus roseus* cell cultures, which finally leads to the production of anticancer metabolites like Vincristine and Vinblastine	Lu et al. (2016), Moerkercke et al. (2015)

(continued)

Table 21.1 (continued)

Plant	Gene	Approach used	Metabolite	Results	Reference
Artemisia annua	farnesyl diphosphate synthase, cytochrome P450-dependent hydroxylase(CYP71AV1), DBR2 (double bond reductase 2)	Global regulation	Dihydroartemisinic acid, Artemisinic acid	AaAOC promoter-GUS transgenic plants showed the overexpression of Dihydroartemisinic acid (125–248%) and Artemisinic acid (172–675%) compared to control plants	Lu et al. (2014)
Ginkgo biloba	Not reported	Elicitors	Bilobalide, Ginkgolides	Both methyl salisylate (MeSA) and Jasmonic acid (JA) synergistically elicited the expression of bilobalide and ginkgolide in the cell culture of *Ginkgo biloba*	Sukito and Tachibana (2016)
Taxus chinensis	Not reported	Elicitors	Paclitaxel	Coculture of *Taxus chinensis* suspension cells with endophytic fungi *Fusarium mairei* in a co-bioreactor results in high-rate paclitaxel anticancer drug production 25.63 mg/L within 15 days	Li et al. (2009)
Taxus chinensis	Not reported	Elicitors	Taxanes	Application of ozone acts as an elicitor in the metabolite production in species of *Taxus*.	Xu et al. (2011)
Andrographis paniculata	Not reported	Elicitors	Andrographolide	Adventitious roots induced from leaf segments of *Andrographis paniculata* in MS medium with 2.7 μM NAA and 30 g/L sucrose showed high rate of andrographolide metabolite production	Praveen et al. (2009)

Panax ginseng	Not reported	Elicitors	Ginsenosides	Vanadate as elicitor enhanced Ginsenosides metabolite production in the cell cultures of *Panax ginseng*	Huang and Zhong (2013)
Colchicum autumnale, Gloriosa superba	S-adenosylmethionine (SAM)-dependent methyltransferase (MT), GsPAL, Gs4CL, GsCCR, GsAER, GsC4H, and GsDAHPS	Overexpression	Alkaloids	Overexpression resulted in transgenic plant production with more alkaloid content	Nett et al. (2020)
Salvia miltiorrhiza	Rosmarinic acid synthase (*RAS*)	CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)/Cas9 (CRISPR-associate) genome editing technology	Rosmarinic acid	Using CRISPR/Cas9 genome editing technology, phenolic acid biosynthetic pathway was modified to produce high amount of Rosmarinic acid	Zhou et al. (2018)
Catharanthus roseus	Feedback-insensitive *Arabidopsis* ASα	Overexpression	Tryptophan, Tryptamin, Lochnericine	Gene overexpression in the hairy root cultures of *Catharanthus roseus* resulted in high-level formation of metabolites	Hughes et al. (2004)
Catharanthus roseus	Feedback-insensitive *Arabidopsis* ASα + *Catharanthus roseus* ASβ; Feedback-insensitive *Arabidopsis* ASα + *C. roseus* TDC, *C. roseus* ASβ	Overexpression	Tryptophan, Tryptamin,		Hong et al. (2006)
Catharanthus roseus	*C. roseus* tryptophan decarboxylase (TDC), *C. roseus* WRKY1	Overexpression	Serpentine		Hughes et al. (2004)
Catharanthus roseus	Feedback-insensitive *Arabidopsis* ASα + *C. roseus* TDC	Overexpression	Tryptamine		Hughes et al. (2004)
Catharanthus roseus	*Arabidopsis* DXS	Overexpression	Serpentine, Ajmalicine		Sander (2009)

(continued)

Table 21.1 (continued)

Plant	Gene	Approach used	Metabolite	Results	Reference
Catharanthus roseus	*Arabidopsis* DXS + C. roseus G10H	Overexpression	Ajmalicine, lochnericine, tabersonine		Peebles et al. (2011)
Catharanthus roseus	*Arabidopsis* DXS + feedback-insensitive *Arabidopsis* ASα	Overexpression	Lochnericine, horhammericine, tabersonine		Peebles et al. (2011)
Catharanthus roseus	*C. roseus* T16H	Overexpression	Lochnericine		Sander (2009)
Catharanthus roseus	*C. roseus* DAT	Overexpression	Horhammericine		Magnotta et al. (2007)
Catharanthus roseus	*C. roseus* WRKY2	Overexpression	Serpentine, Tabersonine		Suttipanta et al. (2011)

ERF, RA, and dehydration responsive element-binding protein (DREB) subfamilies (Girardi et al. 2013). The first and second transcription factors i.e. AP2/ERF exhibit a binding domain comprising 57–66 amino acids, which is conserved. These two are involved in regulating the plant metabolism and combating plant responses to biotic and abiotic stress conditions. Both AP2/ERF factors play a notable role in artemisinin engineering. Artemisinin is a potent herbal medicine used since ancient times for malarial fevers as exhibiting powerful potency against numerous types of malarial parasites which can be a breakthrough for malaria-affected regions globally. Rise in the artemisinin has been reported in transgenic *Artimisia annua* plants after overexpressing ERF factors which are capable of binding to the of *ADS* and *CYP71AV1* motifs (Yu et al. 2012).

The WRKY gene family (which is pronounced "worky" and the term was coined from the highly conserved 60 amino acid long WRKY domains of the transcription factors) belongs to a large group of transcription factors that are found in higher plants which perform diverse functions, including coordinating plant signalling pathways and enhancing plant resilience to biotic and abiotic influences. These also play characteristic roles in plant secondary metabolism (Jiang et al. 2017). W-box(TTGACC/T) of promoters is a specific binding site for transcription factors of the WRKY family. These TFs are involved in regulating developmental and physiological processes of plants, senescence, seed dormancy, defense responses, and stress resistance (Rushton et al. 2010). A surge by about 1.8 times as compared to control plants was shown in the production of artemisinin in upregulated *CYP71AV1 factor* in transgenic *A. annua* plants.

The basic helix–loop–helix (bHLH) TFs, being universal in many eukaryotes, are engaged in an infinite number of regulatory activities including growth, development, maintaining phytohormones, and stabilizing homoeostasis. Several of these compounds are bioactive themselves, e.g., terpenoids, iridoids, and seco-iridoids, which besides exhibiting fundamental and regulating metabolism have shown antimicrobial, anti-inflammatory activities, and foremost anticancer properties (Xu et al. 2016).

The basic region leucine zipper (a dimerization motif) family transcription factors (bZIP TFs) found in plants characteristically accommodate a highly conserved domain, i.e., bZIP, which has two structural features—one having a DNA-binding basic region and the other holding a leucine (Leu) zipper dimerization region. Both having diverse regulators, play critical roles in plant growth, physiological and metabolic pathways, and biotic/abiotic stress mechanisms. A bZIP, i.e., *AabZIP1* was engineered into *A. annua; AabZIP1* upregulated the increase of *ADS* and *CYP71AV1* which promoted the artemisinin in *A. annua* transgenic plants. In comparison to control lines, the artemisinin increased 0.7–1.5 times (Zhang et al. 2015). Hence, all these factors could be prospective metabolic engineering tools for provable persistent production of high-value plant-based pharmaceuticals (Xu et al. 2016).

2.2 RNA Interference (RNAi)

RNA interference (RNAi) is a unique as well as most productive tool in repressing gene expression that indicates suppression at the posttranscriptional level in all eukaryotes (Rukavtsova et al. 2010). RNAi is a similarity- or affinity-based silencing technique in which the pathways for gene expression are achieved through the introduction of double-stranded RNA (dsRNA) which acts upon the target mRNA. This technology has been widely used for the modification of the required metabolites during biosynthesis through downregulation of the competing processes (Pathak et al. 2021). In RNAi, different plant gene constructs are designed in order to suppress the expression of certain genes. A hairpin structure was once thought to be the most practical and effective for silencing, with the double stranded sense and antisense RNA creating a stem displaying integration whereas intermediate single-stranded RNA fragment forming a loop. The transcription of these constructs gives rise to a hairpin RNA structure. It contains dsRNA of a minimum of 100 base pairs that account for inducing effective silencing of genes, while the vector containing a fragment greater than 300 base pairs in size is estimated to be the most appropriate for the same. A significant reduction in about 70–100% of transformants expression of specific genes has been reported when hairpin structures were applied for RNA interference in plants (Wesley et al. 2001; Pathak et al. 2021).

2.3 Enzyme Precursors

Enzymes play the role of diverse protein catalysts that accelerate all biochemical reactions by simplifying the complex molecular dynamics supporting cell function. They are fundamentally essential biological stimulants of metabolic pathways with autonomous, adjustable, and multifunctional activities. Enzymes act competitive with their precursors in all biosynthetic and regulatory pathways. Production of metabolic compounds can be modified and enhanced towards the biosynthesis of the specific metabolites having desired composites by altering or blocking the competitive pathways or targeting the overexpressing genes in the precursor pathway (Verpoorte and Alfermann 2000). Upregulating 1-deoxy-d-xylulose-5-phosphate synthase (GrDXS) gave rise to amplification of terpenoid content in *Pelargonium* spp. (popularly used for essential oil) and *Withania somnifera* (has specific withanolides, which are proved to be immunity boosters) Jadaun et al. 2017. Similarly in other essential oil yielding plants, overexpressing the genes in the metabolic precursor system increased the overall monoterpene concentration. (Mahmoud and Croteau 2001; Muñoz-Bertomeu et al. 2006; Chandran et al. 2020).

3 Genome-Editing Tools for Improvement of Medicinal Plant Properties

Efficient genome editing, like that of conventional plant transformation technology, is entirely reliant on the optimized regenerative procedures. The programmable modifying enzymes give options to make positive alterations with precision and safety on the genomes of food organisms. The favorable sequences of the genomes of other races can be introduced without tedious and expensive crossings and re-crossing, in order to attenuate the already unalterable and unpredictable consequences of the change in environment and to secure food supplies in endangered areas (Carlson et al. 2012). Based on expansion in manipulation techniques at the molecular level, the progression of editing technologies for genetic material has opened up very promising research approaches for reshaping genomes in predominantly all eukaryotes. Genome editing has extraordinary potential prospective and has extended the ability of the scientific community for more precise and accurate contribution from basic research to applied agriculture biotechnology, which will lead to sustainability of crop improvement and hence food security ranging from increased plant resistance to pathogens, drought, extreme heat and cold, water flooding, metal and salt stress in a wide variety of crops to production of higher yields with enhanced carbohydrate, lipid, or protein content or crops with lower fertilizer requirement, water, and nitrogen inputs or also help in reducing food waste and toxins from plant metabolism. While it is technically a genetic modification, in most circumstances full genes and absolutely no genes of other species would not be introduced. A large part of the criticism currently being expressed to GMOs is based on organisms' ownership and the politically inappropriate method seed companies benefit from crop producers. The benefits and fundamental safety of the plants have been obscured by these factors. The combination of ultra-high-throughput techniques blended with traditional transformation technologies has accelerated new opportunities in breeding programs. Furthermore, metabolomic studies employing sequencing, microarray technology in conjunction with bioinformatics and computational biology approaches with molecular markers, linkage mapping, and sequence data have been extremely fruitful for identifying agronomic traits (Mohanta et al. 2017a). However, in order to optimize benefits, particular new and desired agronomic features must be introduced into the corresponding crop plants. To achieve the integration of preferable characteristics, the implementation of complete synthetic biology techniques is necessary, which are commonly known as genome editing tools (MacDonald and Deans 2016). Synthetic biology is a new interdisciplinary subject that combines artificial synthesis of DNA using chemicals with increased knowledge of genomics to allow investigators to quickly produce and assemble catalog directed DNA sequences in new genomes using the principles of engineering to biology. Synthetic biological tools are exact, precise, and predictable. Knowledge of all biological processes, DNA, RNA content, and sequence along with protein-based tools may be used to insert suitable features. (Mohanta et al. 2017b). Here, an overview of all known technologies and their possible applications

for genome editing is given. Plant cell and molecular biology have made enormous practical developments. Acquisition of novel technologies include those customized towards synthesis of DNA; the generation of diversity through combinatorial chemistry using softwares; creation of novel DNA molecules from genes to genomes using directed DNA shuffling and those that augment undistinguished genomes directly from nature called as bioprospecting, laden by biological diversity with a wider range of specificity. Complete genome sequences were deciphered and annotated (Michael and Jackson 2013), while a multitude of genes and their expression were compiled in databases (Wingender et al. 2000). Modern genomics provides methods for regulated manipulation of DNA sequences within the dynamics of structure and function of plant genomes (Petolino 2015).

Genome editing has arisen as a successful genetic engineering technique employing engineered nucleases. This makes them "molecular scissors" or chemically created nucleases for targeting and digesting DNA in precise spots within the genome. These nucleases create double-stranded DNA breaks (DSB) on the specific site, and eventually repair through natural recombination mechanisms using Homologous Recombination (HR) or heterologously dissimilar nonhomologous end joints (NHEJ). Changed sequences after cleavage include insertions or removals resulting in NHEJ gene disruption or integration of HR exogenous sequences. Nucleases can earmark sequences to create breaks under the supervision of protein conjugated DNA interactions or base paired RNA–DNA in the genome editing process (Zhang et al. 2017; Rocha-Martins et al. 2015). Currently, three types of manipulated nucleases have been employed practically for genome editing: zinc finger nucleases (ZFNs—which derived their identity from the zinc finger DNA coupled binding domain), the second ones, transcription activator-like effector nucleases (TALENs which have been derived from specific transcription activator like (TAL) effector DNA binding domain), and third being most relevant clustered regularly interspaced short palindromic repeats (CRISPR/Cas9 derived from the immune system of bacteria, comprise a ribonucleoprotein constituent, Cas9 and guide RNA component. TALENs and CRISPR/Cas9 in particular are currently widely employed in several organisms (Osakabe and Osakabe 2015).

3.1 Zinc Finger Nucleases (ZFNs)

Zinc finger nucleases (ZFNs) are modified restriction enzymes fabricated by fusion of a cleavage domain with binding zinc finger domain. These DNA domains can be designed to choose specific DNA sequences which act upon single sequences in complex genomes that are possible due to endogenous DNA repair machines. ZFN is employed, together with CRISPR/Cas9 and TALEN proteins, as a common method to amend the heterogeneous genomes of complex higher organisms. These nucleases have been used to modify several plant and animal genomes including *Arabidopsis*, tobacco, soy, maize, *Drosophila melanogaster*, *Caenorhabditis elegans*, *Platynereis*

dumerilii, sea urchins, and silkworm. Furthermore, CD4+ human T cell CCR5 gene disrupted by zinc finger nuclease was employed in a mouse hemophilia model in a clinical trial to be as safe to treat HIV/AIDS as possible. ZFNs are also employed to construct the new genetic disease model generation known as isogenic models of human diseases. Modern genomics provides methods for regulated modification of the DNA sequences and a more detailed genome knowledge. ZFNs composed of DNA and nuclease domains can be constructed to acknowledge and hence allow targeted cleavage for specific DNA sequences (Urnov et al. 2010). Several other examples of producing targeted DNA breaks are meganucleases (Stoddard 2011), representative TAL nucleases (Bogdanove and Voytas 2011), and much advanced CRISPR associated endonuclease (Shan et al. 2013b). To encourage diverse repair mechanisms and the potential to act upon specific DNA sequences, these enable various kinds of genomic changes like insertion, deletion, rearrangement, and integrations (Curtin et al. 2012).

ZFNs are nucleases that are united into a defined binding domain of DNA sequence which is very specific with a nonspecific cleavage. DSBs can be produced in precise genomic sites, encouraging cell repair activities of naturally occurring DNA, opening up new pathways for genetic manipulation. ZFN medium genomic loci DSB formation followed by repair by error prone NHEJ might result in gene-specific alterations by insertions or deletions of the base pair. Likewise, the donor DNA templates can be homologous to side-by-side sequence location via a homology-directed repair. ZFNs employed to produce simultaneous DSBs can obtain targeted deletion of the intervening DNA segment. The transgenic integration in ZFN-inducing DSBs can be achieved via NHEJ or HDR, specifically for the site. Genome editing has expanded our knowledge physiologically and genetically for improvement of crop plants. Like traditional plant manipulation technologies, the competence of genome processing is subject to standardized regeneration protocols. As a means for quick evaluation of the ZFN activity in plant populations, high-performance sequencing technologies have been utilized. The functionality of many ZFNs produced to target genes in tobacco was analyzed with pyrosequencing (Townsend et al. 2009). Combined with the aforesaid tools, confirmatory selection tests as well as validation services supplied by some scientific companies, it is likely that new ZFNs for gene targeting testing in various modeling and crop plants might be constructed and validated. Various ways can be utilized to change plant species' genomes utilizing ZFNs. The interpretation of minimum two ZFN monomeric assemblies in the same cell is necessary for selecting a specific native genome sequence not comprising a palindrome-like motif. Now expression may lead to site-specific desired mutagenesis and assembling/substitution of a gene, which is dependent on the donor DNA and plant machinery for repairing it. Using ZFNs as site-specific mutagens was tested for a target location for a well-described QQR ZFN in the transgenic *Arabidopsis* plants reported in the beginning (Bibikova et al. 2001; Lloyd et al. 2005).

Arabidopsis, rice, tobacco, maize, petunia, soyabean, rapeseed, and apple have employed ZFNs up to now, and research is in progress for other crops. For example,

ZFNs were applied by insertion of PAT gene (Phosphinothricin acetyltransferase gene) cassettes to the ZmIPK1 maize gene, resulting in herbicide resistance of mature maize and transposing inositol phosphorus content in seeds (Shukla et al. 2009). It assembled a variety of valuable features, which combined to provide an additional possibility for better production, was the targeted transgenic integration of the ZFN as an established technology (Ainley et al. 2013). Subsequently, ZFNs are employed to find secure areas in the rice genome for integration of genes that serve as reliable locations for subsequent character piling up (Cantos et al. 2014). However, the organization of the ZFNs is a very complex and factually difficult operation that often has little efficiency. ZFN mediated the target in the SSIVa rice gene coding sequence in an attempt to clarify the gene's functioning. Transgenic plants with premature stop codons and alternatives have been generated without prominent expression of SSIVa mRNA, low starch and dwarf-phenotypes. Significantly, the SSIVa gene disorder did not affect other genes related to starch synthesis, as their expression remained indigenous. ZFNs engineered have effectively split and stimulated mutations on the site of SSIVa in rice to affect starch content and factors related to plant height (Jung et al. 2018). One study has shown that modifications in upstream sequence in the DNA binding domain of L1L4 could result in variable phenotypes including fruit organs. These studies provided evidence and emphasize the utility of the modified ZFN method in targeted tomato plant genomes and this could definitely speed up translation research in tomatoes (Hilioti et al. 2016).

3.2 Transcription Activator-Like Effector Nucleases (TALENs)

TALENs offers customized quick and efficient methods for holding together DNA modules with any desired sequence allowing it for a variety of genomic and epigenomic alterations, fused into functional domains. TALE nucleases have been prosperously exploited in the editing of genomes in eukaryotic organisms. Activator-like effectors of transcription, TALENs are fused with the fractionation of FokI endonuclease domain in order to bind target DNA to generate double-strand breaks (Christian et al. 2010; Mahfouz et al. 2011; Miller et al. 2011). Fok1 is an endonuclease, probably protein-based, applied for knocking out the gene of interest by introducing breaks in the destined DNA. DSBs are generally restored in plants by the nonhomologous end joining (NHEJ) system (Shan et al. 2013a; Zhang et al. 2013), leading to minor deletions or inserts (indels) and enhancement in efficiency (Carroll 2011). It is noticeable that the capability of TALENs offer many opportunities. Resembling ZFNs, TALENs are binding proteins which detect certain sequences of DNA fused into a nuclease activator to achieve dissolution (Joung and Sander 2013). TALENs are secretive DNA-binding bacterial proteins that contain a number of preserved 32–34 residual sequenced blocks of two divergent amino acids which significantly determine binding specificities of a single base pair creating selective

combinations of repeated segments with appropriate amino acids of particular DNA-binding domains. Although ZFNs and TALENs are mostly in overlap with applications, due to a robust recognition code, TALENS has the advantage of relative ease of design (Sciences, National Academy of Sciences, Medicine 2017).

The simple modular DNA recognition code discovered with the proteins from TALENs (Boch et al. 2009; Moscou and Bogdanove 2009) expanded an alternative platform for programmable DNA binding protein engineering. After a long time of pioneering zinc-finger protein-based work, a number of effector domains, including nucleases have been accessible for fusing to the said repeats (Mussolino et al. 2011; Mercer et al. 2012). While DNA repeats of TALENs offer greater representation than limited zinc-finger proteins with only a single basic recognition, the cloning of TALE assembly poses an increased technical difficulty because of large and identical repeat sequences. A number of mechanisms are in place for quickly assembling custom TALE arrays to circumvent this problem. These strategies include molecular cloning using "Golden Gate" that assembles several DNA fragments in a regulated manner simultaneously (Cermak et al. 2011), geared up performance solid-phase assembly (Reyon et al. 2012; Briggs et al. 2012), and other cloning procedures (Schmid-Burgk et al. 2013). Several extensive, systematic investigations using various combinatorial approaches have shown that TALE repetitions may be coupled with almost any defined sequence of users. The only limitation to which TALE arrays are cited in studies is that the TALE should be bound with the T nucleotide base (Reyon et al. 2012; Schmid-Burgk et al. 2013). A vast range of plants have shown successful genome editing using TALENs (Martínez-Fortún et al. 2017; Ran et al. 2017). First reported use of TALEN's genome-mediated editing was in rice, where the susceptibility gene for bacterial blight, OsSWEET14, was inactivated, and the resulting mutant rice was found resistant to bacterial blight (Li et al. 2012). TALENs may be used to alter nutritive value of crops such as in soyabean with high omega 9-fatty acid and low polyunsaturated omega 6-fatty acid content caused by interrupting fatty acid desaturase (FAD) genes. This enhances durability and thermal stability of soybean oil (Haun et al. 2014; Demorest et al. 2016). Gene improvements by insertion of TALENs along with donor DNA have also been shown in tomatoes, whereby their replica numbers increased significantly As a result, the upstream incorporation of the anthocyanin regulatory gene upstream enhanced the efficiency of homologous recombination, and purple tomatoes with an elevated level of anthocyanin were obtained (Čermák et al. 2015). A new rice line in the OsBADH2 gene, responsible for the 2-acetyl-1-pyrroline biosynthesis was created by TALEN, resulting in a restore of the contents of the compound, an important part of the natural rice mutant fragrance (Chen et al. 2019; Shan et al. 2015). Knocking out by TALENs has also been reported of the SSR2 gene, the cause of the unwanted steroidal glycoalkaloids, which reduces the chaconine and solanine levels in new potato lines (Yasumoto et al. 2019).

4 Genome Engineering in Medicinally Important Plants Using CRISPR/Cas9 Tool

A unique expansive tool, clustered regularly interspaced short palindromic repeats (CRISPR/Cas9 endonuclease) is a powerful and coherent genome editing strategy. Being efficient and target-specific, this directed mutagenesis provides alternate avenues for use as this activates gene expression instead of directly cutting the DNA. In 1987 a potent CRISPR genome editing technology was established, which was recognized by its excellent benefits as the most important gene editing technique of the century (Martinez-Lage et al. 2018). Due to its many advantages, such as low costs, simplicity, high efficiency, and speed, CRISPR/Cas9 manipulations have taken the advantage over all other earlier known approaches. Type 2 is the most extensively utilized genome editing component of the CRISPR/Cas9 system, consisting of the three major constituents: the CRISPR RNA (cRNA), Cas9 endonuclease, and transactivating crRNA (tracrRNA) (Martinez-Lage et al. 2018). The assembly consists of two components: one being an associated protein called Cas9 that is capable of splitting DNA and the other one a gRNA (guide RNA) that identifies the sequence of DNA to be rectified. The sequences of the considered target genome must first be identified before CRISPR/Cas9 can be used. The guide RNA is then programmed to identify a specific sequence of DNA nucleotides. The role of Cas9 is now to locate the target point and then cut the DNA at that specific locus, allowing modification of the sequence to the existing genome. As a result, CRISPR/Cas9 acts as a DNA redrafting tool for cutting and pasting (Doudna and Charpentier 2014; Barrangou et al. 2007). CRISPR/Cas9 is currently fully sequenced to redirect specific genes in medicinal plants, to survey a synthesis of productive compounds, and to choose properties for enhanced yields and to advance research into biosynthesis and regulatory mechanisms (Ji et al. 2015). Humans have used medicinal plants as food, therapy, and industrial resources from time immemorial. The optimization of plant-derived natural compounds of biological meaning is achieved through classic biotechnology as well as new techniques like next generation sequencing (NGS). Previously, protein-based editing equipment such as ZFNs and TALENs were promoted for genome manipulation at transcription levels (Dey 2020).

The major aim of CRISPR/Cas9 in plants is to improve disease resistance by removing susceptibility genes and overexpressing genes. Human beings undergo evolution through genetic variations with gradual changes as it allows best adaptability of a population in response to changing environments. Modern genetic modification involves scientific techniques, which either add or silence additional DNA in gene sequences of organisms. The potential to modify the gene of an organism is diverse, especially if concerned with the consequences in humans. Its implementation as a genome-editing system focuses on generation of heritable, targeted mutations in transgene-free plants or knocking out of genes interpreting selectively via mutations. Since the discovery of *Nicotiana tabacum* protoplasts in 1988, the first gene targeting experiment, the DNA double-strand breakings have

increased implementing targets. Later in the next decade, researchers attempted to standardize targeted gene editing. Several independent groups developed a CRISPR/Cas9 system for a number of major crops: rice, wheat, and model plants *Arabidopsis* and tobacco by 2013.

Plant breeders now have an option to accelerate the translation of target-specific sequence modification, providing a rapid alternate measure for agricultural crop refinement for the first time in their history. Since then, continuous advancement has made genomics editing a widely used low-cost, convenient genetic manipulation tool in CRISPR/Cas9 approach such as CRISPR/CPf1 and replacement of nucleotides. Genome editing has altered relevant agronomic traits in a variety of crops. This approach also has advanced hybrid production techniques, and has helped in removing unwanted features and adding desired features to elite variables. It precisely amends crop characteristics as desired and consequently CRISPR/Cas9 has the potential to improve global food safety as well as sustainable agriculture.

CRISPR/Cas9 is extensively used for genomic editing in plants because of the ease of engineering in the number of different species to date (Malzahn et al. 2017). During cold acclimation, a large chromosomal deletion is attributed to the dispensable functionality of tandem arrayed CBF genes (C-repeat binding factor) in model plants (Zhao et al. 2016). It was also reported that the tetraploid cotton genome could be edited (Li et al. 2017; Chen et al. 2017). In rice genomes, CRISPR/Cas9 has been extensively used for functional analysis. Furthermore, male sterility induced by the environment is designed to enhance hybrid breeding (Li et al. 2016; Zhou et al. 2016). Also, disease-tolerant rice (Wang et al. 2016) and knocking out host genes in *Arabidopsis* (Pyott et al. 2016) were established. Two prominent bioenergy crops, poplar and switchgrass, are polyploids with high-frequency single nucleotide polymorphisms (SNPs) that hinder gene editing. This powerful tool has been employed for target genes implicated in lignin production of these two species, using their multiplexing capacities and their specificity (Carroll and Somerville 2009; Okada et al. 2010). The technology CRISPR/Cas9 has enabled crop value and quality of food to be improved by upgrading their nutrient status. In tomatoes, lycopene is a plant component with antioxidant and favorable medicinal characteristics. The success of increasing the quantity of lycopene in tomatoes is predicted to also help improve the micronutrient content of plants by CRISPR/Cas9 technology (Pillay 2020). In a model of purple calli in carrot (Klimek-Chodacka et al. 2018), torenia (Nishihara et al. 2018), petunia (Yu et al. 2021), and black rice (Jung et al. 2019). The carrot cell culture targeted by blocking carrot flavanone-3-hydroxylase gene (F3H) for anthocyanin biosynthesis signifies the application of a CRISPR/Cas9 system. The anthocyanin pathway that expresses a purple-colored calli F3H gene encoding flavanone-3-hydroxylase was blocked with multiplexing vectors of CRISPR/Cas9. This led to white calli growth and confirmed the functioning and visual scorable marker of the gene for the screening (Klimek-Chodacka et al. 2018).

5 Conclusion and Future Perspectives

Metabolic engineering and genome editing are promising technologies that can contribute to the sustainable production of food for the welfare of the expanding population. The use of genome modification in plants remains a matter of biosafe, social, and ethical concern. The biggest issue is the danger of unwanted genetic modifications caused by off-target changes in plants. Although genome editing has several benefits as compared to typical plant breeding, its implementation in medicinal plants is still challenging. Molecular or genetic investigations in medicinal plants are difficult, preventing genes that cause desirable features to be identified. For the identification of genes with desired characteristics, sequencing of medicinal plants of interest will be important. These can be genetically engineered by a set of optimized tools and techniques with preferable recognized characteristics in crops without a genome reference (Xu et al. 2019). Progress in metabolic engineering in medical plants is aimed at important customized attributes, such as increasing root metabolites, biotic and abiotic resistance to pathogens and physical stress, and improved shelf life. These characteristics might be multifactorial and are difficult to improve by plant breeding. *Artemisia annua* was replicated into tobacco with high biomass for conjunctional supertransformation for artemisinic acid production utilizing the precursor to artemisinin (Alok et al. 2020). Genome editing can give an enhanced and effective approach for secondary metabolite in vitro production. CRISPRs, ZFNs, and TALENs are all accomplished to produce site-specific DSBs with different specificity and efficiency levels. The previous usage of these systems has demonstrated surprising new capabilities and enabled the development of model systems in an extensive range of organisms. The information coupled with the big data from metabolomics studies can be exploited in future breeding programs opening avenues towards the development of new cultivars with best nutraceutical values and improved agronomic traits.

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Chapter 22
Applications of Genome Editing Techniques for the Improvement of Medicinal Plants

Reema Mishra, Preeti Agarwal, and Aparajita Mohanty

1 Introduction

Medicinal plants have long been used in therapeutics and are an important component of Ayurveda, Homeopathy and Unani system of medicine (Ravishankar and Shukla 2007). They play an important role in curative as well as preventive medical therapy (Niazian 2019). These plants are rich in secondary metabolites like alkaloids, flavonoids, glycosides, steroids and amines, which are the key bioactive compounds that contribute to their medicinal properties. These phytoconstituents form the basis for the use of these plants in ethnomedicines and commercial pharmaceutical drugs (Jain et al. 2019; Li et al. 2020).

About 80% of the world's population largely rely on ethnomedicines for their health care requirement (Ekor 2014). The demand for these medicinal plants is increasing because of their ease of availability, affordability, efficacy and no side effects in comparison to synthetic drugs (Ekor 2014; Okoye et al. 2014). Medicinally important phytoconstituents can be directly extracted from plants and can also be chemically synthesized to produce metabolites as well as their derivatives with related uses (Altemimi et al. 2017). Plant tissue culture (PTC) technique is another substitute for the production of these metabolites that are otherwise difficult to be isolated via direct extraction from plant. PTC has been exploited to produce a variety of bioactive phytochemicals from *Catharanthus roseus*, *Taxus baccata*, *Coleus forskohlii*, *Hypericum perforatum* etc. (Chandran et al. 2020). However, production by plant cell culture has various limitations including the fact that it is a long and tedious procedure with a low yield of these metabolites (Chen et al. 2019). These

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M. K. Swamy, A. Kumar (eds.), *Phytochemical Genomics*,
https://doi.org/10.1007/978-981-19-5779-6_22

limitations can be overcome by modifying the delivery systems and plant regeneration time, use of new vectors for dicot as well as monocot plant transformation and employing innovative approaches like the use of nanotechnology, germ cells, shoot apex and pollen-facilitated transformation (Dey 2021). Nonetheless, the increased demand for these metabolites can be achieved by increasing yield via conventional breeding or genetic engineering approach. The introduction of next-generation sequencing techniques has shown immense potential in analyzing and identifying the genes and enzymes involved in secondary metabolite biosynthetic pathways in medicinal plants. Further, the recent advent of targeted genome editing (GE) techniques has revolutionized biotechnology-based manipulation of biosynthetic pathways for improved synthesis of secondary metabolites (Pouvreau et al. 2018; Dey 2021; Rehman et al. 2021).

GE tools have the potential to make desired changes in the genome of an organism. These tools use site-specific nuclease (SSN), a programmable nuclease made up of DNA-binding domains (sequence-specific in nature) which is fused to a DNA cleavage domain (non-specific in nature) that targets specific gene sequences (Urnov et al. 2010; Carroll 2011; Zaidi and Mansoor 2017). These nucleases facilitate site-directed mutagenesis and offer an advantage over techniques where mutagenesis is random (Osakabe et al. 2010). In the last few years, a number of GE techniques like customized homing endonucleases (meganucleases), oligonucleotide-directed mutagenesis-ODM, zinc-finger nucleases-ZFNs, transcription activator-like effector nucleases-TALENs and clustered regularly interspaced short palindromic repeats-CRISPR/Cas9 for genome modifications have been extensively used for genome manipulation at transcriptional level.

These tools have been widely used to improve nutritional value as well as productivity of crop plants and enhance plant biotic and abiotic stress responses. GE techniques have proved to be a powerful tool, not only to enhance agricultural yield but also in the production of secondary metabolites by modifying metabolic pathways and developing plants with optimized secondary metabolite profiles (Dey 2021). Till date, application of GE to medicinal plants is limited due to insufficient transcriptome studies and unavailability of whole genome sequence data which is essential to avoid any off-target effects (Alok et al. 2020). However, CRISPR/Cas9 has been successfully tried in *Camelina sativa*, *Dendrobium officinale*, *Dioscorea zingiberensis*, *Nicotiana tabacum*, *Papaver somniferum* and *Salvia miltiorrhiza* (Alagoz et al. 2016; Kui et al. 2017; Li et al. 2017; Jiang et al. 2017; Mercx et al. 2017; Morineau et al. 2017; Feng et al. 2018; Ozseyhan et al. 2018; Marchev et al. 2020; Dey 2021).

The present chapter discusses the various GE tools, their comparison, limitations and regulatory measures. Further examples of improved medicinal plants obtained by using these tools and future prospects of these techniques for improvement of medicinal plant properties are highlighted.

2 Genome Editing Tools and Their Comparison

Development and innovation in GE tools have provided ample opportunities to edit the genomes for basic and applied research using artificial nucleases, thus enabling specific modification of genomes. There are several tools which have been developed for precise genome editing in plants. In 1996, it was demonstrated that protein domains of "zinc fingers" (ZF) in association with endonuclease domains of FokI can be used as site-specific nuclease (the ZFNs), which can precisely cleave the DNA molecules in vitro (Kim et al. 1996). This was followed by development of other GE methods like TALENs and very recently CRISPR/Cas9. All of these GE tools are based on the application of chimeric nucleases that efficiently and precisely edit the genome by inducing double-strand breaks (DSBs) in the DNA followed by a repair mechanism (Fig. 22.1). The breaks can be repaired either by error-prone NHEJ (non-homologous end joining) repair mechanism or in some cases by HR (homologous recombination) mechanism (Kamburova et al. 2017).

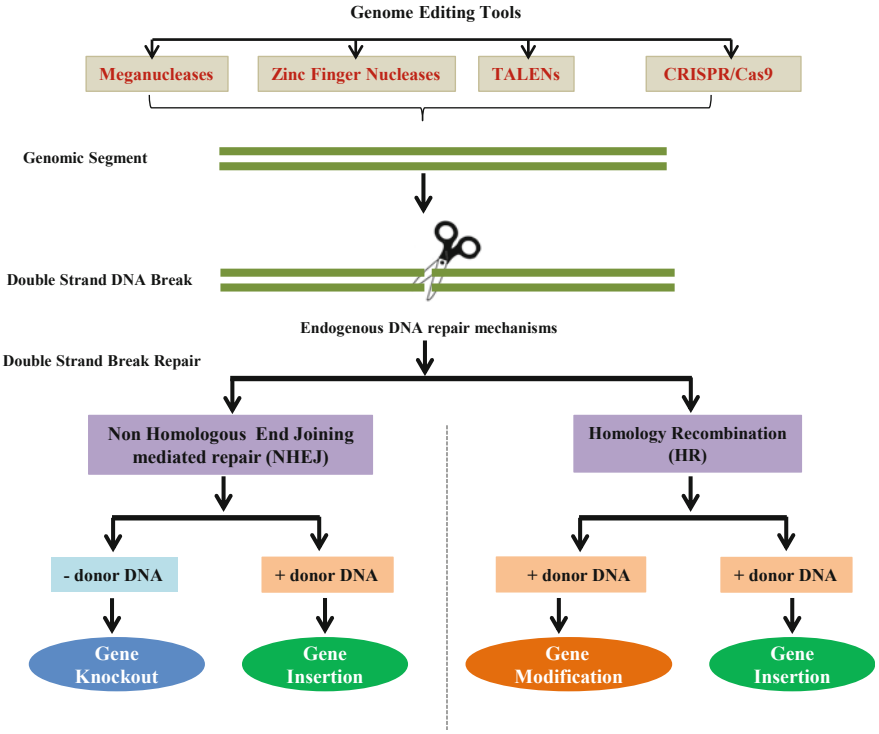

Fig. 22.1 Mechanism of targeted genome editing in plants and different types of genome modifications depending on the availability of a repair template and repair mechanism

2.1 Meganucleases

Meganucleases (also referred to as homing endonucleases-HE) are longest and extremely specific DNA cleaving enzymes. They are found in archaea, prokaryotes and unicellular eukaryotes. Meganucleases comprise DNA binding and DNA cleavage domains. They can precisely replace, remove and modify desired sequences more efficiently. These are naturally occurring restriction enzymes that efficiently cleave the dsDNA at the required recognition sites, which are about 14–40 bp long (Silva et al. 2011; Carroll 2017). They work by inducing DSB at the recognition site and this break is repaired by HR-mediated repair mechanism. This is followed by the insertion of meganucleases coding sequence into the desired gene. Homing endonucleases are referred to as "homing" as they assist in the lateral mobility of genetic elements within an organism. HE genes may be mobile genetic elements or they may be found inside a mobile genetic element. They are encoded as genes embedded in group I and group II introns or intein and permit the horizontal transfer of genes (Silva et al. 2011). Till date various customized meganucleases have been used in several applications of genetic engineering (Stoddard 2011).

In comparison to ZFN and TALENs, meganucleases are smaller in size and thus are compatible with only some viral vectors where small-sized coding sequences are required (Dunn and Pinkert 2014). However, they possess certain limitations and thus they are not much preferred over other GE tools. For instance, both the DNA-binding and cleavage domains of meganucleases extend over each other and therefore the catalytic activity of the enzyme is hindered. Also, meganucleases display higher mutation rate in comparison to other GE tools (Stoddard 2011).

Further, due to the non-availability of customizable components and the absence of protocols required for designing the constructs for specific recognition of particular DNA sequence, meganucleases are less attractive and are less preferred for genome engineering.

2.2 Oligonucleotide-Directed Mutagenesis (ODM)

This genome editing tool is used for targeted mutagenesis and has been successful in mammals, yeast, bacteria and plants. It provides fast, accurate and non-transgenic breeding approach for improving any desired agricultural trait (Sauer et al. 2016). It uses specific 20–100 bp long oligonucleotides whose sequence bears homology to the target genome sequences except a change of single base pair. This introduces specific mutation in the genome for site-directed specific editing of the desired gene. These synthetic oligonucleotides that bear homology to a specific sequence of the target gene, when transiently introduced into the cells, bind to the targets and activate the repair machinery of the cells. The repair process works by identifying the only mismatch in the DNA strand and then that same mismatch is copied into the target sequence. This results in single base editing in the genome and consequently the

desired trait in the plant is expressed (Abdurakhmonov 2016; Kamburova et al. 2017).

2.3 Zinc Finger Nucleases (ZFNs)

ZFNs are the first generation of GE techniques which use designed chimeric nucleases. Chimeric nucleases are fusion proteins which comprise of multiple DNA-binding domains derived from zinc finger (ZF)-bearing transcription factors. The Cys_2His_2 zinc finger DNA-binding domain is a commonly present DNA-binding domain in eukaryotes. Each ZF consists of 30 amino acids, folded in a conserved ββα configuration (Gaj et al. 2013). Each ZF protein recognizes a 3 nucleotide bp in the DNA. The ZFN monomer consists of customized Cys_2His_2 zinc finger domain at the N-terminal region and bears a non-specific FokI DNA cleavage domain at the C-terminus. For ZFN activity, dimerization of FokI domain is critical (Kim et al. 1996). A ZFN dimer consists of 2, 3 or 4 zinc finger domains that recognize 18 or 24 bp of target sequence (Kamburova et al. 2017). ZFNs first recognize the two flanking sequence sites (i.e. on forward strand and on the reverse strand) and then bind on both side of the sequence site. After binding, dimerization of the FokI domain occurs followed by cleaving of the DNA at required site, thus creating a DSB with 5′ overhangs (Urnov et al. 2010). Subsequently the cells repair DSBs by NHEJ or HR pathway (Rehman et al. 2021). There are numerous benefits of using ZFNs but it has its own limitations too. Firstly, it is difficult to assemble the ZF domains that can specifically bind to a stretch of nucleotides with precision. Another disadvantage is the constraint of target site selection. It can also introduce off-target nicks. To reduce this, a pair of ZFNs should be used which have variant FokI domains (Doyon et al. 2011; Gaj et al. 2012).

The designing and use of ZFNs include their modular design, linking together the individual ZFs and optimization of ZFs for accurate targeting of DNA sequences. Nowadays engineered zinc fingers are available commercially. This helps to bypass structuring and validation of ZFN. ZFN technology is found to be very efficient and can facilitate designing of mutant and creating transgenic plants (Gaj et al. 2013). The ZFNs have been used for inactivation, modification and insertional disruption of target genes in *Arabidopsis*, tobacco and maize (Osakabe et al. 2010; Petolino et al. 2010; Zhang et al. 2010; Ainley et al. 2013; Gaj et al. 2013).

2.4 Transcription Activator-Like Effector Nucleases (TALENs)

TALENs are natural proteins derived from pathogenic bacteria belonging to the genus *Xanthomonas*. TALEN is a fusion of TALE-DNA binding domain and a DNA cleavage domain. The DNA-binding domain can be manipulated to bind to any

target DNA sequence, whereas the DNA cleavage domain functions as a nuclease to produce nicks at the DNA region specified by TALE-DNA binding domain. The DNA-binding domain consists of tandemly organized repeats of 34 amino acids. Two adjacent amino acids (i.e. at positions 12 and 13) vary in these tandem repeats and are termed as RVD (repeat variable diresidue) (Deng et al. 2012; Mak et al. 2012). The variability in RVDs has a strong implication in the recognition of specific DNA sequences.

One major advantage of TALENs is that it is easy to design and can be constructed in a short period of 2 days. Also several constructs can be made to compile a library of TALENs for targeting a complete set of genes in a genome (Cermak et al. 2011; Miller et al. 2011; Reyon et al. 2012; Kim et al. 2013). Similar to ZFNs, modular TALE repeats are coupled for identification of stretches of DNA sequences. However, there is no requirement of re-engineering of the linkage between repeats for creating long TALEs which would help in targeting one specific site in a genome, like that of ZFNs. Several effector domains are currently accessible and they can be linked to TALE repeats to achieve targeted genetic manipulations (Rehman et al. 2021). Another advantage of TALENs is that the TALE array can bind to large sequence (greater than 18 bp) in comparison to ZFNs (which can bind to 9–18 bp) (Smith et al. 2014; Suzuki et al. 2014). Further, TALENs when introduced into target cells produce lesser cell toxicity in comparison to ZFNs (Reyon et al. 2012; Guilinger et al. 2014; Rehman et al. 2021).

A limitation for TALE arrays is that the binding sites for TALE should have thymine (T) base at their start site. Studies have shown that the N-terminus of DNA-binding protein has tryptophan (at 232 position) which interacts with the thymidine. This interaction determines the binding efficiency of TALENs. This limitation can be overcome by generating mutant alternatives of TALEN at the N-terminus domain which have affinity for other DNA bases also (Mak et al. 2012; Lamb et al. 2013). Another limitation of TALENs is its comparatively larger size (~3 kb) than ZFNs (~1 kb). This large size hinders its delivery and expression in the target cells. This limits their use in therapeutic applications, where delivery into viral vectors is with restricted cargo size or in the form of RNA molecules (Yang et al. 2013). Moreover, large size TALENs show less specificity. Further, for designing TALENs, it is necessary to re-engineer a new protein independently for every target. This is in contrast to CRISPR which is easier to design and simpler to use. Till date, TALENs have been successful in GE of various animal and plant species like *Arabidopsis*, wheat, rice, potato and tomato (Xiong et al. 2015).

2.5 *Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)*

Recently the CRISPR/Cas system is viewed as an effective GE tool, and particularly promising is the CRISPR/Cas9 editing system for inducing targeted genetic modifications. It consists of Cas9 nuclease and a guide RNA (gRNA). The gRNA

distinctively binds to the target sequence of the genome and guides Cas9 to incise the target site, thus resulting in a DSB. In nature, CRISPR method is used by bacteria to confer protection against attacking foreign DNA by RNA-mediated DNA cleavage (Seed 2015). They target specific DNA sequences for cleavage, and this mechanism depends on CRISPR loci (unique sites found in the bacterial genome). These loci are made up of operons which code for Cas9 protein and a repetitive range of repeat sequences called spacers. The spacer sequences are short fragments which are derivatives of foreign DNA. After recombination process, they integrate into the bacterial genome and then use them to express the synthetic guide RNA (sgRNA) (Zhang et al. 2014; Rehman et al. 2021). In CRISPR/Cas9 method, the identification of target site is dependent upon the interaction between the gRNA and target DNA site. The gRNA and Cas9 complex has nuclease activity, mediated by Cas9 endonuclease that creates incision on DNA sequence (Deltcheva et al. 2011; Cong et al. 2013; Kamburova et al. 2017). Thus overall CRISPR/Cas9 functions in three stages: (1) expression of Cas9-nuclear localized protein, (2) generation of gRNA (20 nt) that is corresponding to the target gene (3) essential requirement of an NGG PAM ("N" can be any nucleotide base; PAM—Protospacer Adjacent Motif) recognition site near 3′ end of the target region which is involved in association of CRSIPR/Cas9 complex with the target region.

CRISPR/Cas9 tool has an advantage over ZFNs and TALENs that it can simply aim at any genomic sequence by varying the 20-bp protospacer. Another advantage is that multiple gRNAs can be used to target multiple sites simultaneously within the same cell. Thus at the same time, multiple genes can be mutated (Cong et al. 2013; Mali et al. 2013; Gilles and Averof 2014; Park et al. 2014; Rehman et al. 2021). In plants, CRISPR/Cas9 has been the most preferred GE tool. However, there are limitations too which hinder its applicability. The large size of the CRISPR/Cas9 system makes it unsuitable for packaging into viral delivery vectors. The drawback of this GE tool is that it introduces many accidental off-target alterations in the genome in comparison to its variants (Zhang et al. 2016; Hua et al. 2019). Another disadvantage is, it requires a 5′-NGG-3′ PAM sequence flanking a 20 bp target sequence where it only identifies the NGG PAM site. However, the xCas9 variant is found to be more target efficient, shows greater DNA specificity, less off-target mutation and displays a wide range of PAM compatibility (NG, GAT and GAA). This is the most preferred tool and has been applied to several plant species including model plants (*Arabidopsis*, *Nicotiana* etc.), medicinal plants (*Papaver somniferum*, *Salivia miltiorrhiza* etc.), crop plants (rice, wheat, maize, sorghum etc.) and fruits (apple, banana, orange etc.). Further, CRISPR/Cas9 has been efficiently used to engineer a number of secondary metabolic pathways and particularly for increasing the yield of secondary metabolites in medicinal plants (Sander and Joung 2014; Voytas and Gao 2014; Niazian 2019; Alok et al. 2020; Dey 2021; Shabir 2021).

CRISPR/Cas9 system requires an *Agrobacterium*-facilitated transformation method for engineering a mutant transgenic plant and has proved to be successful in various plants like in *P. somniferum*, *Taraxacum kok-saghyz*, *S. miltiorrhiza,* and *S. pimpinellifoilium* (Alagoz et al. 2016; Iaffaldano et al. 2016; Li et al. 2017; Manghwar et al. 2019). CRISPR/Cas9 system has been also utilized for increasing

the yield of secondary metabolites in medicinal plants (Niazian 2019; Dey 2021; Shabir 2021). A large number of CRISPR variants have been developed for efficient genome editing like spCas9-NG, base editing (BE), Cas 13, Cas12a (Cpf1) and xCas9 (Manghwar et al. 2019). In medicinal plants, the selection of right target region in their genome is the most important requirement for genome editing (Alok et al. 2020). Another important factor that determines efficiency of the CRISPR/Cas9 system is the vector and delivery method required for transfer of gRNA and Cas9. The delivery of the CRISPR construct is done via *Agrobacterium*, gene gun (gold particles) and PEG-mediated methods. *Agrobacterium*-mediated transformation is frequently used for medicinal plants. There are reports of use of PEG-mediated delivery in protoplasts of *Arabidopsis thaliana*, rice, tobacco and lettuce. The transgenics showed mutation frequencies of about 46% (Woo et al. 2015; Zhang et al. 2016). The PEG-mediated transformation method is reported in an ornamental medicinal orchid named as *Phalaenopsis* (Alok et al. 2020). About 30 different plants have been effectively edited using CRISPR/Cas9 system; however, application of this method is found to be limited in medicinal plants because of the lack of their genome information. The whole genome sequence information of opium and *Artemisia annua* are available and thus it has opened up the prospects for engineering metabolic pathways in these plants using CRISPR/Cas9 method (Guo et al. 2018; Shen et al. 2018). Therefore, CRISPR/Cas method and its versions hold great potential in understanding the gene functions, targeted editing, single base substitution and multiplex editing of important genes. Consequently, improvement of nutritional value, increased yield of secondary metabolite in medicinal plants and enhanced resistance of plants to biotic and abiotic stress can be achieved (Kumar et al. 2021).

2.6 Comparison of Genome Editing Tools

The introduction of and rapid developments in GE tools (meganucleases, ZFNs, TALENs and very recently CRISPR/Cas) have necessitated a comparative analysis for their efficient and appropriate applications. A schematic representation comparing the features of the GE tools is presented in Fig. 22.2.

Meganucleases are obtained from genetic elements of microbes and comprise of DNA binding and DNA cleavage domains. ODM is composed of exogenous polynucleotide (chimeraplast), while ZFNs are derived from eukaryotic gene regulators that consist of ZF domain coupled with nonspecific FokI nuclease domain. TALENs derived from *Xanthomonas* comprises TALE-DNA binding domain and non-specific FokI nuclease domain. The catalytic domain of meganucleases is present in the DNA binding site while ODM does not possess any catalytic domain. For ZFNs and TALENs, FokI restriction endonuclease domain acts as the catalytic domain. The most popular CRISPR is obtained from immune system of bacteria and archaea. It has crRNA (non-coding RNA) and Cas9 proteins as its core components (Kamburova et al. 2017; Iqbal et al. 2020). Cas9 has two endonuclease domains

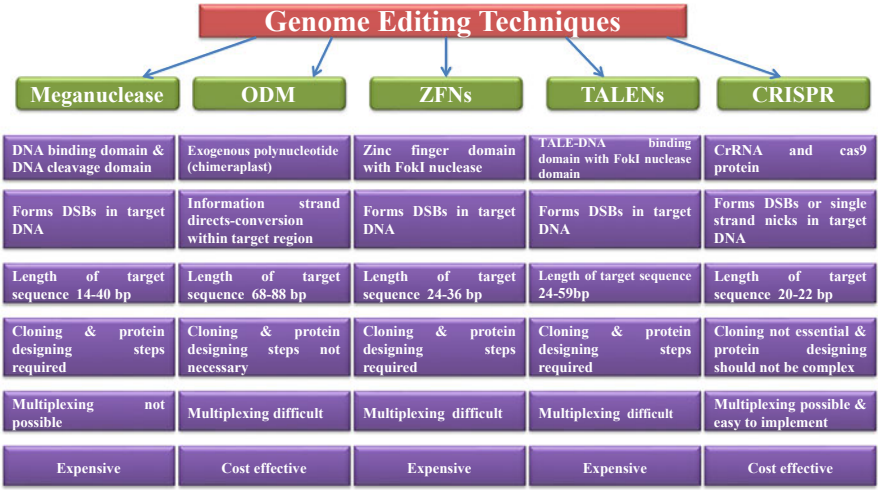

Fig. 22.2 Comparison of important features of genome editing techniques. Abbreviations: *ODM* oligonucleotide directed mutagenesis; *ZFNs* zinc finger nucleases; *TALENs* transcription activator-like effector nucleases; *CRISPR* clustered regularly interspaced short palindromic repeats; *DSB* double-strand break

(RuvC and HNH) which act as its catalytic domain. RuvC initiates the cleavage of the DNA strand (sequence is not complementary to the gRNA) and HNH domain of Cas9 cleaves the DNA strand (that is complementary to the gRNA) (Sajid et al. 2017). All the three tools (meganucleases, ZFNs, TALENs) work by introducing DSBs in target DNA while CRISPR induces either DSB or single-strand breaks in the target DNA. However, in ODM, the information strand guides conversion (s) within the target region (Kamburova et al. 2017; Iqbal et al. 2020). The small size of meganucleases and ZFN permit the use of viral vectors and facilitate easy delivery, while Cas9 is too large for smaller capacity viral vectors. Cas9 shows moderate ease of in vivo delivery in contrast to the TALENs which exhibit difficulty of in vivo delivery. Multiplex gene editing can be easily achieved by CRISPR for facilitating multiple mutations at the same time in contrast to ZFNs and TALENs. Moreover, multiplexing is not possible by meganucleases, and ODM too has its own technical difficulties, making it unfit for multiplexing (Kamburova et al. 2017; Iqbal et al. 2020).

Each of these tools has its own targeting limitations, like in meganucleases the re-engineering for new specificities is quite challenging. For TALENs, presence of 5 ˈT of the target sequence for each TALEN is very critical and acts as its limiting factor. Similarly, for CRISPR, the target DNA for Cas9 should essentially contain 5'-NGG-3' PAM (Nemudryi et al. 2014; Kamburova et al. 2017). It has been found that meganucleases, ZFN and TALENs are highly sensitive to methylation compared to CRISPR and therefore CRISPR can target methylated regions of DNA as well. CRISPR and TALENs show less cytotoxicity than ZFNs and meganucleases. Meganucleases induce the highest mutation rates, while CRISPR exhibits the lowest mutation rate in comparison to other tools (Kamburova et al. 2017; Iqbal et al. 2020).

Using CRISPR, it is possible to create large-scale libraries, while it is technically difficult with TALENs and ODM and impossible to create libraries with ZFN (Kamburova et al. 2017). ZFN is difficult to design and screen. It is more laborious and comparatively costlier than TALENs. TALENs are highly effective, more specific, moderately easy to construct and screen as compared to ZFNs. CRISPR, on the other hand, is less laborious, less time-consuming, more cost-effective, specific, efficient and easy to design and screen in comparison to others. Meganucleases are extremely difficult to construct and screen, are time-consuming, laborious and very expensive (Kamburova et al. 2017; Sajid et al. 2017; Xu et al. 2020b).

3 Application of Genome Editing in Medicinal Plants

The therapeutic properties of medicinal plants are attributed to their secondary metabolites like phenols, terpenoids, alkaloids etc. These varied phytochemicals have diverse functions and they exhibit various pharmacological properties. Genome editing techniques have been used to manipulate genes involved in secondary metabolite biosynthesis pathways. Medicinal plants that have been successfully edited for secondary metabolite production (Table 22.1; Fig. 22.3) are being discussed below.

3.1 Camelina sativa

C. sativa belongs to the family Brassicaceae and is a traditional oil producing crop that is grown in Europe and Central Asia. It possesses various advantageous traits such as adaptability to diverse environmental conditions and tolerance to insects (Murphy 2016; Ergönül and Özbek 2020). It is also considered to be highly nutritious and an ideal feed for the livestock. Owing to its numerous benefits, it is used in cosmetics and pharmaceutical sector as well as in bio-fuel industry (Murphy 2016; Dey 2021). Several studies have shown that it can help in decreasing the cholesterol level and is rich in linoleic (omega-6), α-linolenic (omega-3) fatty acid, and bioactive compounds like phenolics and tocopherols (Ergönül and Özbek 2020). It has antioxidant, anti-inflammatory, anticancer properties and is also recommended as an enhancer sensitive to insulin (Dey 2021). CRISPR/Cas9-based genome editing has been effectively applied to hexaploid *C. sativa* to enhance the content of oleic acid via knocking out *FAD2* (Fatty Acid Desaturase2) genes (Jiang et al. 2017; Morineau et al. 2017). Study by Ozseyhan et al. (2018) has shown that knock out of *FAE1* (Fatty Acid Elongase1) resulted in decline in very long-chain fatty acids (VLCFAs) and improved the α-linolenic acid/oleic acid content. The United States Department of Agriculture has also given a free pass to CRISPR/Cas9-derived *C. sativa*, which has an enhanced content of omega-3 oil (Waltz 2018; Sabzehzari et al. 2020).

Table 22.1 Genome-edited medicinal plants with editing details for their secondary metabolite production

Medicinal plant	Targeted gene (s)	Genome editing technique	System used for Cas9 delivery	Mutation rate (%)	Mutation type	References
Camelina sativa	*FAD2*	CRISPR/Cas9	*Agrobacterium*-mediated transformation	–	Deletions Insertions	Jiang et al. (2017)
Dendrobium officinale	*C3H*, *CCR*, *C4H*, *IRX*, *4CL*	CRISPR/Cas9	*Agrobacterium*-mediated transformation	10–100	Deletions Substitution Insertions	Kui et al. (2017)
Dioscorea zingiberensis	*Dzfps*	CRISPR/Cas9	*Agrobacterium*-mediated transformation	60	Deletions	Feng et al. (2018)
Nicotiana tabacum	*XylT* and *FucT*	CRISPR/Cas9	*Agrobacterium*-mediated transformation	–	Deletions Insertions	Mercx et al. (2017)
	BBL	CRISPR/Cas9	*Agrobacterium*-mediated transformation	–	Insertions	Schachtsiek and Stehle (2019)
Papaver somniferum	*4'OMT*	CRISPR/Cas9	*Agrobacterium*-mediated transformation	80–85	Deletions	Alagoz et al. (2016).
Salvia miltiorrhiza	*SmCPS1*	CRISPR/Cas9	*Agrobacterium rhizogenes*-mediated transformation	42.3	Deletion	Li et al. (2017)
	SmRAS	CRISPR/Cas9	*Agrobacterium rhizogenes*-mediated transformation	50	Insertion Deletion	Zhou et al. (2018)
	SmLAC	CRISPR/Cas9	*Agrobacterium rhizogenes*-mediated transformation	–	Insertion Deletion	Zhou et al. (2021)

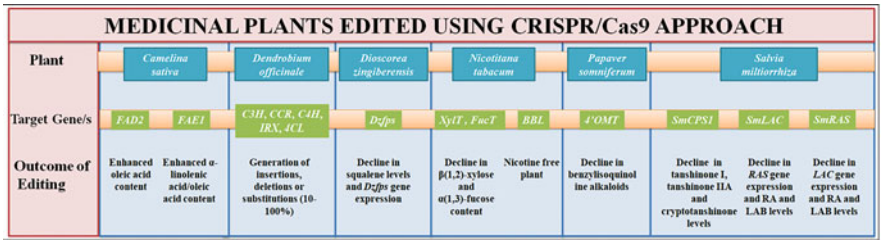

Fig. 22.3 Medicinal plants (edited using CRISPR/Cas9 technology) with the altered target gene (s) and editing outcomes

3.2 Dendrobium officinale

It is an herbaceous orchid belonging to the family Orchidaceae. It is a medicinal herb with different pharmacological properties (immunomodulating, anti-inflammatory, antidiabetic, anticancerous, antimicrobial, antiherpetic, antifungal, aquaporin-5 inducing etc.; Teixeira da Silva and Ng 2017). The main phytochemical constituents of *D. officinale* are alkaloids, phenanthrene, bibenzyls and polysaccharides (Cakova et al. 2017; Tang et al. 2017). Plant extract is used for stomach nourishment and for increasing fluids in body. It is also used as tonic and astringent (Cakova et al. 2017). It is considered to be a model system for gene manipulation in the family because of the availability of its sequenced genome and very well developed transformation system (Yan et al. 2015).

Lignocellulose plays an important role in imparting taste to *D. officinale*. In order to reduce its content Kui et al. (2017) employed CRISPR/Cas9-facilitated genome editing system via *Agrobacterium*-mediated transformation and targeted five genes namely *COUMARATE 3-HYDROXYLASE* (*C3H*), *CINNAMOYL COENZYME A REDUCTASE* (*CCR*), *CINNAMATE 4-HYDROXYLASE* (*C4H*), *IRREGULAR XYLEM5* (*IRX*) and *COUMARATE: COENZYME A LIGASE* (*4CL*) involved in lignocellulose biosynthesis. CRISPR/Cas9 editing system could produce edits like deletions, substitution and insertions in the target genes at a rate of 10–100% (Kui et al. 2017) suggesting it to be an efficient approach for genome manipulation.

3.3 Dioscorea zingiberensis

Dioscorea zingiberensis (family Dioscoreaceae) commonly called “Peltate Yam” is an important medicinal plant cultivated in China. The rhizome of this plant is used as food and in traditional Chinese medicines for treatment of stomach distension, cough, loss of appetite, sprains, cardiovascular diseases etc. (Zhang et al. 2018). The dominant phytoconstituent of *D. zingiberensis* was identified as disogenin, a steroidal sapogenin present majorly in the rhizome. Disogenin is an important

precursor for synthesis of steroidal hormones and exhibit multiple pharmacological properties like anti-helminthic, anti-inflammatory, neuroprotective, anti-thrombosis, cardioprotective and anti-allergic. (Feng et al. 2018; Zhang et al. 2018).

Farnesyl pyrophosphate synthase is an important enzyme required in the diosgenin biosynthetic pathway. It is required for the synthesis of E-isomer farnesyl pyrophosphate (FPP). Feng et al. (2018) applied CRISPR/Cas9 editing method for mutating farnesyl pyrophosphate synthase encoding gene of *D. zingiberensis* (*Dzfps*). Expression constructs were transformed using *Agrobacterium*-mediated transformation, and decline in *Dzfps* transcripts and squalene (an intermediate in disogenin biosynthesis pathway formed after condensation of 2 FPP molecules) content was detected in the transgenic plants in comparison with wild type plants. CRISPR/Cas9 system created deletions in the target gene (*Dzfps*) with the mutation rate of 60% (Feng et al. 2018).

3.4 Nicotiana tabacum

N. tabacum is an herbaceous plant that belongs to the family Solanaceae. It is the main source of tobacco. The main secondary metabolites are alkaloids, terpenoids, flavonoids and phenylpropanoids. There are various reports of molecular farming of *N. tabacum* and its associated species for the production of recombinant proteins like xylanase, proteases, glycoenzyme α-galactosidase, glycohormone erythropoietin-EPO, IgG antibody VRC 01 and many more that have substantial implication in pharmaceutical and industrial sectors (Dey 2021). CRISPR/Cas9 technology has been effectively applied in BY-2 suspension cells of *N. tabacum* to knock down the *XylT*-β (1,2)-xylosyltransferase gene and *FucT*-α(1,3)-fucosyltransferase gene so as to inactivate them and produce glycoproteins without having plant-specific glycans (Mercx et al. 2017). Schachtsiek and Stehle (2019) also reported the non-transgenic nicotine-free tobacco by targeting *BBL* (berberine bridge enzyme-like) genes via CRISPR/Cas9 tool.

3.5 Papaver somniferum

Papaver somniferum commonly known as opium poppy belongs to the family Papaveraceae. Therapeutically it is used as tranquilizer, sedative, analgesic, for treatment of headache, asthma, cough etc. (Masihuddin et al. 2018). The major phytochemical responsible for its narcotic and analgesic actions is an alkaloid. It has different types of medicinally important alkaloids, one of which is benzylisoquinoline alkaloids (BIAs). BIAs consists of morphine (analgesic), papaverine (vasodilator), sanguinarine (antimicrobial), codeine (antitussive) and noscapine (anticancer) (Hagel and Facchini 2013; Singh and Sharma 2020).

Using type II CRISPR/SpCas9 system via non-homologous end-joining genome repair, *4'OMT2* (coding for 3-hydroxy-N-methylcoclaurine 4′–methyltransferase protein) gene important for regulating BIA biosynthesis in *P. somniferum* was targeted. Leaf tissue was infiltrated with Cas9 and sgRNAs (synthetic and viral based) constructs using *Agrobacterium*-mediated transformation. InDel sequence analysis was performed to detect mutation in the clones. Transgenic plants displayed decline in BIA (e.g. noscapine, morphine, thebaine, S-reticuline) levels suggesting efficient knock out of *4'OMT2* gene (Alagoz et al. 2016).

3.6 Salvia miltiorrhiza

Salvia miltiorrhiza, a medicinal herb commonly called as Danshen or Chinese red sage, belongs to the family Lamiaceae. Dried roots of *S. miltiorrhiza* have been used to cure cardiovascular diseases, cerebral infarction, blood stasis relief and to control the blood flow (Lin and Hsieh 2010). The major phytochemicals in *S. miltiorrhiza* include lipophilic compounds called as diterpenoids (e.g. tanshinones) and phenolic acids (water soluble) namely salvianolic acid, lithospermic acid, flavonoids and rosmarinic acid (RA) (Chun-Yan et al. 2015). These compounds have been shown to confer various pharmacological properties (antioxidant, cardioprotective, anti-inflammatory, antifibrotic etc.; Wu and Wang 2012; Chun-Yan et al. 2015).

Using CRISPR/Cas9 genome editing technology, Li et al. (2017) targeted *S. miltiorrhiza* diterpene synthase gene (*SmCPS1*, a gene involved in tanshinone biosynthetic pathway). *Agrobacterium rhizogenes*-mediated transformation was performed to deliver CRISPR/Cas9, and sgRNA expression vectors and homozygous (3) as well as chimeric mutants (8) were generated. In the homozygous mutants, tanshinone I, tanshinone IIA and cryptotanshinone could not be detected, while in the chimeric mutants, measurable but reduced levels was observed (Li et al. 2017).

In another report, *S. miltiorrhiza* rosmarinic acid synthase gene (*SmRAS*), important for the synthesis of phenolic acids, was edited using CRISPR/Cas9 genome editing system. RAS enzyme has been shown to be a crucial enzyme in RA biosynthesis pathway and it results in the build-up of lithospermic acid B (LAB). *Agrobacterium rhizogenes*-mediated transformation of constructs generated 2 heterozygous, 5 biallelic, and 1 homozygous mutant. HPLC–MS/MS analysis of the transgenic hairy roots showed decline in *RAS* gene expression and RA and LAB levels and increase in 3,4-dihydroxyphenyllactic acid (RA precursor) content (Zhou et al. 2018). Laccase enzyme has been reported to play a crucial role in the conversion of RA to salvianolic acid B (SAB). Using CRISPR/Cas9 genome editing technology, Zhou et al. (2021) generated mutant of laccase genes (*SmLACs*) by targeting the conserved domains. They observed decline in the expression of laccase genes along with decrease in RA and SAB levels in the edited *S. miltiorrhiza* transgenic lines (Zhou et al. 2021).

From all these studies it can be inferred that CRISPR/Cas9 genome editing system is an effective platform to mutate genes, which will pave the way for

uncovering their novel functions. It can also help in generating new plant varieties with increased levels of valuable secondary metabolites.

4 Limitations Associated with Application of Genome Editing Techniques

With the help of GE techniques rapid progress in the improvement of plants is being achieved. For acceptance of any new technology adequate data regarding its efficiency and safety are essential. Therefore, it is also important to address the limitations in order to find ways to overcome them and enhance the efficiency of genome editing in plants. Some of the limitations of applications of the genome editing techniques are discussed below.

4.1 Off-Target Effects

Genome editing techniques target the genes efficiently but they are confronted with the generation of unwanted mutations at sites in the genome which are not actually targeted (off-target) questioning the usage of these techniques for therapeutic applications. Off-target edit is caused due to non-specific and unintentional mutation caused at the untargeted region because of the sequence resemblance to targeted region (Graham et al. 2020). These unintended mutations can lead to undesirable changes in the genome-edited plants. Significant research has been done to minimize the effects of off-target edits like improvement in sgRNA design approaches, base editing, controlling the Cas9-sgRNA concentration and delivery of ribonucleoprotein (reviewed by Zhang et al. 2015; El-Mounadi et al. 2020; Dey 2021).

4.2 Absence of an Efficient Transformation and Regeneration System

In medicinal plants, genes have been amended using CRISPR/Cas9 genome editing methodology via *Agrobacterium*-mediated transformation. However, for numerous medicinal plants, an efficient genetic transformation and regeneration system in tissue culture has not been established. The other challenges include prolonged tissue culture methods, low transformation efficiency and induction of somaclonal variations. To overcome these limitations alternative platforms like use of ribonucleoproteins (DNA free editing system) and viral vectors or in vitro transcription, magnetofaction etc. for efficient direct delivery of Cas9 or sgRNA expression constructs needs to be explored (Manghwar et al. 2019; Mao et al. 2019; Vats et al. 2019).

4.3 Challenges with Polyploid Genomes

Due to large genome size, heterozygous nature and multiple sets of chromosomes in plants having polyploid genomes, site-directed mutagenesis becomes a challenging task. Also in polyploids, it is difficult to generate knock-out mutants of homologous genes having high sequence similarity, and a large number of constructs need to be designed to edit the multiple copies of target genes present (Vats et al. 2019). Codon optimization of Cas9 protein, guide RNA designing, use of efficient promoters and GC content optimization of the target sequence can be manipulated to increase mutagenic efficiency in polyploid plants (Zaman et al. 2019; Schaart et al. 2021).

4.4 Precise Editing of the Genome

CRISPR/Cas9 technique mutates the genes at specific sites by generating DSBs that are repaired by NHEJ or in some cases via HDR. NHEJ produces InDels (insertions and deletions) leading to frameshift mutation in the target gene which ultimately abolishes its function. NHEJ is error prone, so the accuracy is less. HDR repair pathway also results in insertions and substitutions but it is associated with the drawbacks of low efficiency, selection marker re-deletion, positive selection problems and it fails to detect biallelic targets also (Xu et al. 2020a). Other efficient techniques for accurate and precise target genome editing techniques like base editing (Rees and Liu 2018) and prime editing (search and replace genome editing; Anzalone et al. 2019) need to be explored and employed.

4.5 Genome-Edited Plants vs. Genetically Modified Organisms

Despite several advantages of genome editing techniques, they are associated with certain biosafety issues (Yang 2020). There is an on-going debate whether genome-edited plants should be considered equivalent to genetically modified organisms (GMOs; transgenic plants). Medicinal plants have also been genetically engineered for synthesis of secondary metabolites which are important as food supplements, therapeutic products, pigments, insecticides etc. (Siahsar et al. 2011; Cardoso et al. 2019). Though genetically engineered medicinal plants have been utilized in diverse fields, still food and environment safety have become the topics of debate and strict, extensive regulatory measures have been levied on GMOs. People have debated that genome editing platforms should not be considered the same as GMOs. Firstly because, the mutations generated by genome editing techniques are small indels as compared to large gene fragments transformed in case of GMOs. Such mutations

(indels) are often found in naturally grown plants. Secondly, transgene free gene-edited plants can also be generated (Mao et al. 2019).

5 Regulatory Measures for Gene-Edited Plants

Genome editing does not pose any significant threat to human health, environment and economy according to the majority of experts (Lassoued et al. 2019). As discussed in the previous sections, realistic scientific regulatory approaches should be designed to facilitate the release of these edited plants easily and rapidly. With an increase in number of genome-edited plants, the global scenario of regulations will also continue to evolve and vary in different countries. The main question remains the same if they should be included in the category of GMOs. Market-oriented genome-edited plants are ready in many countries but many of them have no clear regulatory measures to be followed.

United States Department of Agriculture (USDA) has relatively lenient regulations for cases wherein exogenous transgene is not introduced into the genome of the GMO. Gene-edited plants if included in this category could evade strict, cost-extensive and time-consuming regulatory obstacles. The USA released a CRISPR-edited, browning-resistant white mushroom (*Agaricus bisporus*) in the market without any regulations (Waltz 2016). Similarly, CRISPR-edited crops namely maize, camelina, *Setaria viridis* (bristlegrass) and *Glycine max* (soybean) have also been released in the US market (Jaganathan et al. 2018).

Based on the Japanese Cartagena Act, Japanese government does not include products obtained from genome-edited plants (side-directed nuclease-1; SDN-1 type modification) under "living modified organisms" (Tsuda et al. 2019). The overall regulatory status of genome amended crop plants in Argentina (Lema 2019), Canada (Ellens et al. 2019), Australia (Mallapaty 2019) and other countries seems to be optimistic (Braddick and Ramarohetra 2020; Menz et al. 2020).

The European Court of Justice in July 2018 ruled a controversial decision that gene-edited plants should be approved as per the rigorous regulations designed for GMOs only (Court of Justice of the European Union 2018; Hjort et al. 2021). Department of biotechnology, Ministry of Science and Technology, Government of India (2020) has also drafted regulatory guidelines and framework for the approval of plants developed using genome-editing techniques. They have proposed a tiered approach for categorizing the genome-edited organisms into three groups. Group I includes organisms with single or few base pair edits or deletions as in SDN-1. Group II harbours organisms with few/several base pair edits like SDN2, while group III combines the organisms with synthetic/foreign DNA as in SDN-3. Risk assessment in Group I and Group II organisms will be less stringent when compared with group III organisms.

Therefore, the existing regulatory measures for GMOs should not be imposed on gene-edited plants as their modification system is different. If required, there is need

to draft policy measures wherein gene-edited plants should be accepted without following strict norms which might cause delay in their applicability.

6 Conclusions and Future Perspectives

The on-going COVID pandemic has taught the world that in order to satisfy the medical demands of the ever-growing human population; alternate strategies for strengthening the healthcare sector are essential. Many synthetic medicines are available in the market but their consumption is associated with side effects. Medicinal plants synthesize a huge array of phytochemicals which have been used as natural therapies for curing many diseases. Over the last few years, different methodologies have been designed to enhance the production of these phytochemicals from medicinal plants. Genome editing techniques (meganucleases, ZFN, ODM, TALENs), especially CRISPR/Cas, have proved useful to silence or mutate one or more genes involved in secondary metabolite synthetic pathways and thus opening exciting avenues for phytochemical engineering in medicinal plants. These techniques have several advantages over the conventional plant breeding and genetic engineering techniques as they are rapid, less laborious, specific and easy to implement.

However, till date genomes/genes of limited medicinal plants have been manipulated using genome editing techniques. Therefore, facilitating research in this direction will help in harnessing the complete potential of these techniques for identifying novel genes of complex secondary metabolic pathways which will ultimately impact the mankind. Engineering of metabolic pathways necessitates the simultaneous editing of multiple genes. CRISPR/Cas9 system has the benefit of multiplexing, making it an ideal candidate for multiple target sites manipulation. One of the most common and effective approaches to study gene function is to generate gene knock-outs. Using genome editing techniques, genome-wide mutant libraries of respective medicinal plants can be made, which will be of immense significance in assigning gene functions. Genome editing techniques can also be used to regulate the expression of genes, which will further help in the dissection of different mechanisms for medicinal plant improvement.

Another aspect of research that needs to be addressed to fully exploit genome editing techniques is the availability of genome sequences of medicinal plants. Till date, complete genome sequences of very few medicinal plants are available posing a challenge for designing genome editing approaches for secondary metabolite production. Therefore, more medicinal plant genomes need to be sequenced and for that dedicated funding is also required. Simultaneously new bioinformatics tools should be developed for identification of genes of biosynthetic pathways for production of therapeutic phytoconstituents. There is also a requirement for an effective plant transformation system for delivering genome-edited expression constructs as well as in vitro regeneration system for generating gene-edited medicinal plants. Also, vector/transgene less editing should be given preference to avoid stringent

regulatory hurdles that might delay their release in the market. The strategy of subcellular morphology transformation (similar to using CRISPR/Cas9 technique for increasing the production of terpenoids in yeast, by expanding the endoplasmic reticulum, by Arendt et al. (2017)) can also be employed to medicinal plants for enhancing the synthesis of important phytochemicals. Amalgamation of artificial polyploidy and CRISPR/Cas9 gene editing via *Agrobacterium rhizogenes*-mediated transformation of plant hairy roots as proposed by Niazian (2019) can be an ideal approach to enhance the synthesis of medicinal plant-derived bioactive compounds. These proficient research approaches will help in generating new plant varieties with increased concentration of pharmaceutically relevant phytoconstituents and will also help in elucidating their metabolic networks for further research.

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Chapter 23
Genome Editing in Medicinal Plants Using CRISPR/Cas9 Tool

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1 Introduction

Medicinal plants are rich sources of bioactive organic compounds called phytochemicals that can be used for therapeutic purposes, and act as potential precursors for drug synthesis. Medicinal plants offer a diverse range of pharmacological effects, which depends on the presence of phytochemical constituents in one or more organs of the plant. The therapeutic properties of these phytochemical substances are scientifically established in diverse medicinal plants and are proven to play a defensive role in various chronic diseases (Hussein and El-Anssary 2019). Understanding the plant chemical composition is critical to determine the medicinal value. Plant phytochemical substances constitute primary metabolites that are involved in the basic plant metabolism and secondary metabolites are the metabolic intermediates that offer specialized functions. Secondary metabolites are known to have various biological properties such as anti-biotic, anti-fungal, and anti-viral effects. Since ancient times, they are known to play a significant role in traditional and herbal

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M. K. Swamy, A. Kumar (eds.), *Phytochemical Genomics*,
https://doi.org/10.1007/978-981-19-5779-6_23

medicine in alleviating various diseases (Makkar and Becker 2009). Studies on these potential pharmacological compounds in modern medicine have resulted in the production of drugs to treat diverse human diseases from migraines to cancer (Hussein and El-Anssary 2019). Secondary metabolites from the medicinal plants are used in the drugs to treat various diseases like diabetes, liver disease, HIV, typhoid, malaria, arthritis, cancer, and even COVID19 (Oladeji 2016; Bhat 1995; Greenwell and Rahman 2015; Choudhary et al. 2015; Bhuiyan et al. 2020).

Every year, consumer preference for traditional medicine is increasing, and this increase can be appreciated from the global herbal market. The global trade of medicinal and aromatic plants is US$800 million per year and is expected to increase with an estimated value of US$50 trillion by 2050 (Volenzo and Odiyo 2020). Although their global market is huge, a vast amount of medicinal plants is unexplored and not documented yet. In addition, a bulk amount of the material trade is from the wild-harvested sources, and only a very small number of species are cultivated (Di Pierro et al. 2012). Moreover, several environmental factors including biotic and abiotic stress affect the production of secondary metabolites in medicinal plants. Although plant in vitro culture technology is being used for the production of phytochemical secondary metabolites, it has various limitations including the higher cost and the laborious establishment of step-to-step protocols for biomass and medicinal compounds (Cardoso et al. 2019). Genetic manipulation of hairy roots provides an alternative method for secondary metabolite production; however, it is limited by the unavailability of transformation protocols for various plants (Hussain et al. 2012). Hence, the commercial production of secondary metabolites with medicinal value by conventional methods, or tissue culture and genetic transformation methods are not adequate for the increasing demand. Alternative advanced high-throughput technologies are required for the efficient production of phytochemical medicinal compounds to meet the high demand in pharmaceutical industries and to improve the quality and quantity. Recent genome editing technologies are proved to be a potential tool to alter the targeted site in the genome and thereby emerged as a promising tool to engineer the metabolic pathways for the secondary metabolite production in medicinal plants.

Genome editing technologies including transcription activator-like effector nucleases (TALENS), zinc-finger nucleases (ZFNs), and clustered regularly interspaced short palindromic repeats/CRISPR associated9 (CRISPR/Cas9) can be employed to effectively harness the plant genome by precise alteration of DNA sequences. However, TALENS and ZFNs have the limitations of limited efficiency and technical complexities (Akinyemi et al. 2018). CRISPR/Cas9 is a simple, highly efficient, and robust technology that has greatly advanced metabolic and pathway engineering thereby improving the quality and production of secondary metabolites. CRISPR/Cas9 system is originally an adaptive immune system in prokaryotes protecting from DNA viruses by specifically cleaving the double-stranded DNA. This CRISPR/Cas9 is a precise site-directed mutagenesis tool by introduces small heritable mutations for RNA-guided genome editing. CRISPR/Cas9 has been successfully employed in various crops to improve yield and stress tolerance. The use of CRISPR/Cas9 has been limited to only a few medicinal plants due to the lack of

sufficient whole-genome sequence information in many medicinal plants. However, recently various secondary metabolic pathways in medicinal plants have been engineered by CRISPR/Cas9 facilitating the production and quality of pharmaceuticals (Dey 2021). CRISPR/Cas9 has the potential of being an efficient tool for engineering customized medicinal plants for the promising medicinally important secondary metabolite production (Niazian 2019). This chapter presents the application of the CRISPR/Cas9 system in medicinal plants as a potential genome editing tool to improve the quality and production of secondary metabolites.

2 Importance of Bioactive Compounds in Medicinal Plants and the Effect of Biotic and Abiotic Stress on Secondary Metabolite Production

Medicinal plants have long been used as traditional medicines to treat numerous diseases with important chemical compounds derived from medicinal plants (Tavakoli et al. 2021). Compounds derived from the metabolic pathways in the leaves, stems, and roots of medicinal plants are used in the pharmaceutical industry to manufacture drugs (Zehra et al. 2019). A wide range of phytochemicals with their diverse bioactivities present in the medicinal plants enable a single medicinal plant with multiple uses where the bioactive compounds extracted from the different organs of the plant are often used to treat different diseases (Dawurung et al. 2021). Several plant-derived drugs, semi-synthetic and synthetic drugs based on secondary metabolites have been manufactured. Various examples of these plant-based drugs such as the origin of the analgesic activity of aspirin are from the plant genera *Salix spp* and *Populous spp*, which is related to salicin. Other examples with a potential impact on medicine include the paclitaxel (Taxol), isolated from the bark of the Pacific yew tree *Taxus bravifolia* (Taxaceae) used in the treatment of refractory ovarian cancer (Littleton 2007). Hypericin and pseudohypericin are the two compounds isolated from *Hypericum perforatum* (Guttiferae), which exhibited activity against various retroviruses including HIV. These compounds are significantly important due to their role in the stabilization of the structure of HIV capsid and the inhibition of the release of reverse transcriptase, thereby preventing the uncoating process (Mukherjee 2019). Some of the medicinally important secondary metabolites from various medicinal plants are listed in Table. 23.1.

Secondary metabolites play a key role in the adaptation of plants to changing environments and stress conditions. However, the production of these phytochemical compounds is affected by both biotic and abiotic stresses. Severe environmental factors such as biotic and abiotic stress conditions severely affect the metabolic pathways responsible for the production of secondary metabolites (Bohnert et al. 1995). Medicinal plants exposed to biotic and abiotic stress show the initial increase in secondary metabolite production as a part of defense mechanism; however, the production of secondary metabolites gradually decreases with the increased levels of

Table 23.1 List of some medicinally important secondary metabolites from various medicinal plants and their medicinal uses

S. No	Medicinal plant name	Compound name	Medicinal uses	References
1	*Catharanthus roseus*	Vincristine	Anticancer, lymphoblastic leukemia, and rhabdomyosarcoma	Gutierrez-Camino et al. (2018)
2	*Leonurus sibiricus* L	Chlorogenic acid, caffeic acid, and ferulic acid	Antimicrobial potential and cytotoxic activity	Sitarek et al. (2018)
3	*Artemisia annua*	Artemisinin	Fevers, inflammation, headaches, bleeding, and malaria	Liu et al. (1999)
4	*Salvia miltiorrhiza*	Tanshinone and phenolic acid	Cardiovascular and cerebrovascular diseases	Jiang et al. (2019)
5	*Panax ginseng*	Ginsenoside	Antioxidant and anti-inflammatory effects	Zhang et al. (2004)
6	*Catharantus roseus*	Terpenoid and indole alkaloids	Antidiabetic, bactericide, and antihypertensive	Almagro et al. (2015)
7	*Ocimum basilicum*	Rosmarinic acid	Asthma, multicellular inflammation, and cough	Bais et al. (2002)
8	*Tanacetum parthenium*	Diacetylene	Polymorphonuclear leukocyte	Brown et al. (1997)
9	*Aconitum napellus*	Aconitine	analgesic	Michael (2015)
10	*Atropa belladonna*	L-hyoscyamine	Parasympathomimetic	Michael (2015)
11	*Camptotheca acuminate*	Camptothecin	Tumor therapy	Michael (2015)
12	*Lactuca virosa*	Sesquiterpene lactones	Used in ailments, burns, diarrhea, and influenza	Selen et al. (2020)
13	*Cinchona pubescens*	Quinidine	Antiarrhythmic	Michael (2015)
14	*Coffea arabica*	Caffeine	Act as a stimulant	Michael (2015)
15	*Colchicum autumnale*	Colchicine	Gout treatment	Michael (2015)
16	*Cytisus scoparius*	Sparteine	Antiarrhythmic	Michael (2015)
17	*Silybum marianum*	Silymarin	Protecting liver against snakebite, insect stings, and mushroom poisoning	Karimi et al. (2011)
18	*Erythoxylum coca*	Tropane alkaloids	Asthma, chronic bronchitis, pain, and flu symptoms	Kathrin and Kayser (2019)
19	*Withania somnifera*	Withanolides	Asthma, diabetes, hypertension, stress, arthritic diseases, and cancer	Narendra et al. (2011)

(continued)

Table 23.1 (continued)

S. No	Medicinal plant name	Compound name	Medicinal uses	References
20	*Catharanthus roseus*	Vinblastine	Anticancer and relieving muscle pain	Gupta et al. (2017)
	Cichorium intybus	Sesquiterpenoids and hyoscyamine	Cardiovascular disease and cancer	Chadwick et al. (2013)
21	*Datura stramonium*	Hyoscyamine, atropine, and scopolamine	Anticholinergic syndrome	Sebastian et al. (2017)
22	*Colubrina asiatica*	Asiaticoside	Treatment of various skin conditions such as leprosy, lupus, varicose ulcers, eczema, psoriasis, diarrhea, fever, and amenorrhea diseases	Kashmira et al. (2010)
23	*Digitalis lanata*	Digitoxin and digoxin	heart insufficiency	Michael (2015)
24	*Andrographis paniculata*	Andrographolide	Anti-inflammation, anti-cancer, anti-obesity, and antidiabetes	Yan et al. (2019)
25	*Salvia sclarea*	Diterpenoid and triterpenoid	Ulcers, gout, diarrhea inflammation, and rheumatism	Bonitoa et al. (2011)
26	*Echinacea purpurea*	Caffeic acid derivatives	Reduce inflammation, anti-cancer, and reduce diabetes	Murthy et al. (2014)
27	*Digitalis lanata*	Cardenolides	Used in the treatment of heart diseases	Morsy (2016)
28	*Solanum aculeatissimum*	Steroidal saponin	Anti-inflammatory, antitumor, antidiabetic, antifungal, and antibacterial	Kohara et al. (2005)
29	*Astragalus membranaceus*	Astragalosides	Treatment of viral & bacterial infections, Anti-aging, inflammation, as well as cancer.	Zhang et al. (2020)
30	*Fagopyrum tataricum*	Rutin	Antitumor, antioxidant, anti-inflammatory, hepatoprotective, anti-diabetic activities,	Rui et al. (2016)
31	*Ambrosia artemisiifolia*	Thiarubrine	Cytotoxic, antimicrobial, antiviral, molluscicide, antiprotozoal, and hepatoprotective	Kiss et al. (2012)
32	*Chondrodendron tomentosum*	Tubocurarine	Muscle relaxant	Michael (2015)

(continued)

Table 23.1 (continued)

S. No	Medicinal plant name	Compound name	Medicinal uses	References
33	*Lycopodium clavatum*	Huperzine A	Alzheimer treatment	Michael (2015)
34	*Physostigma venenosum*	Physostigmine	Alzheimer treatment	Michael (2015)
35	*Pilocarpus joborandi*	Pilocarpine	Glaucoma treatment	Michael (2015)
36	*Psychotria ipecacuanha*	Emetine	Treatment of amebae infections	Michael (2015)
37	*Strophantus gratus*	Ouabain	Heart insufficiency	Michael (2015)
38	*Taxus brevifolia*	Paclitaxel (Taxol)	Tumor therapy	Michael (2015)
39	*Cannabis sativa*	Tetrahydrocannabinol	Analgesic	Michael (2015)
40	*Catharanthus roseus*	Dimeric Vinca alkaloids	Tumor therapy	Michael (2015)
41	*Ginkgo biloba*	Ginkgolide	Anti-inflammatory, anticancer	Chandra et al. (2020)
42	*Hypericum perforatum*	Hypericin, Hyperforin	Antidepressants and antioxidant	Zanoli (2004)
43	Boraginaceae	Rosmarinic acid	Anti-inflammatory agent	Luo et al. (2020)
44	*Camelina sativa*	ω-3 fatty acid, PUFA	Anti-inflammatory disease severe wound healing and heart disease	Campos et al. (2013)
45	*Dendrobium officinale*	Alkaloids,phenanthrenes, glycoside	Cardioprotective, antitumor, gastrointestinal protective, anti-diabetes	Chen et al. (2021)
46	*Dioscorea Zingibe rensis*	Diosgenin (steroidal saponin)	Anticancer, hypercholesterolemia, inflammation	Jesus et al. (2016)
47	*Ocimum basilicum,* and *Salvia miltiorrhiza*	Rosaminic acid	Anti-inflammatory and anti-apoptotic	Luo et al. (2020)
48	*Lithospermum ruderale and Salvia miltiorrhiza*	Lithospermic acid	Antioxidant, cardiovascular diseases, and hepatitis	Andrey et al. (2020)
49	*Vitisvinifera hairy roots*	Stilbenoids are non-flavonoid	Anti-microbial, antifungal, and cardioprotective	Koh et al. (2021)
50	*Salvia miltiorrhiza*	Tanshinone	Involves in arrhythmic effects and protection against ischemia perfusion	Li et al. (2017)

(continued)

Table 23.1 (continued)

S. No	Medicinal plant name	Compound name	Medicinal uses	References
51	*Dioscorea zingiberensis*	Squalene	Used as adjunctive therapy in a variety of cancers	Kelly (1999)
52	*Erythroxylum coca*	Cocaine	Analgesic and stimulant	Michael (2015)
53	*Galanthus woronowii*	Galanthamine	Alzheimer treatment	Michael (2015)
54	*Papaver somniferum* (Opium poppy)	Benzyl isoquinoline alkaloids	Antiparasitic and antimalaria	Rubio-Pina and Vazquez-Flota (2013)
55	*Papaver somniferum*	Morphine	Anti-oxidant, antimutagenic, anticarcinogenic effects, and antiinflammatory	Hao et al. (2015)
56	*Chelidonium majus*	Celidonine	Anti-jaundice, and pain-relieving	Sylwia et al. (2018)
57	*Piper methysticum*	Kavain	Anti-microbial, and antiinflammation	Meenakshi (2019)
58	*Pilocarpus jaborandi*	Pilocarpine	Treatment of glaucoma	Cho et al. (2013)
59	*Atropa belladonna*	Atropine	Anti-cholinergic	Rajput (2013)
60	*Nicotiana tabaccum*	Nicotine	It activates the brain, nervous system, treats headache, and sinusitis	Charlton (2004)
61	*Zingiber officinalis*	Ginger	Treating nausea, dysentery, heartburn, and flatulence	Ann and Zigang (2011)
62	*Curcuma longa*	Turmeric	Anti-inflammatory, liver diseases, skin cancer, smallpox, and chickenpox	Prasad and Aggarwal (2011)
63	*Carica papaya*	Papain	Anti-inflammatory drug	Tarun and Yash (2015)
64	*Piper methysticum*	Shikimic acid	Treatment of influenza A & B	Amalia and Ramón (2012)
65	*Rauvolfia reserpina*	Reserpine	Hypertonia treatment	Michael (2015)
66	*Sanguinaria canadensis*	Sanguinarine	Antibacterial, and antiviral	Michael (2015)

stress. Drought, salinity, high temperature, floods, and low temperature are the environmental conditions that adversely affect secondary metabolite production (Ramakrishna and Ravishankar 2011; Isah 2019; Jan et al. 2021). Water deficit stress is the major factor that influences secondary metabolite production. Plants

exposed to water deficit stress show decreased water content in the cell leading to decreased metabolism and thereby resulting in the lesser secondary metabolite production (Ramakrishna and Ravishankar 2011; Indrajeet and Rajesh Kumar 2018). Similarly, salt stress causes dehydration reducing the water content in the cytoplasm and vacuole leading to decreased secondary metabolite production. Since the medicinal plants grow in diverse environmental conditions, biotic and abiotic stresses are the major global factors that affects the production of the medicinally important phytoactive compounds useful for the pharmaceutical industry (Ramakrishna and Ravishankar 2011; Indrajeet and Rajesh Kumar 2018; Isah 2019) (Table 23.2).

3 CRISPR/Cas9 Mechanism

CRISPR/Cas9 is a robust, breakthrough technology that enables the generation of the desired heritable mutations in a specific site in the plant genome. CRISPR has the potential to modify the target trait of interest in a site-specific manner in a span of a few generations in contrast to traditional breeding (Ma et al. 2016). CRISPR offers various advantages such as the genomic alterations that are stable and are heritable to offspring, which is a key factor for plant breeding. Other advantages include the complete knockout of the target gene, and a partial knockout can be achieved by dosage difference in RNAi (Liu et al. 2016). CRISPR/Cas9 mediates genome editing by inducing DNA-double-stranded breaks in the genome (Fig. 23.1). Guide RNA (gRNA) and Cas9 protein together form a complex in CRISPR/Cass9 system. The gRNA sequence of the CRISPR/Cas9 system consists of 20 nucleotides that are complementary to the target DNA. The Cas9 protein component constitutes the catalytic activity that can induce cuts in the double-stranded DNA. gRNA is a synthetic fusion of the bacterial CRISPR RNA (CrRNA) and trans-activating CRISPR RNA (traRNA) (Shalem et al. 2015). Type II CRISPR system contains the combination of CrRNA and traRNA along with Cas9 protein which is being widely used in recent research. SPACER is a small sequence which is attached adjacent to gRNA through which cas9 binds to the gRNA, which guides the cas9 to induce cuts in the double-stranded DNA. The site where the cut is introduced is called as PAM site. Cas9 induces cuts in the double-stranded DNA immediately after the formation of gRNA and Cas9 complex (Hille and Charpentier 2016; Tang et al. 2019). Undesired nucleotides present near the double-stranded break modify the DNA sequence and/or are subsequently removed which hinder the following translation and protein synthesis leading to gene silencing. The double-stranded breaks are further repaired by either the non-homologous end joining (NHEJ) pathway or homology-directed repair (HDR) pathway (El-Mounadi et al. 2020). In the NHEJ repair pathway, the Ku protein attaches to the double-stranded DNA break ends, and further DNA protein kinase catalytic subunits (DNA-PKCs) and Artemis proteins together bind the DNA break ends to form the complex that facilitates the phosphorylation leading to DNA synthesis. This double-stranded DNA is further

Table 23.2 Application of CRISPR/Cas 9 system in the production/alteration of secondary metabolites in medicinal plants

S. No	Plant name	Gene name	Secondary metabolite	References
1	*Camelina sativa* (L.) Crantz	*FAD2*	ω-3 fatty acid (Oleic acid) PUFA	Jiang et al. (2017)
2	*Camelina sativa* (L.) Crantz	CsFAD2	ω-3 fatty acid (Oleic acid) PUFA	Morineau et al. (2017)
3	*Dendrobium officinale* kimura and Migo	*C3H*, *C4H*, *4CL*, *CCR*, and *IRX I*	Alkaloids, phenanthrenes, Polysacharides, bibenzyls, essential oils, and glycosides	Kui et al. (2017)
4	*Dioscorea zingiberensis* C. H. Wright	*Dzfps*	Diosgenin (Steroida l saponin) and squalene	Feng et al. (2018)
5	*Nicotiana tabacum* L.	Two *XylT* and four *FucT*	Alkaloids, flavonoids, terpenoids, phenylpropanoids and IgG2 antibodies	Mercx et al. (2017)
6	*Nicotiana tabacum* L.	*NtPDS* and *NtPDR6*	Alkaloids, flavonoids, terpenoids, phenylpropanoids	Gao et al. (2015)
7	*Papaver somniferum* L.	4′OMT2	Benzylisoquinoline alkaloids (BIAs)	Alagoz et al. (2016)
8	*Salvia miltiorrhiza* Bunge	*SmCPS1*	Phenolic acid, diterpenoids and tanshinone	Li et al. (2017)
9	*Salvia miltiorrhiza* Bunge	*SmRAS* gene	Phenolic acid *viz.* RA and lithospermic acid B	Zhou et al. (2018)
10	*Salvia miltiorrhiza*	*SmPAL*, *SmTAT*, *SmC4H*, and *SmRAS*	Production of lithospermic acid	Yang et al. (2017)
11	*Salvia miltiorrhiza*	*SmRAS*	Rosemaric acid and lithospermic acid	Zheng et al. (2018)
12	*Salvia miltiorrhiza*	*SmLAC*	The gradual reduction of the accumulation of RA, SAB, phenolic acid biosynthesis	Zhou et al. (2021)
13	*Solanum lycopersicum*	γ-aminobutyric acid pathway modified	GABA content	Li et al. (2018)
14	*Taraxacum* species.	*1-FTT*	More rubber content	Iaffaldanoa et al. (2016)
15	*Papaver somniferum* (Opium poppy)	*OMT2*	Reduction of BIAs	Yagiz et al. (2016)
16	*Camelina sativa*	CsFAD2	Low oleic acid production	Jiang et al. (2017)

converted into blunt-end DNA by DNA ligase (Mateos-Gomez et al. 2017). This repair pathway can introduce InDel mutations, causing the coding frameshift resulting in premature stop codon initiating the nonsense-mediated decay (NMD)

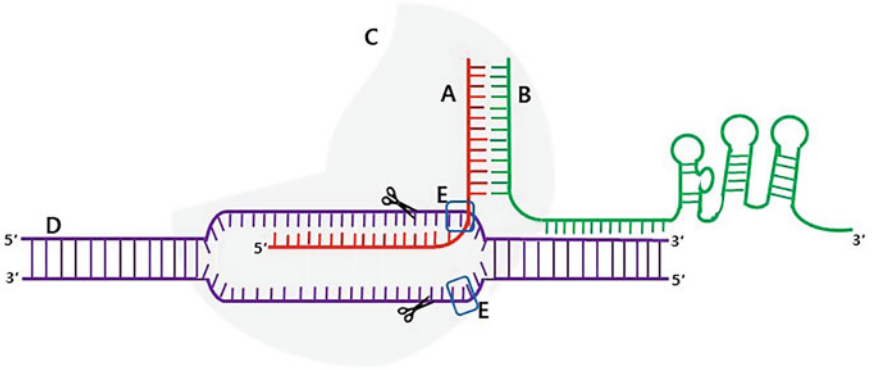

Fig. 23.1 Targeted genome editing by CRISPR/Cas9 system. Components involved include (**a**) crRNA; (**b**) tracrRNA; (**c**) Cas9 endonuclease; (**d**) double-stranded target DNA; (**e**) Protospacer adjacent motif (PAM) sequence. (Modified from Bhatta and Malla 2020)

of the transcript, inducing loss-of-function mutations (Bernheim et al. 2017; Tang et al. 2019). Whereas the HDR repair pathway uses homologous donor DNA sequences from sister chromatids or foreign DNA to create accurate insertion and substitution between DSBsites or two DSBs for further modifications. This repair mechanism occurs at the S and G2 phases of the cell cycle (Branzei and Foiani 2008). MRE11-Rad50-Nbs1 (MRN complex) complex binds at the 5′end of the double-strand break ends and forms the 3′ overhangs. Later the replication protein A (RPA) binds to the single-strand DNA to prevent the nuclease activity and recoiling of DNA (Symington 2014). RAD-51 protein is involved in the searching of homologous DNA and eventually leads to the invasion to complete the homology-directed repair (Tang et al. 2019).

4 Role of CRISPR/Cas9 in the Improvement of Bioactive Pharmaceutical Compound Production

Secondary metabolites or phytochemical compounds produced in medicinal plants can be used to treat various ailments in humans and to improve immunity against various diseases. The medicinal plants, hence, can also be called green chemical factories (Soltani et al. 2018; Andrey et al. 2020). Medicinal plants and plant products are being used as prolific sources of food, nutraceuticals, pharmaceuticals, and biomaterials for human beings. To improve and accelerate the production of these secondary metabolites which are the potential ingredients in drug manufacture, advanced genetic technologies such as CRISPR/Cas9 are highly essential. CRISPR/Cas9 facilitates the addition, deletion, or silencing of multiple genes in the plant genome leading to the development of the desirable novel traits. CRISPR/Cas9 has the potential of genome editing that enables the elucidation of secondary metabolite

pathways, increased production of secondary metabolites with improved quality. The phytochemical profile of the medicinal plants can be modulated through CRISPR/Cas9 system for efficient commercial production (Tang et al. 2017; Jan et al. 2021).

Downregulation or functional gene knockout or knock-in using the CRISPR technology not only reduces unwanted products but also induces desirable metabolic pathways to produce target secondary metabolites (Mercx et al. 2017; Shabir 2021). Usually, important secondary metabolites from plant parts is done through organogenesis in plant tissue culture media, which is later multiplied by fermentation technique, and finally compounds are analyzed by NMR, MVDA, and HPLC. Earlier various biotechnological applications have been used to modulate plant secondary metabolite pathways to increase the production of valuable secondary metabolites of therapeutic, dietary, and industrial use (Dey 2021). Similarly, after genome editing by the CRISPR system, explants are grown on suitable media for callus induction, which later undergoes the fermentation process and finally, quality analysis is performed (Zhou et al. 2021; Andrey et al. 2020).

Rosaminic acid (RA) is a water-soluble polyphenol that is abundantly present in the culinary herbs of *Ocimum basilicum* and *Ocimum tenuiflorum*. These are the esters of caffeic acid and 3,4-dihydroxy phenyl lactic acid and are present more in Lamiaceae and Boraginaceae families. These are used as a flavoring agent in foods and beverages and are used as an anti-inflammatory, anti-apoptotic, anti-tumorigenic agents (Luo et al. 2020). L-phenylalanine and L-tyrosine are the precursors of the RA accumulation pathways (Xiao et al. 2011). Such medicinally important RA can be produced in transgenic plants expressing *C4H* and *SmTAT* genes from *Salvia miltiorrhiza* by hairy root culture (Huang et al. 2009). Lithospermic acid (LA) is a polycyclic phenolic carboxylic acid extracted from the roots of the flowering plants *Lithospermum ruderale* and *S. miltiorrhiza* (Andrey et al. 2020). *MYC2* and helix loop helix transcription factors are involved in lithospermic acid production. Upregulation of *SmPAL*, *SmTAT*, *SmC4H*, and *SmRAS* genes resulted in the increased production of LA in the root system (Yang et al. 2017). LA is an antioxidant and is highly effective against cardiovascular diseases, hepatitis, and HIV-1nucleocapsid protein (Begum and Gogo 2020; Mori et al. 2020; Andrey et al. 2020).

Stilbenoids are non-flavonoid polyphenols, which are used to treat a wide variety of diseases. So far 60 Stilbenoids have been isolated from 28 Cyperaceae species of all parts of plants. These compounds are divided into two types based on dihydro benzofuran rings (Dávid et al. 2021). Overexpression of *PAL* and *STS* genes in *Vitis vinifera* hairy roots increases the production of stilbenoids, which possess antimicrobial, antifungal, and anti-inflammation activities as well as for cardioprotection, (Akinwumi et al. 2018; Koh et al. 2021). The *SmRAS* gene plays a key role in a water-soluble phenolic acid biosynthetic pathway. In 50% of transgenic plants, significant variations were observed in hairy roots due to genome editing by the CRISPR system. Further, analysis of secondary metabolites of these transgenic lines revealed that the synthesis of RA, LA, and ROS marinic acid gradually decreased and 3,4 dihydroxy phenyl lactic acid was increased (Zheng et al. 2018).

Laccase is a copper-containing glycol protein multifunctional gene related to monolignol oxidation. It is involved in cell elongation, lignin polymerization, and phenolic acid biosynthesis (Marcella et al. 2020; Balasubramanian et al. 2016). The slavonic acid B (SAB) and phenolic compounds, which are regulated by the *SmLAC* gene, are used to treat cardiovascular disease. The *SmLAC7* and *SmLAC20* genes are also involved in the synthesis of phenolic compounds that are used in the medicinal field. *SmLAC* gene knockout through the CRISPR system resulted in the gradual reduction of the accumulation of RA, SAB, phenolic acid biosynthesis, development of hairy roots, and lignin formation. This study suggested that these genes are responsible for the production of phenolic compounds which are of higher medicinal importance (Zhou et al. 2021). *S. miltiorrhiza* committed *diterpene synthase* (*SmCPS1)* gene is involved in tanshinone biosynthesis. CRISPR gene-edited plants showed both increased and decreased production of tanshinone. The tanshinone phenolic compounds exhibited pharmacological activities such as arrhythmic effects and protection against ischemia perfusion. It was observed that Geranylgeranyl diphosphate (GGPP) acts as a precursor for tanshinone and taxol production (Li et al. 2017).

N-glycan residues are usually linked to proteins produced by plants such as β-1,2-xylose and α-(1,3)-fucose, both of which cause immunogenicity and allergenicity in humans. These two glycan residues were hence silenced or knocked out by the CRISPR/Cas9 system to produce more pharmacologically important IgG2 antibodies in nicotine tobacco BY-2 cells. Gene-edited plants produced higher levels of IgG2 antibodies and the absence of N-glycans was confirmed by mass spectrophotometry and PCR (Mercx et al. 2017). Squalene is an organic compound produced as a biochemical intermediate involved in cancer therapy and it works as a natural antioxidant (Kelly 1999; Fatma 2013). The *DZfps* gene was mutated by the CRISPR system in *D. Zingiberensis* to reduce squalene content, and the squalene content produced by mutated plants was confirmed by gene expression and GC-MS analysis (Feng et al. 2018). Oleic acid is an omega fatty acid developed by gene editing used in the treatment of inflammation, severe wound healing, and heart disease (Campos et al. 2013). Mutant of *FAD2-2* gene in soybean caused the production of lesser oleic acid in some transgenic lines and more in some plants. (Al Amin et al. 2019). γ-aminobutyric acid is a non-proteinogenic amino acid that acts as an antioxidant, antimicrobial, and anti-inflammatory agent and is involved in the treatment of antidiabetes, (Ngo and Sang 2019). γ-aminobutyric acid pathway in tomatoes was modified via CRISPR/Cas9 to increase the GABA content (Li et al. 2018). *Fructan 1-fructosyltransferase* (*1-FTT*) gene is modified by using CRISPR/Cas9 technology for the production of more rubber content (Iaffaldanoa et al. 2016). Benzyl isoquinoline alkaloids (BIAs) are the secondary metabolites used in the pharmacological industry as antiparasitic and antimalarial agents (Rubio-Pina and Vazquez-Flota 2013). Knockout of *4 OMT2* gene in the opium poppy resulted in the complete reduction of BIAs content in mutant lines (Yagiz et al. 2016). The *Camelina sativa* FAD2 (CsFAD2) gene was modified by CRISPR/Cas9 in *Camelina sativa,* which resulted in low oleic acid production (Jiang et al. 2017).

Agrobacterium rhizogenesis is used to increase the production of secondary metabolites in medicinal plants. Totally 31 alkaloids including 8 isomers were identified in the hairy root culture of *R. stricta* and extracted metabolites from these plants were analyzed by GC-MS and HPLC. Similarly, *Papaver bracteatum* is a medicinal plant with alkaloids like baine, codeine, and morphine; noscapine and papaverine are extracted from this plant (Rehman et al. 2021; Akhgari et al. 2015). Fatty acids such as palmitic acid, palmitoleic acid, stearic acid, and oleic acid are produced in higher amounts by gene knockout in *Camelina sativa* by CRISPR/Cas9. These compounds play a crucial role in the pharmacological industry (Lyzenga et al. 2019). The *C3H*, *C4H*, *4CL*, *CCR*, and *IRX* gene knockouts through CRISPR/Cas9 in *D.officinale* resulted in the mutation in lingo cellulose biosynthetic pathway leading to the development of novel orchid variety (Kui et al. 2017). These phytocompounds are used as antioxidants, renoprotective, and gastroprotective. The 6 glycosyltransferase genes in *Nicotiana* were modified by CRISPR/Cas9 to produce a core-1, 3-fucose and β-1,2-xylose-free recombinant protein (Jansing et al. 2019).

5 Limitations of CRISPR/Cas9 in Medicinal Plants

Although genome editing by CRISPR/Cas9 is an efficient and robust technology in diverse organisms, it is hindered by a few limitations in medicinal plant research. Often, off-target mutations which resemble the on-target effects result in the limiting of this technology for therapeutic purposes (Iaffaldanoa et al. 2016). Insertion of random Indels at the target locus occurs in many of the CRISPR edited plants due to the error-prone nature of the NHEJ repair pathway (Feng et al. 2013). Lower transfection efficiency is a major drawback of CRISPR/Cas9, limiting its application, owing to the lack of an efficient transfection system (Dey 2021). The unavailability of transformation and regeneration protocols and also the genetic and molecular resources such as molecular markers, vectors, and promoters in most of the medicinal plants restricts its usage (Altpeter et al. 2016). Besides these, the polyploid and unstable genomes of medicinal plants with huge genome size, copy number, and lack of information of genome sequence are the major bottlenecks of wider application of CRISPR/Cas9 genome editing for the improvement of valuable secondary metabolites in medicinal plants (Vats et al. 2019; Hoff et al. 2018).

6 Conclusions and Future Perspectives

Medicinal plants possessing highly valuable pharmacological and nutraceutical compounds require a deeper understanding of secondary metabolite pathways and upscaling of these important metabolites, which can be achieved through the

potential CRISPR/Cas9 genome editing. CRISPR/Cas9 system is a powerful technology that has evolved to achieve the desired results by modifying the genome of crops and medicinal plants. This technology can generate gene knockout, gene knock-in, point mutation, and gene replacement in the medicinal plant's genome, which enables the regulation of metabolic pathways and secondary metabolites. CRISPR/Cas9 editing allows efficient metabolic engineering to increase the production of desirable compounds such as alkaloids, terpenoids, coumarins, tannins, flavonoids, saponins, phenols, and glycosides to meet the increasing demand of these phytochemicals. Although CRISPR/Cas9 is a revolutionary technology, its wider applicability is hindered in most medicinal plants owing to the lack of whole-genome sequence information. Recent advancements of multi-omics and NGS technologies extending to medicinal plants serve a future hope of genome sequence information of these immensely valuable medicinal plants that can pave a path for the deeper elucidation of metabolic pathways and upscaling of the precious phytochemical with pharmaceutical importance.

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Chapter 24
Deciphering the Potential of RNAi Technology as Modulator of Plant Secondary Metabolites with Biomedical Significance

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Abbreviations

COMT	Caffeic acid O-methyltransferase
dsDNA	Double-stranded DNA
dsRNA	Double-stranded RNA
hpRNA	Hairpin RNAs

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M. K. Swamy, A. Kumar (eds.), *Phytochemical Genomics*,
https://doi.org/10.1007/978-981-19-5779-6_24

mRNA	Messenger RNA
Pre-miRNA	Precursor of miRNA
PTGS	Posttranscriptional Gene Silencing
RdRP	RNA dependent RNA polymerase
RISC	RNA-induced silencing complex
RNA	Induced silencing complex
RNAi	RNA interference
RNAse	Ribonuclease
SA	Salicylic acid

1 Introduction

Biotechnological tools and applications have made it possible to genetically modify plants in a more precise and faster way. Biotechnological tools, including in vitro regeneration and genetic transformation, are specifically essential for both multiplication as well as enhancing medicinal plants genetically (Liew and Yang 2008). Another such example is RNA interference (RNAi) technology or posttranscriptional gene silencing (PTGS) that helps in regulating the expression of genes. This has further laid a massive impact on the manipulation of secondary metabolites of medicinal plants that are used as pigments, drugs, fragrances, pesticides, and even food additives. In current times, medicinal plants are considered to be highly indispensable as life-saving drugs. The use of products and supplements extracted from medicinal plants has increased significantly, with more than 80% of people across the globe relying on such plants for their primary health care needs (Ekor 2014). This chapter emphasizes the basic mechanisms and applications of RNAi technology on medicinal plants. In addition, different examples have been discussed related to the gene knockdown approach that is often used as a useful means to enhance the biosynthesis of secondary metabolite within medicinal plants.

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2 Description and Discovery of RNAi

RNA interference (RNAi) or PTGS is defined as a biological response which is conserved to double-stranded RNA (dsRNA) where the mRNA cognate to dsRNA is degraded. This process of dsRNA-mediated gene silencing is characterized by a high degree of specificity (Caplen et al. 2000). Widespread uses of this gene silencing system have improved and led to greater exploitation of medicinal plants to be commercially valuable and to produce plant-derived drugs and flavoring agents. The mechanism in which mRNA is degraded from a specific gene was discovered in 1998 (Fire and Mello 2006). The presence of the RNA molecules on double-stranded pairs in the cell activates the mechanism of RNAi. During this, dsRNA is responsible for activating the biochemical machinery that further degrades the mRNA molecules carrying genetically identical double-stranded RNA. Once mRNA molecules are disappeared, corresponding genes silence, leaving behind no protein of the encoding type. This conserved mechanism occurs in several eukaryotes, including fungi, plants, as well as animals (Grishok et al. 2000). The co-suppression where gene silencing is mediated by transgene was among one of the initial RNAi-related phenomena to be demonstrated in plants.

3 Components of the Process of RNAi

3.1 RNA-Induced Silencing Complex (RISC)

This is a type of endonuclease that is a mixture of proteins and siRNA, which further targets and degrades mRNAs within the cells complementing the siRNA strand. Once it is found, the RISC activates the RNAse enzyme, thereby separating the targeted RNA (Borgio 2009). RISC is usually associated with 20–23 bp siRNA, which helps to decrease the levels of translation (Hammond et al. 2000). It can, therefore, be stated that RISC is a catalyst that cleaves a single phosphodiester bond of mRNA.

Dicer: This is a ribonuclease that helps in the conversion of dsRNA to short ds-RNA fragments (siRNA) of uniformed size. It contains helicase domain, thereby splitting dsRNA at a distance of 21–25 bp and producing siRNA with 2-nt 3′ overhangs and 5′ phosphorylated ends (Bernstein et al. 2001). This component is used as a catalyst in the first step of the RNAi pathway. The ability to breakdown mRNA into the siRNA guide strand is possessed by a catalytic component of dicer, namely Argonaute (Jaronczyk et al. 2005).

3.2 RNA-Dependent RNA Polymerase (RdRP)

It is a versatile RNA enzyme that assists in genome replication, thereby helping in the transcription of single-stranded (ss) RNA into dsRNA. Despite the divergence in sequences, its core structural features are conserved and resemble a cupped right hand-shaped structure (Venkataraman et al. 2018).

3.3 Primary and Secondary siRNA

A variety of primary siRNAs are produced from the catalysis of dsRNA, which acts as a sole determinant of RISC specificity. However, massive RNA degradation response by microinjecting 400–500 bp dsRNA without the presence of amplified dsRNA is difficult to explain (Fire et al. 1998). Many studies have analyzed that RNAi can target sequences of RNA outside the dsRNA-inducer molecule. Furthermore, Sijen et al. (2001) analyzed that in the case of transitive silencing, the endogenous unc-22 gene is also silenced. This, therefore, suggests that interaction with the unc-22::GFP transcript is responsible for expanding target sequences for silencing. The accumulation of siRNAs homologous to sequences of unc-22 has been verified by many subsequent RNase protection experiments (Sijen et al. 2001; Escobar and Dandekar 2003). The RdRP rrf-1 mutant was analyzed to be essential for the production of "secondary" siRNAs.

This led to the proposal of a model that depicts that primary siRNAs are induced from direct destruction of introduced double-stranded RNA molecules, which has pairing capability with homologous mRNA. This results in the production of more dsRNAs, besides synthesizing a huge population of secondary siRNAs. In some cases, these siRNAs are amplified and can go beyond the boundaries of the original dsRNA trigger, making it to be highly indispensable for PTGS. However, a similar mechanism like that of transitive silencing seemed to operate in plants, generating huge amounts of dsRNA from the integrated transgenes. Escobar and Dandekar (2003) further stated that there is a possibility for systemic silencing in plants to be caused due to amplification effect wherein a mobile signal molecule from the local PTGS initiation site primes de novo synthesis of dsRNA from homologous mRNA templates in detached tissues (Escobar and Dandekar 2003).

4 The Mechanism Behind the Actions of RNAi

The figure mentioned below depicts the basic mechanism of RNAi phenomena in plants, which consists of multiple steps (Fig. 24.1). The generation of dsRNA with the use of RdRP is included in RNAi, which activates the transcription of "aberrant ssRNA" from a transgene of a transgenic plant. This, in turn, triggers the process of

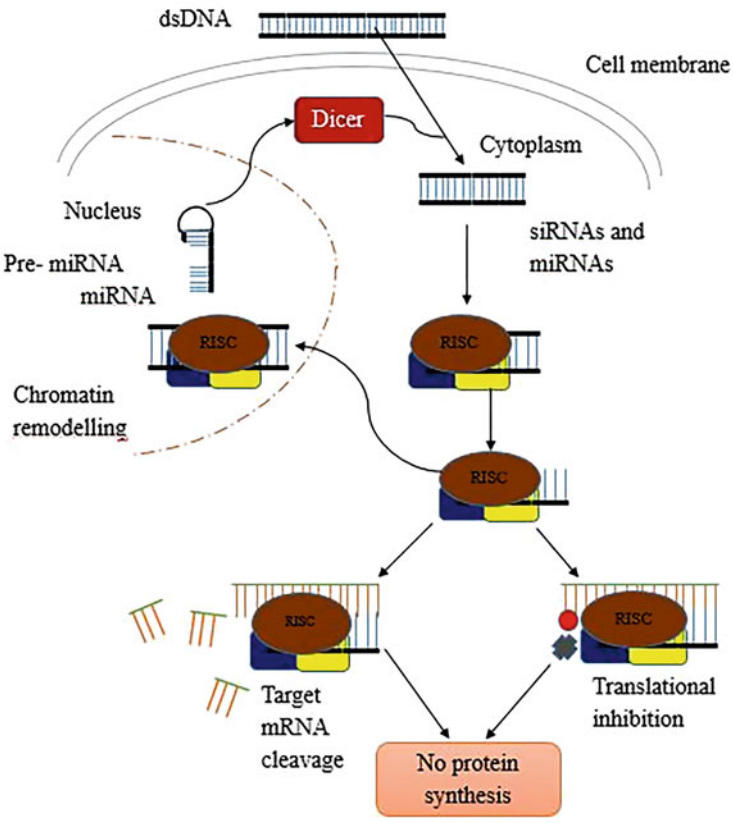

Fig. 24.1 Molecular mechanism of gene silencing via RNAi technology

generating dsRNA by RdRP, besides activating the RNAi pathway (Waterhouse et al. 2001). This further involves microRNA (miRNA); in plants and animals it is responsible for the regulation of gene expression (Hannon 2002; Aukerman and Sakai 2003). Precursor of miRNA (pre-miRNA) is a type of small hpRNA (hairpin RNAs) that has "bulges" in its stem. Dicer processes every dsRNA, hpRNA, and pre-miRNA into 21–25 nt RNA duplexes. The Dicer enzyme targets when the dsRNA enters the cell. Dicer cuts the dsRNA into 21–25 siRNAs when it is activated by ATP. These, in turn, are embodied into a nuclease complex, which is labeled as RISC. In the next step, these incorporated siRNAs are unwound (Kusaba 2004). Complex activation is attained due to the antisense strand that remains in RISC, which further cleaves mRNA. This cleaved mRNA is complementary to the siRNA. Posttranscriptional silencing of genes via the use of RNAi technology has been diagrammatically represented in Figs. 24.1 and 24.2.

Generally, heterologous genes are introduced in plants to synthesize a novel compound out of it. Many of the steps in the biosynthesis of such plant compounds can be regulated by the application of RNAi technology to reduce levels of

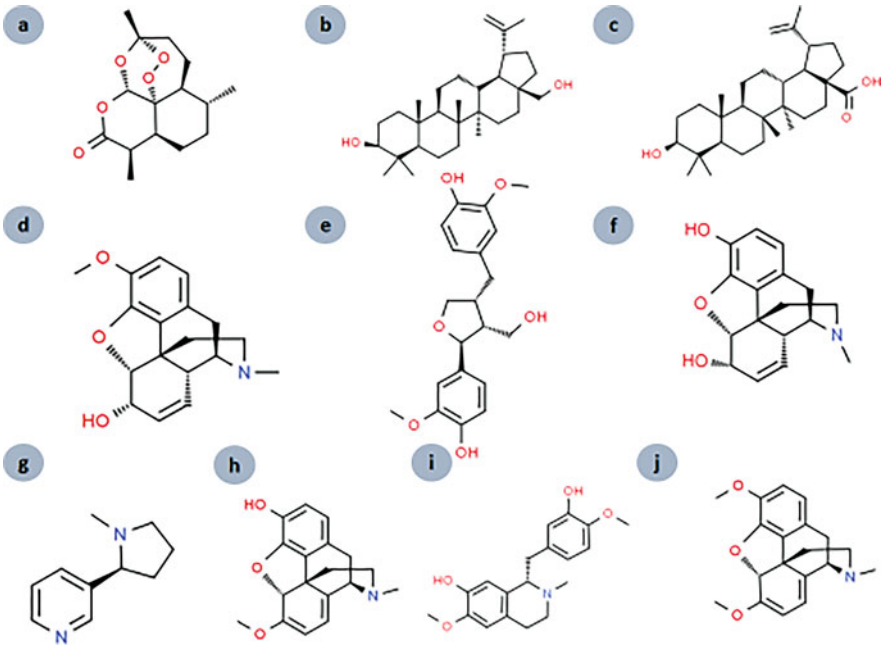

Fig. 24.2 Chemical structures of the plant-derived natural products modulated using RNAi; (**a**) artemisinin, (**b**) botulin, (**c**) betulinic acid, (**d**) codeine, (**e**) laricresinol, (**f**) morphine, (**g**) nicotine, (**h**) oripavine, (**i**) S-reticuline, and (**j**) thebaine. (Figures were retrieved from https://www.chemspider.com/)

undesirable compounds. Some of the successful use of RNAi-regulated gene-silencing for the improvement of different medicinal plants are discussed below. Few applications of RNAi-induced silencing in several medicinal plants are summarized in Table 24.1.

5 Applications of RNAi in Medicinal Plants

5.1 Centella asiatica *(L.) Urb. (Apiaceae)*

From the studies of Sharma et al. 2020, it has been reported that in *Centella asiatica* the RNAi-*DXR* and CaHMGR-RNAi construct was introduced to silence the genes called *1-deoxy-D-xylulose*-5-*phosphate reductoisomerase* (*DXR*) and *3-hydroxy-3-methylglutaryl-CoA reductases (HMGR)*, respectively. The *DXR* and *HMGR* are key regulatory enzymes in MEP and MVA pathways, respectively. Therefore, this experiment further helped to understand that the biosynthesis of terpenoid saponins called centelloids within *Centella asiatica* is dependent on these two genes (Sharma et al. 2020; Kalita et al. 2018).

Table 24.1 Summary of RNAi-mediated silencing and its application in different medicinal plants

Plants	Target gene or transcription factor silenced by RNAi	Observation	References
Centella asiatica (L.) Urb. (Apiaceae)	*1-deoxy-D-xylulose-5-phosphate reductoisomerase* (*DXR*)	Silencing these two genes downregulates biosynthesis of centelloids	Sharma et al. (2020)
	3-hydroxy-3-methylglutaryl-CoA reductases (HMGR)		Kalita et al. (2018)
Artemisia annua L. (Asteraceae)	*Cinnamate-4-hydroxylase* (CH4)	Downregulation this gene leads to increase in artemisinin content	Kumar et al. (2016)
	1-*deoxy-D-xylulose*-5-*phosphate reductoisomerase* (*DXR*)	Silencing this gene helped to understand pathway responsible for biosynthesis of artemisinin	Wang et al. (2018)
	AaPDR3 gene	Reduction of β-caryophyllene content and a gradual increase in artemisinin	Fu et al. (2017)
	Squalene synthase SQS	Silencing this gene increases artemisinin	Ali et al. (2017)
	AaHY5	AaHY5 positively regulates the biosynthesis of artemisinin	Hao et al. (2019)
Panax notoginseng (Burkill) F.H.Chen (Araliaceae)	*Cycloartenol synthase* (*CAS*)	Increased concentration of saponins	Yang et al. (2017)
Rehmannia glutinosa (Gaertn.) DC. (Plantaginaceae)	*P-coumarate-3-hydroxylase (C3H*)	Silencing this gene leads to downregulation of allelopathic phenolic biosynthesis in roots of *R. glutinosa*.	Yang et al. (2020)
Isatis indigotica Fortune ex Lindl. (Brassicaceae)	*IiWRKY34*	RNAi silencing of this gene negatively regulates biosynthesis of lariciresinol	Xiao et al. (2020)
Brassica napus L. (Brassicaceae)	*BnMYB43*	RNAi inhibition of this gene decreases the development and growth of oilseed rape but improves resistance against *Sclerotinia sclerotiorum.*	Jiang et al. (2020)
Panicum virgatum L. (Poaceae)	*Caffeic acid O-methyltransferase* (COMT)	Ferulate 5-hydroxylase (F5H) downregulation lowers S lignin biosynthesis but increases guaiacyl (G) unitsx	Wu et al. (2019)
Papaver somniferum L. (Papaveraceae)	*Codeinone reductase (COR)*	(S)-reticuline increased and codeine, morphine, thebaine, and oripavine decreased	Allen et al. (2004)
Populus sp. (Salicaceae)	*MYB134*	RNAi-inhibition of this gene leads to reduced	

(continued)

Table 24.1 (continued)

Plants	Target gene or transcription factor silenced by RNAi	Observation	References
		accumulation of condensed tannin (CT)	Gourlay et al. (2020)
Betula platyphylla Sukaczev (Betulaceae)	*S-nitrosoglutathione reductase (GSNOR)*	Inhibition of this gene results in increased biosynthesis of betulin and upregulates expression of gene encoding lupeol synthase (LUS)	Fan et al. (2018)
	BpCAS (Cycloartenol synthase)	*BpW* (lupeol synthase gene) and *BpY* (β-amyrin synthase gene) expression were enhanced along with betulinic acid	Yin et al. (2020)
	Bp-AS (β-amyrin synthase)	*BpW* expression was enhanced but *BpY* expression was severely suppressed	
Nicotiana tabacum L. (Solanaceae)	Genes encoding ornithine decarboxylase, aspartate oxidase, and arginine decarboxylase	Silencing these genes by RNAi results in reduced nicotine levels, concentration of putrescine is also regulated by these genes	Martinez et al. (2020)
	NtHDG2	Silencing this gene leads to decreasing flavonols concentration by 20.9%	Wang et al. (2020)

5.2 Artemisia annua *L. (Asteraceae)*

From this plant, a sesquiterpenoid endoperoxide compound is extracted i.e., Artemisinin, which is very useful against malaria. The *cinnamate-4-hydroxylase* (CH4) gene was downregulated by the action of RNAi technology, which was accompanied by the increase in artemisinin content along with salicylic acid (SA) (Kumar et al. 2016). RNAi-mediated suppression of the *DXR* gene helped to understand that it plays a crucial role in the biosynthesis of artemisinin in *A. annua* (Wang et al. 2018) The RNAi technology has been widely used in *Artemisia annua* to improve its medicinal properties. *AaPDR3*-RNAi transgenic Artemisia plant was grown by knocking out the *AaPDR3* gene which results in a reduction of β-caryophyllene content and a gradual increase in artemisinin within these transgenic plants (Fu et al. 2017). Similarly, suppressing the expression of the Squalene synthase *SQS* gene contributes to increasing artemisinin (Ali et al. 2017). Furthermore, a transcription factor called AaHY5 was also found to be positively regulating the biosynthesis of artemisinin and this was experimentally proven by the action of RNAi suppression of the *AaHY5* gene (Hao et al. 2019).

5.3 Panax notoginseng *(Burkill) F.H.Chen (Araliaceae)*

This is a well-known medicinal plant that is extensively used in China. The biosynthesis of triterpene within this plant was an improvement by the application of RNAi technology. After the transformation of a gene encoding for farnesyl pyrophosphate synthase (FPS) another gene called *cycloartenol synthase* (*CAS*) was silenced by RNAi technology. The observation depicted that these transgenic lines of *Panax notoginseng* produced an increased concentration of saponins (Yang et al. 2017).

5.4 Rehmannia glutinosa *(Gaertn.) DC. (Plantaginaceae)*

It belongs to the *Scrophulariaceae* family and is used widely as a medicinal plant in China. Its roots contain many kinds of pharmacologically active compounds. However, in this plant, by the use of RNAi suppression technology, it was observed that the *P-coumarate-3-hydroxylase (C3H*) gene is responsible for the synthesis of phenolic acid/phenylpropanoid. RNAi-mediated suppression of *P-coumarate-3-hydroxylase* (*C3H*) leads to downregulation of allelopathic phenolic biosynthesis in roots of *R. glutinosa* (Yang et al. 2020).

5.5 Isatis indigotica *Fortune ex Lindl. (Brassicaceae)*

Within the transcriptome of this plant 64 *IiWRKY* genes were identified. Moreover, in *IiWRKY34* expression was found to be significantly higher in tetraploids than in diploids, which is further positively correlated with lariciresinol accumulation. Overexpression of the genes and RNAi studies revealed that *IiWRKY34* regulates lariciresinol production, whereas its overexpression promotes root growth along with drought and salt stress tolerance (Xiao et al. 2020).

5.6 Brassica napus *L. (Brassicaceae)*

According to the studies of Jiang et al. 2020; RNAi inhibition of the *BnMYB43* gene family expression has been demonstrated to decrease the development and growth of oilseed rape. It further lowers the yield, and impair lodging resistance. However, this inhibition improves resistance against *Sclerotinia sclerotiorum.* These findings demonstrate that *BnMYB43*, as a key factor in the growth-defense trade-off, positively controls plant shape, yield potential, and vascular lignification while negatively affecting resistance against *S. sclerotiorum* (Jiang et al. 2020).

5.7 Panicum virgatum *L. (Poaceae)*

In angiosperms, ferulate 5-hydroxylase (F5H) regulates the hydroxylation of coniferaldehyde and coniferyl alcohol for the production of syringyl (S) lignin. The F5H downregulation in COMT-RNAi (caffeic acid O-methyltransferase-RNAi) transgenic *Panicum virgatum* (switchgrass) plants hampered S lignin production, which further results in increasing of the guaiacyl (G) units and decreasing the 5-OH G units. On the other hand, when F5H was overexpressed in COMT-RNAi transgenic plants, it decreased G units and increased the concentration of 5-OH units. Whereas, S lignin biosynthesis deficit was restored or partially compensated, depending on the amount of downregulation of COMT in *Panicum virgatum* (Wu et al. 2019).

5.8 Papaver somniferum *L. (Papaveraceae)*

In this plant, a particular gene called *COR* which codes for *codeinone reductase* has been silenced using RNAi technology. After gene silencing, the transgenic plants accumulated the precursor alkaloid (S)-reticuline (which takes place seven enzymatic steps upstream of codeinone reductase) at the expense of codeine, morphine, thebaine, and oripavine. The unexpected buildup of (S)-reticuline implies a feedback mechanism that prevents general benzylisoquinoline synthesis intermediates from accessing the morphine-specific branch. Transcript levels of seven additional enzymes in the pathway were unchanged, both downstream and upstream of (S)-reticuline. Therefore, in *Papaver somniferum*, RNAi-mediated substitution of morphine along with reticuline, which is a non-narcotic alkaloid, could be possible (Allen et al. 2004).

5.9 Populus sp. *(Salicaceae)*

RNAi-mediated inhibition helped to understand the function of a particular gene called *MYB134* within this plant. When the expression of this gene is knocked out via RNAi it leads to reduced accumulation of condensed tannin (CT). Therefore, *MYB134* is responsible for the synthesis for the biosynthesis of CT. However, in the transgenic *Populus sp.*, accumulation of CT within the roots was not affected, therefore, implying the presence of additional regulators of CT in roots and stressing the intricacy of CT regulation in *Populus sp*. To evaluate the effect of downregulation of CT during oxidative stress resistance, MYB134-RNAi and control leaves were subjected to methyl viologen which is a reactive oxygen producer. When compared to wild-type leaves, MYB134-RNAi plants suffered considerably greater photosystem II damage, as observed by lower chlorophyll fluorescence. The

leaves of MYB134-RNAi also had higher levels of hydrogen peroxide, which is a reactive oxygen species (ROS), than wild-type leaves. Therefore, it implies that CT can act as an antioxidant and further protect plants against oxidative stress (Gourlay et al. 2020).

5.10 Betula platyphylla *Sukaczev (Betulaceae)*

In this plant, a gene responsible for encoding *S-nitrosoglutathione reductase (GSNOR)* was RNAi-silenced. This leads to increased biosynthesis of betulin by at least two times in transgenic lines when compared to the wild-type plant. Furthermore, in GSNOR-RNAi transgenic plant, the expression of another gene that encodes for lupeol synthase (LUS) was also upregulated. LUS is a key enzyme that helps in the biosynthesis of betulin. These findings confirmed that GSNOR-RNAi inhibition mediates betulin synthesis at both the genetic and pharmacological levels (Fan et al. 2018).

In another experiment, it was reported that RNAi-mediated silencing of *BpCAS* (Cycloartenol synthase) and *Bp-AS* (β-amyrin synthase) dramatically reduced the expression of triterpenoid synthesis-related genes. *BpW* (lupeol synthase gene) and *BpY* (β-amyrin synthase gene) expression were enhanced in *BpCAS* gene silencing birch. *BpW* expression was enhanced in *Bp-AS* silencing birch, but *BpY* expression was severely suppressed. The betulinic acid content of *BpCAS* silenced birch was considerably enhanced. Also, betulinic acid, oleanolic acid, total triterpenoids, and soluble sugar levels rose substantially in *BpCAS* silencing birch (Yin et al. 2020).

5.11 Nicotiana tabacum *L. (Solanaceae)*

Downregulation of ornithine decarboxylase, aspartate oxidase, and arginine decarboxylase resulted in reduced nicotine levels within the leaves of the respective plants. Transgenic with RNAi-silenced aspartate oxidase had the lowest nicotine levels within the leaves. Furthermore, putrescine, which is a primary polyamine involved in the biosynthesis of nicotine, was found to have a qualitative relationship with nicotine concentration in transgenic plants produced by RNAi-mediated silencing of ornithine decarboxylase and arginine decarboxylase (Martinez et al. 2020).

Wang et al., 2019 reported that NtHDG2-RNAi transgenic lines were created by RNAi-mediated silencing of the gene *NtHDG2*, which belongs to the class IV of the HD-ZIP family. Furthermore, in the NtHDG2-RNAi plants, the contents of flavonols decreased by 20.9–52.7% compared to that of wild-type plants. Therefore, *NtHDG2* increases the accumulation of flavonol in *Nicotiana* leaves by regulating the expression of *NtMYB12* which is a regulatory gene, along with three structural genes viz., *NtF3′H, NtPAL*, and *NtF3GT* which are also involved in the production of flavonoids (Wang et al. 2020).

6 Conclusions

RNA interference (RNAi) has the potential to be a very powerful tool for gene silencing applications. RNAi has been used widely in the scientific community since its discovery by Fire et al. (1998). RNAi is still in its developing stage in the field of medicinal plant research. As previously stated, the RNAi and gene disruption techniques differ in concept, and therefore each has its own set of advantages and disadvantages. RNAi is beneficial in research and helps in the biosynthesis of essential pharmacological compounds by medicinal plants, which could further lead to innovative and quick applications. In this review, a few examples are given where RNAi technology has been used to either identify several genes responsible for different pharmacological properties in medicinal plants or to study the effects by silencing a particular gene. Further, the complete effects of RNAi technology in medicinal plants should be understood so that an inducible RNAi system along with an efficient inducer and promoter can be developed. As a result, the potential of RNAi technology in improving medicinal plants has begun to be recognized.

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Chapter 25
Regulatory Noncoding RNAs: An Emerging Paradigm for Understanding Phytochemical Biosynthesis and Functioning

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1 Introduction

The heterogeneity of phytochemical biosynthetic pathways, along with their immense influence on both plants and animals, demands an extensive delineation of the underlying regulatory aspects of the same. Though several key protein-coding genes relevant for distinct secondary metabolite pathways have been identified and characterized, there is still a lack of information about how these genes are modulated through noncoding RNAs (ncRNAs). Recently, the functional annotation of numerous noncoding transcripts, including small noncoding RNAs (sRNAs) and long noncoding RNAs (lncRNAs), unveiled their role in regulating genes that are vital for both primary and secondary metabolisms in plants (Xie and Fan 2016). With the advent of upgraded sequencing technologies, diverse repositories of regulatory noncoding RNAs in plants have been exposed, in which micro RNAs (miRNAs) and small interfering RNAs (siRNAs) represent two potential riboregulators that regulate the gene expression at both transcriptional and post-transcriptional level (Guleria et al. 2011). Among them, miRNAs are a class of small ncRNAs well-known for their post-transcriptional gene regulation, which ultimately leads to RNA silencing. Nevertheless, a least explored facet of miRNA-mediated gene regulatory mechanism also exists, which is associated with the production of phased secondary small-interfering RNAs (phasiRNAs) that can act either by cis or trans (tasiRNAs) mechanisms on their targets. Lately, many studies propound the role of these tiny regulators in biosynthesis as well as aggregation of phytochemicals in plants. Recent studies also reinforce the regulatory role of lncRNAs, which with their mRNA-like structure can interact with both coding as well as noncoding transcripts involved in

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M. K. Swamy, A. Kumar (eds.), *Phytochemical Genomics*,
https://doi.org/10.1007/978-981-19-5779-6_25

plant growth and defense. Besides, certain lncRNAs can also act as endogenous target mimics (eTMs) complementary to miRNAs, which bind with the regulatory miRNAs that otherwise target genes allied with phytochemical pathways (Xie and Fan 2016; Narnoliya et al. 2019; Zhu et al. 2019).

Plant phytochemicals possess enormous utility, due to their nutritional values, pharmacological properties, and application in the production of significant industrial compounds like cosmetics, flavors, dietary additives, and drugs (Isah 2019). However, naturally these compounds are produced in a minor concentration under specific environmental conditions, due to the high energy consumption required for their production (Gupta et al. 2017). The diverse regulatory elements contributing to this stringent regulation of phytochemical biosynthesis can be exploited in metabolic engineering to uplift the synthesis of the same. The everlasting demand for enhanced phytochemical production presently culminates at genetic manipulation approaches mediated mainly by regulatory noncoding RNAs. Compared to the conventional RNA interference (RNAi) approach, novel silencing approach mediated by artificial miRNAs (amiRNAs) and synthetic-tasiRNAs (syn-tasiRNAs) tend to have numerous advantages that tackle the limitations of the former technique. High sequence specificity accompanied by lesser off-targeting and a single promoter ample for regulating multiple artificial sRNAs that perform multiplex silencing make these techniques ideal for metabolic engineering (Zhang 2014). Taking lncRNAs into consideration, the functional interpretation of these noncoding RNAs are quite challenging due to their poor sequence similarity and complex methods of action. Also, as the functional aspects of lncRNA depend on their secondary structure rather than their primary sequence, the functional annotation as well as application of identified lncRNAs in related species remains questionable (Bazin and Bailey-Serres 2015). Nevertheless, methods like CRISPR/Cas9-mediated gene-editing technology are gradually exposing a promising approach to produce loss-of-function mutants of lncRNAs in plants (Li et al. 2018). Moreover, the novel research area of synthetic biology in developing human cell associated artificial lncRNAs (AlncRNAs) proposes the scope for their assured implementation over several aspects of plant growth, development, and metabolism in the coming years (Yao et al. 2020).

Withal, an in-depth knowledge regarding the basic mechanism of regulatory-non coding RNAs and their targeting potency associated with phytochemical biosynthesis is essential to exploit their utility in developing plant varieties with desirable and adequate concentration of a single secondary metabolite or a combination of them. This chapter summarizes the expanding information about the role of small and long noncoding RNAs in regulating the multitude of elements associated with phytochemical biosynthesis and their accumulation in plants. Also, the composite connections between sRNAs, lncRNAs, and distinct genes, along with the application of artificial noncoding RNA-mediated techniques are discussed, which provides a new angle of interpretation to delineate the entirety of phytochemical biosynthesis that can be further exploited for developing novel approaches of their targeted manipulation.

2 Regulatory Noncoding RNAs in Plants

Different classes of noncoding RNAs in plants are recognized as active molecules that can regulate gene expression at both transcriptional and post-transcriptional level, which influence various aspects of plant growth, development and responses to stresses and external cues (Yu et al. 2019). MicroRNAs are a major set of regulatory RNAs known for their pleiotropic nature of regulation (Xie et al. 2010). These endogenous RNAs of 18–24 nucleotides have pivotal roles in growth, development, and responses to abiotic and biotic stress, via post-transcriptional regulation of gene expression. Regulation of miRNA target genes is based on the complementarity between the miRNA and target gene sequence. The complex formation between miRNAs and target sequence could result in mRNA cleavage or translational repression or sometimes lead to the generation of secondary small interfering RNAs. The miRNAs are recently established as potential bioactive molecules with the property of transferability across species and cross-kingdom gene regulation (Gualtieri et al. 2020).

Small interfering RNAs (siRNAs) are established as essential regulators of growth, development, and immune responses in plants. The evidence for a group of secondary siRNAs, whose production is triggered by miRNA or other siRNA-mediated target cleavage and RNA-dependent RNA polymerase (RDR) activity, is recently found in plants (de Felippes 2019). Typically, these siRNAs seem to exist in 21 or 24 nt size, and what strikes the most is their phased pattern of expression, hence named phased secondary siRNAs/phasiRNAs. Apart from the conventional cis activity mediated by these phased siRNAs, there exists a subset called trans-acting siRNAs or tasiRNAs that can interact in trans and hence target those transcripts that are not their source of origin (Fei et al. 2013).

Another important type of noncoding RNA is long noncoding RNAs (lncRNAs), which exhibit mRNA-like structures and gene regulatory functions in plants. lncRNAs are conventionally known as transcripts longer than 200 bp, which lack the property to build a full-length protein. The function of lncRNAs varies from serving as a decoy that mimics specific regions of the target protein, a scaffold which recruits multiple proteins together for the formation of functional complexes, a guide for other small RNA complexes to the target sites, or signal enhancer to produce downstream regulatory RNAs (Franco-Zorrilla et al. 2007; Heo and Sung 2011; Matzke and Mosher 2014). Presently, many studies exposing the role of circular RNA (circRNA), a typical noncoding RNA with covalently closed loop structure, which can act as miRNA decoys in plants have been established (Zuo et al. 2016; Wang et al. 2017; Tang et al. 2018; Zhang et al. 2020). A genome-wide study in *Salvia miltiorrhiza* revealed numerous circRNAs with miRNA-binding sites, among which some of them had genes regulating biosynthesis of secondary metabolites like terpenes and growth regulators like brassinosteroids and gibberellins as their precursors (Jiang et al. 2021). The so-far identified mode of noncoding RNA-mediated regulations is summarized in Fig. 25.1.

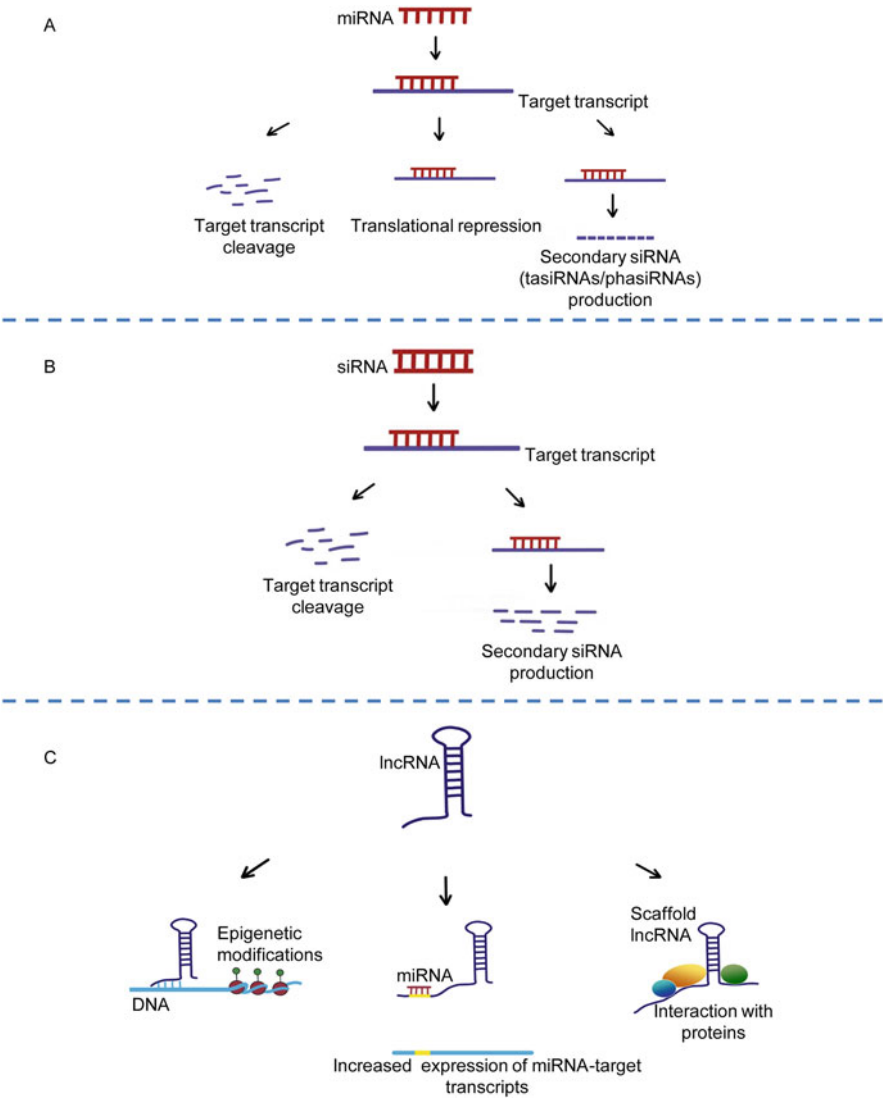

Fig. 25.1 A. miRNA induces cleavage or translational repression of its targets, and at times initiates secondary siRNA production B. siRNAs either cleave their complementary target transcripts or trigger secondary siRNA production C. lncRNAs enhance epigenetic modifications, act as target mimics/miRNA decoys, and as scaffolds for diverse protein interactions

Despite the role of these regulatory noncoding RNAs upon various stresses and developmental stages, their vital part in regulating the biosynthesis and functioning of secondary metabolites has gained significant attention recently.

3 miRNA-Mediated Regulation on the Biosynthesis of Secondary Metabolites

miRNAs are recently established as riboregulators of biosynthesis and accumulation of various secondary metabolites in plants (Bulgakov and Avramenko 2015; Gupta et al. 2017). Recent studies have found the involvement of miRNAs in the regulation of diverse metabolites such as flavonoids, alkaloids, terpenoids, and lignin in various species of plants (Adjei et al. 2021). Flavonoids are a class of secondary metabolites of low molecular weight phenylpropanoids, extensively spread over the plant kingdom with a variety of significant molecular functions as signalling molecules, modulators of phytohormonal activities, plant–microbe interactions, and stress responses (Taylor and Grotewold 2005; Lepiniec et al. 2006; Santelia et al. 2008; Buer et al. 2010). Various flavonoid metabolites are produced through the central phenylpropanoid pathway, sharing multiple enzymes and substrates in common. A few miRNAs were found to be involved in the regulation of flavonoid biosynthesis. An earlier computational study with 323,318 ESTs of *Helianthus*, identified miRNAs specifically belonging to miR2911 family involved in the modulation of tocopherol production in the plant (Barozai et al. 2012). The biosynthesis and activity of anthocyanins, an important subclass of flavonoids, are under tight regulation of miRNAs across the plant kingdom. It was identified that miR156-mediated post-transcriptional control over *squamosa promoter binding protein-like* (SPL) genes in *Arabidopsis thaliana* directly linked to the anthocyanin accumulation in the stem part. Overexpression of miR156 leads to increased accumulation of anthocyanins by targeting *SPL9*. The study also established the *SPL9* gene as a negative regulator of anthocyanin accumulation via disruption of the MYB-bHLH-WD40 protein complex (Gou et al. 2011). Another transcriptomic study in persimmon (*Diospyros kaki*) fruits revealed that the targets of certain differentially expressed miRNAs were implicated in the accumulation of proanthocyanidins or tannins. Among the identified miRNAs, miR858 and miR156 were antagonistically involved in regulating genes related to the biosynthesis of proanthocyanidins. It appeared that miR858 acts as a positive regulator while miR156 acts as a negative regulator of proanthocyanidin production (Luo et al. 2015). Further studies in *A. thaliana* demonstrated that MYB transcription factors related to flavonoid biosynthesis were regulated by miR858a. The control over flavonoid biosynthesis by the specific miRNA was proved by overexpression and mutation studies (Sharma et al. 2016). In addition, the significant role of miR858a in regulating flavonoid biosynthesis was exposed in *Osmanthus fragrans*, which was modulated by the negative correlation of miR858a and genes such as *MYB1*, *CHI* (*chalcone isomerase*), *CHS* (*chalcone*

synthase), and *FLS* (*flavonol synthase*), which are significant for flavonoid synthesis (Shi et al. 2021). Moreover, a recent transcriptome study in Himalayan mayapple (*Podophyllum hexandrum*) unraveled a few miRNAs, which are predicted to regulate several polypropanoid and flavonoid biosynthesis pathways. miR1438 modulates a gene, *caffeoyl-CoA O-methyltransferase*, related to several pathways linked to secondary metabolisms like phenylalanine metabolism, phenylpropanoid biosynthesis, flavonoid biosynthesis, stilbenoid, diarylheptanoid, and gingerol biosynthesis. Additionally, miR1873 and miR5532 identified in the same study were predicted to regulate *dihydroflavonol 4-reductase C* and *2-hydroxyisoflavanone dehydratase* respectively, which are directly linked to flavonoid biosynthesis pathways (Biswas et al. 2016). Another study in the same species identified miRNAs, their corresponding targets and downstream metabolic pathways related to the production of podophyllotoxin, which is a high value secondary metabolite known for its anticancerous properties. The study revealed that miR396b, miR2673a, miR828b, and miR2910 can be utilized as suitable candidates for improving the podophyllin content in *P. hexandrum.* The identified miRNAs target UDP-glycosyltransferase, flavonol synthase, glyceraldehyde 3-phosphate dehydrogenase, peroxidase, malate dehydrogenase, phosphoenolpyruvate carboxylase, WRKY 37, and MYBF1 transcription factor transcripts which are part of shikimic acid pathway and phenyl propanoid pathway related to the production of podophyllotoxin (Kumar et al. 2018). Recently, a research study identified methyl jasmonate responsive novel miRNAs, regulating genes related to the podophyllotoxin biosynthesis pathway. The 66 novel miRNAs identified in the study target *s-adenosyl-L-methionine-dependent methyltransferase*, *cytochrome p450*, *flavonol synthase/flavanone 3-hydroxylase*, *4-coumarate: ligase*, and *phenylalanine ammonia-lyase*. Through regulation of these genes, the identified novel miRNAs are linked to the biosynthetic pathways of other secondary metabolites such as phenylpropanoid, alkaloids, and terpenoids (Biswas et al. 2021). miRNA-mediated regulation of flavonoid pathways was reported during salt stress conditions, indicating their involvement in metabolic flux reprogramming during the stress conditions. The study identified differentially expressed miRNAs and corresponding targets from salinity-exposed *Halostachys caspica* and their involvement in salt stress-related pathways, such as calcium signalling pathway, MAPK signalling pathway, plant hormone signal transduction, and flavonoid biosynthesis (Yang et al. 2015). An miRNA coexpression study of *Camellia sinensis* revealed that gallated catechin, a renowned flavanol, was negatively regulated by miR156, but positively enhanced by miR166 and miR172 as well. The study exposed the potent role of miRNAs in modulating flavor compound biosynthesis in *C. sinensis*, the tea plant (Li et al. 2021).

Terpenoids are the biggest class of volatile compounds synthesized out of C5 precursors in plants (Dudareva et al. 2013). In silico studies in many plant species identified various miRNAs with a possible role in the regulation of terpene biosynthesis and their biological activities. In *Salvia sclarea*, a preliminary transcriptome analysis revealed the miRNAs and targets related to terpenoid and phenylpropanoid biosynthetic pathways (Legrand et al. 2009). The *SPL9* gene, which was reported to be regulated by miR156, was found to act as a transcriptional activator of

sesquiterpenoid biosynthesis via binding to the *terpene synthase 21* (*TPS21*) gene (Yu et al. 2015). Thus, miR156 was identified to act as a modulator of both flavonoids and terpenoids through the regulation of a specific gene. Another study in the medicinal herb *Picrorhiza kurroa* identified an miRNA, miR4995, one of the predicted targets encodes for 3-deoxy-7-phosphoheptulonate synthase enzyme, that takes part in picroside biosynthesis pathway. The reduction in the target transcript leads to higher biosynthesis and accumulation of picroside (Vashisht et al. 2015). A transcriptome study in *Ferula gummosa* unraveled the role of miRNAs inherent in miR2919, miR838, miR5021, miR5658, and miR5251 families in regulating terpene biosynthesis. The study also disclosed potentiality of miRNAs in the regulation of terpene-regulating transcription factors such as, SPL7, SPL11, and ATHB13 (Sobhani Najafabadi and Naghavi 2018).

Terpene trilactones are a class of unique terpenoids present in *Ginkgo biloba* L. known for their high medicinal value. Recently several studies identified miRNAs from *G. biloba.* It was revealed that four conserved miRNAs and five novel miRNAs from *G. biloba* have possible role in biosynthesis pathways of terpene trilactones. These miRNAs might have potential role in terpene trilactones accumulation by targeting transcription factor genes, *bHLH, WRKY*, and *AP2,* which act as regulatory genes in the pathway of terpene trilactones biosynthesis (Ye et al. 2020).

Additionally, several miRNAs of *Xanthium strumarium*, regulating enzymes involved in sesquiterpene biosynthesis, were identified and validated. These include miR7539, miR5021, and miR1134 regulating 1-deoxy-D-xylulose 5-phosphate synthase (DXS), 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR), isopentenyl diphosphate (IPP)/dimethylallyl diphosphate (DMAPP) synthase (IDS), and isopenteyl diphosphate isomerase (IDI) (Fan et al. 2015). An ethnopharmacologically important plant *Artemisia annua* is the only natural source of artemisinin (ART), a sesquiterpene lactone, which is a largely used medicine to treat malaria. miRNA families, namely miR159, miR172, and miR166, targeted *cytochrome P450 reductase* gene, which possesses a crucial role in artemisinin biosynthesis (Khan et al. 2020).

Alkaloids are a class of nitrogen-containing low molecular weight compounds with diverse roles in plants. These organic compounds with great structural diversity are mainly derived from amino acids and have been extensively used as pharmaceuticals, stimulants, narcotics, and poisons (Kurek 2019). Opium alkaloids, the most widely used alkaloids, are modulated by pso-miR13, pso-miR2161, and pso-miR408 in opium poppy (*Papaver somniferum*). The study found that pso-miR13 regulates *7-O-methyltransferase* gene, involved in biosynthesis of morphinan alkaloids. Similarly, pso-miRNA2161 targets S-adenosyl-L-methionine, 30-hydroxyN-methylcoclaurine 40-O-methyltransferase 2 enzyme and pso-miR408 regulates reticuline oxidase-like protein, where both these enzymes are part of benzylisoquinoline alkaloids (BIA) biosynthesis pathways (Boke et al. 2015). In addition, target mimicry studies also helped to identify miRNA-mediated alkaloid regulation. The study by Li et al. (2015), through target mimicry of nta-miRNA27, established the role of this specific miRNA in nicotine biosynthesis via regulating *quinolinate phosphoribosyl transferase 2* gene. miR156-mediated regulation over

the *SPL9* gene also modulates the biosynthesis of alkaloids. The *SPL9* controls jasmonate synthesis, and enhanced jasmonate expression stimulates the biosynthesis of glucosinolates (Mao et al. 2017). Thus, miR156 remains as a regulator of all the major class of secondary metabolites. Glucosinolate synthesis was found to be regulated by miR826 and miR5090 of *A. thaliana* via controlling the expression of a common target gene *AOP2*, which encodes a 2-oxoglutarate-dependent dioxygenase (Liang et al. 2012; He et al. 2013). Study in *Curcuma longa* exposed the combined regulation mediated by miRNAs on biosynthesis of secondary metabolites like curcuminoid, flavonoid, alkaloid, and terpenoid. Among the identified miRNAs, when flavonoid pathway was found to be regulated by miR2919, both terpenoid and alkaloid pathways were found to be influenced by miR5021 (Singh and Sharma 2017). An miRNA expression study in *Solanum tuberosum* manifested the miRNA-TOR (target of rapamycin) interplay, which is a least explored realm of plant biology. TOR inhibition in *S. tuberosum* enhanced the activation of several miRNAs influencing diverse secondary metabolite pathways like miR4376-3p, miR5303c, miR7983-5p in phenylpropanoid biosynthesis, miR8045 in terpenoid-quinone biosynthesis, miR7997a in isoquinoline alkaloid biosynthesis, and miR5303 in carotenoid biosynthesis (Deng et al. 2021).

Medicinal plants are rich in a wide range of plant secondary metabolites that can be explored for their applicability in the production of medicines, insecticides, drugs, toxins, dyes, etc. However, the regulation of biosynthetic pathways of these secondary metabolites are not completely understood in the medicinal plants. Emerging studies have been focused on identification of miRNAs involved in biosynthesis, mechanism of action as well as degradation pathways of the secondary metabolites (Sabzehzari and Naghavi 2019). *Lonicerae japonicae*, commonly known as honeysuckle is a medicinal plant familiar for its anti-inflammatory and hypolipidemic properties. A recent study by Liu et al. (2017) reported a few novel miRNAs which might regulate genes involved in flavonoid biosynthesis in the honeysuckle plant. Additionally, miRNA profiling on *Murraya koenigii* L., a subtropical medicinal plant native to Asia which produces therapeutic compounds including, carbazole alkaloids, identified miRNAs associated with the secondary metabolite production. The study identified 142 conserved and 7 novel miRNAs from *M. koenigii* regulating target genes involved in the terpenoid backbone biosynthesis pathway and flavonoid biosynthesis pathway (Gutierrez et al. 2021). The transcriptome study of in vitro root and leaf culture in *Withania somnifera*, a putative medicinal plant of solanaceae family, divulged the role of miR5140, miR159, miR477, and miR530 in supplementing withanolide production. These studies might be crucial for the overproduction of highly valuable secondary metabolites (Srivastava et al. 2018).

4 Secondary siRNA-Mediated Regulation on the Biosynthesis of Secondary Metabolites

Apart from the conventional roles of plant miRNAs in mediating post-transcriptional cleavage and translational repression, another remarkable function exhibited by these tiny regulators is to trigger secondary regulatory pathways comprising siRNAs (Bulgakov and Avramenko 2015). The secondary siRNAs produced by such initiator-miRNAs are renowned for possessing their role in plant growth, development, responses to stress, and in distinct metabolic activities by regulating a multitude of both coding as well as noncoding transcripts (Tirumalai et al. 2019).

The biosynthesis of phenylpropanoids, a recognized class of plant phytochemicals with an indispensable role in several aspects of plant development, is partly regulated by the coordinated activity of tasiRNAs along with their trigger miR828 and *MYB* genes. A cascade of miR828-AtTAS4-siR81(¡)-*MYB*s directs the regulation of anthocyanins, a member of the flavonoid family produced by phenylpropanoid pathway. Interestingly, the miR828 overexpression lines of *A. thaliana* display a reduced expression of several *MYB* genes like, *AtMYB82, AtMYB75/PAP1, AtMYB90/PAP2,* and AtMYB113, which in turn cause the suppression of enzyme-coding genes that are crucial for anthocyanin biosynthesis, leading to the declined accumulation of the same (Deng and Lu 2017). While the *MYB* gene regulation by miR828b-mediated direct silencing is extensively found in gymnosperms, monocots, and dicots, their regulation executed by tasiRNAs produced from miR828 triggered TAS4 or MYB transcripts are narrowed to dicots. These dual regulatory mechanisms in anthocyanin biosynthesis are presumed as a pertinent evolutionary event during monocot–dicot divergence (Luo et al. 2012). Analogous to the above-mentioned regulatory mechanism in anthocyanin biosynthesis, the metabolism of carbohydrates in certain tissues of cotton is also found to be regulated by miRNA-*MYB*/*TAS4*-tasiRNA pathway (Guan et al. 2014). Carbohydrates are inevitable compounds for secondary metabolites, since they can integrate into numerous phytochemicals via glycosidation linkages (Hussein and El-Anssary 2019). Remarkably, the study in cotton proposed that miR828 triggers the production of tasiRNAs from *GhMYB2D* gene to inhibit the fiber development in cotton by repressing carbohydrate metabolism, so that the sugar molecules can be utilized for other growth and developmental activities in plants (Guan et al. 2014). *TRANSPARENT TESTA19* (*TT19*) is critical for confiscating the accumulation of anthocyanin and is considerably regulated by R2R3-MYB transcription factor PRODUCTION OF ANTHOCYANIN1 (PAP1/MYB75). Both *TT19* and *PAP1* expression in *A. thaliana* are partly influenced by certain elements inherent in the RDR6-SGS3-DCL4-sRNA pathway, where TAS4-siRNA81(−), a typical tasiRNA, targets certain transcripts like *PAP1, PAP2,* and *MYB113* under specific metabolic conditions. This study in *A. thaliana* exposes the scope for a monitoring system devised of siRNAs that can regulate the central carbon metabolism, which is fundamental for the formation of phytochemical precursors (Jiang et al. 2020). Other than the miRNA-triggered secondary siRNA production, there are specific primary siRNAs that

initiate the production of secondary siRNAs. One event of that kind is represented by secondary siRNAs triggered by *CHS* gene-derived primary siRNAs that generally code for chalcone synthase, a key enzyme required for diverse products of secondary metabolism in plants. The siRNAs derived from *CHS7* and *CHS8*, specifically in the seed coat, but not in cotyledons or other vegetative tissues, expose the contribution of siRNAs in regulating the metabolic activities in a tissue-specific manner (Tuteja et al. 2009). Though the significance of secondary siRNAs in phytochemical biosynthesis is well recognized, comprehensive research is still needed to expose novel siRNA candidates that can be implicated for the development of improved plant varieties with desired secondary metabolite production.

5 lncRNA-Mediated Regulation on the Biosynthesis of Secondary Metabolites

Recently, the comprehensive role of lncRNAs in plants is extensively scrutinized due to their several regulatory impacts on diverse cellular activities via histone modification, chromatin remodeling, and functioning as miRNA target mimics (Jha et al. 2020). The flavor and aroma of a beverage product oolong tea (*Camellia sinensis*) relies markedly on the differentially expressed lncRNAs and their targets associated with flavonoid, terpenoid, and jasmonic acid/methyl jasmonate. Strikingly, two lncRNAs, LTCONS_00054003 and LTCONS_00060939, exhibited a positive correlation with their target genes, *4CL* (4-coumarate CoA ligase) and *CHI*, that are vital for flavonoid metabolism. Furthermore, the competence of eTM-based regulation of JA/MeJA biosynthesis pathway and terpenoid metabolic pathway were manifested by LTCONS_00026271-novel_miR44, LTCONS_00020084-miR169d-5p_1 and LTCONS_00026271-novel_miR44-*LOX,* LTCONS_00020084-miR169d-5p_1-*ACX* pairs respectively (Zhu et al. 2019). Similarly, a set of lncRNAs acting as miRNA target mimics in rose-scented Geranium, displayed a discernible role in regulating significant genes of terpene and tartrate biosynthesis pathways. In addition, several enzymes like geranylgeranyl diphosphate synthase, terpene synthase, and hydroxymethylglutaryl-CoA reductase of terpene pathway together with polygalacturonase and hexokinase of tartarate pathway were established to be directly targeted by lncRNAs as well (Narnoliya et al. 2019). Biosynthesis of nicotine, a chief alkaloid found in *Nicotiana* species, is also partly regulated by the very same mechanism of eTM. Here, an lncRNA named nta-eTMX27, acts as the decoy for miRNA nta-miRX27. Thus, the effect of nta-miRX27 diminishes, which otherwise targets *QPT2*, a significant gene in nicotine biosynthesis that codes for quinolinate phosphoribosyl transferase (Xie and Fan 2016).

The transcriptome analysis of sea buckthorn (*Hippophae rhamnoides)*, a fruit rich in diverse bioactive compounds like secondary metabolites, vitamins, and antioxidants, unraveled the action of cis or trans-acting lncRNAs on components of distinct

phytochemical pathways, including the biosynthesis of carotenoids and flavonoids. The study exposed the potent role of lncRNAs in regulating enzymes associated with first-committed steps and rate-limiting steps in distinct phytochemical pathways. For instance, the interaction of lncRNA TCONS_00082246 with phytoene synthase and TCONS_00085219 with chalcone synthase reveals the regulatory impact of these lncRNAs on first-committed steps of carotenoid and flavonoid biosynthesis, respectively (Zhang et al. 2018). Unmasking the impression of lncRNAs in modulating significant phytochemical pathways of sea buckthorn can be further utilized to expose the concealed factors contributing to fruit ripening and pigmentation in other fruit producing species.

Though some heavy metals are required for plants, certain others like cadmium (Cd) are considered as threats to both animals and plants. The potential of lncRNAs, namely XLOC_058523, XLOC_104363, and XLOC_059778, to modulate OS11G0552000, a gene coupled with phenylpropanoids and phenylalanines, was observed in rice roots under Cd-stress. These heavy metal-induced lncRNAs and their associated phytochemical pathways, proposes the possibility of their role in inducing several transporter proteins to mediate the elimination of toxic concentration Cd from the cell (Chen et al. 2018). An extensive study integrating the identified Cd-stress-induced lncRNAs and other genetic elements allied with them can be exploited to supplement the network of regulatory components involved in other heavy metal stress responses, which can be further utilized to reduce the effect of these toxic substances on both plants and animals. The competency of lncRNAs to act upon salinity stress was monitored in *Pistacia vera*, which brought out interesting evidence of lncRNA_ PveLR34269 targeting Laccase genes. These genes can enhance the monolignol polymerization vital for the biosynthesis of lignin, where lignin accumulation apparently has been a characteristic feature of cells adapted to salinity (Chun et al. 2019; Jannesar et al. 2020). Hence, additional in-depth understanding of the functional attributes of regulatory lncRNAs and detection of novel lncRNA candidates related to phytochemical biosynthesis can contribute to their precise exploitation for specific phytochemical production with the desired amount in plants. Another facet of interdependence between lncRNAs and phytochemicals unravels the modulation of cancer-related lncRNAs by bioactive secondary metabolites. Abnormal regulation of lncRNAs can sometimes lead to cancer, due to the activation of either oncogenic lncRNAs or suppression of tumor suppressor lncRNAs. Several studies uncovered the role of phytochemicals like baicalin, curcumin, resveratrol, genistein, and berberin in regulating the expression of lncRNAs like CCAT1 (colon cancer-associated transcript 1), HOTAIR (HOX antisense intergenic RNA), XIST (X-inactive specific transcript), PCAT29 (prostate cancer-associated transcript 29), TTTY18 (testis-specific transcript, Y-linked 18), and CASC2 (cancer susceptibility candidate 2) to impart anticancer effects (Kalhori et al. 2021). The noncoding RNAs and their regulatory impact on specific phytochemical biosynthesis discussed in this chapter are summarized in Table 25.1.

Table 25.1 Regulatory noncoding RNAs involved in phytochemical biosynthesis

Phytochemical	Sources	Regulatory noncoding RNAs	Functions	References
Flavonoids	*Helianthus*	miRNA2911	Tocopherol production	Barozai et al. (2012)
	Arabidopsis thaliana	miR156	Anthocyanin accumulation	Gou et al. (2011)
	Diospyros kaki	miR858 miR156	Tannin accumulation	Luo et al. (2015)
	Podophyllum hexandrum	miR1438 miR1873 miR5532 miR2673a miR828b	Flavonoid biosynthesis	Biswas et al. (2016)
	Arabidopsis thaliana	miR828 AtTAS4-siR81(¡)	Anthocyanin accumulation	Deng and Lu (2017), Jiang et al. (2020)
	Glycine max	CHS-derived siRNAs and secondary siRNAs	Isoflavone and anthocyanin biosynthesis	Tuteja et al. (2009)
	Camellia sinensis	LTCONS_00054003 LTCONS_00060939	Flavonoid biosynthesis	Zhu et al. (2019)
	Hippophae rhamnoides	TCONS_00085219 TCONS_01039552 TCONS_00061167 TCONS_00061354	Flavonol and anthocyanin biosynthesis	Zhang et al. (2018)
	Oryza sativa	XLOC_058523 XLOC_104363 XLOC_059778	Flavonoid biosynthesis	Chen et al. (2018)
	Lonicerae Japonicae	U4992168 U2743257	Flavonoid biosynthesis	Liu et al. (2017)
	Curcuma longa	miR2919	Flavonoid biosynthesis	Singh and Sharma (2017)
	Camellia sinensis	miR156, miR166, miR172	Catechin biosynthesis	Li et al. (2021)
	Osmanthus fragrans	miR858	Flavonoid biosynthesis	Shi et al. (2021)
Terpenoids	*Pogostemon cablin*	miR156	Sesquiterpenoid biosynthesis	Yu et al. (2015)
	Picorhiza Kurroa	miR4995	Picroside biosynthesis	Vashisht et al. (2015)
	Xanthium strumarium	miR7539 miR5021 miR1134	Sesquiterpene biosynthesis	Fan et al. (2015)
	Camellia sinensis	LTCONS_00026271 novel_miR44 LTCONS_00020084 miR169d-5p_1	Terpenoid biosynthesis	Zhu et al. (2019)
	Hippophae rhamnoides	TCONS_00082246	Carotenoid biosynthesis	Zhang et al. (2018)

(continued)

Table 25.1 (continued)

Phytochemical	Sources	Regulatory noncoding RNAs	Functions	References
	Pelargonium graveolens	lnc_TR36323| c1_g1_i1	Terpene biosynthesis	Narnoliya et al. (2019)
	Ginkgo biloba	miR167 miR163e novel miR_2024 miR_457 miR_218 miR_2642	Terpene trilactones metabolism	Ye et al. (2020)
	Curcuma longa	miR5021	Terpenoid biosynthesis	Singh and Sharma (2017)
	Ferula gummosa	miR2919 miR838 miR5021 miR5658 miR5251	Terpene biosynthesis	Sobhani Najafabadi and Naghavi (2018)
	Artemisia annua	miR159 miR172 miR166	Artemisinin biosynthesis	Khan et al. (2020)
	Solanum tuberosum	miR5303	Carotenoid biosynthesis	Deng et al. (2021)
	Withania somnifera	miR5140 miR159 miR477 miR530	Withanolide biosynthesis	Srivastava et al. (2018)
Alkaloids	*Papaver somniferum*	pso-miR13 pso-miR2161 pso-miR408	Morphinan alkaloids, benzylisoquinoline alkaloids	Boke et al. (2015)
	Nicotiana tabacum	miRX17 miRX27 miRX20 miRX19	Nicotine biosynthesis	Li et al. (2015)
	Arabidopsis thaliana	miR156	Glucosinolate accumulation	Mao et al. (2017)
	Arabidopsis thaliana	miR826 miR5090	Glucosinolate accumulation	Liang et al. (2012), He et al. (2013)
	Nicotiana tabacum	nta-eTMX27 nta-miRX27	Nicotine biosynthesis	Xie and Fan (2016)
	Curcuma longa	miR5021	Isoquinoline alkaloid biosynthesis	Singh and Sharma (2017)
	Solanum tuberosum	miR8045	Isoquinoline alkaloid biosynthesis	Deng et al. 2021)
Lignin	*Pistacia vera*	lncRNA_ PveLR34269	Lignin biosynthesis	Chun et al. (2019), Jannesar et al. (2020)

6 Using Artificial ncRNAs for Enhanced Plant Phytochemicals

Artificial miRNAs (amiRNAs) and synthetic tasiRNAs (syn-tasiRNAs) are two emerging tools that can mediate selective silencing of desired transcripts. These techniques have exceptional advantages over the conventional double-stranded RNA (dsRNA)-induced silencing method. Unlike dsRNA, the amiRNA and syn-tasiRNA constructs have least chance of off-target silencing and transitive silencing. Also, the necessity of only a single promoter to direct a single transgene cassette of numerous amiRNAs or syn-tasiRNAs makes their implementation over diverse pathways in intricate metabolic engineering (Zhang 2014). AmiRNAs are produced by altering the miRNA and miRNA* sequences in the miRNA-precursors to generate amiRNA/amiRNA* duplexes that can specifically silence their target transcript (Samad et al. 2017). Syn-tasiRNAs, also known as artificial tasiRNAs (atasiRNAs), are produced by replacing tasiRNA sequences from the TAS DNA (tasiRNA precursors) with desired sequences of interest. When such modified TAS genes are inserted into plants, they follow the conventional tasiRNA biogenesis pathway to produce syn-tasiRNAs targeting their respective target sequences (Sanan-Mishra et al. 2021).

AmiRNA-incorporated vectors have successfully downregulated two monolignoid biosynthetic genes of *Corchorus olitorius* (jute), namely coumarate 3-hydroxylase (*C3H*) and ferulate 5-hydroxylase (*F5H*). The transgenic jute with F5H-amiRNA and C3H-amiRNA insertion exhibited a reduced lignin content along with enhanced digestibility, without any hinder on their developmental and defense activities (Shafrin et al. 2015). Likewise, tobacco flavonol synthase (*NtFLS*) genes were silenced using an amiRNA named amiFLS, to affirm the role of rutin in defense response against *Spodoptera litura*. The *ATMYB12* gene-overexpressed tobacco lines had an increase in rutin production, which leads to an enhanced resistance against *S. litura*. Whereas, transgenic tobacco with both *AtMYB12* and amiFLS exhibited a decline in rutin production that in turn diminished the insect resistance mediated by the flavanol rutin (Misra et al. 2010). Another successful gene silencing approach triggered by amiRNAs in a model marine diatom, *Phaeodactylum tricornutum,* involved the suppression of phytoene synthase (*PSY*) gene, which results in the decline of carotenoid production in the same (Kaur and Spillane 2015). An increased production of β-carotene, a renowned carotenoid compound was achieved by amiRNAs that silence autophagy-related genes, *ATG1* and *ATG8* in *Chlamydomonas reinhardtii.* This study complemented that amiRNA-mediated regulation of crosstalk between autophagy-carotenoid biosynthetic pathway not only enhanced the expression of diverse carotenoid compounds but also triggered the production of saturated and monosaturated fatty acids, which may act as potent biodiesel source (Tran et al. 2019). Nevertheless, amiRNA-mediated gene silencing sometimes confronts with certain difficulties, where incorporating multiple precursor-miRNAs in a single transgene construct may hinder the amiRNA processing during their maturation and even affects their downstream targeting.

On the other hand, a single TAS locus can integrate multiple syn-tasiRNAs that can specifically target either multiple sites of the same gene or efficiently silence diverse genes in the same or different gene family. Besides, introducing atasiRNA constructs along with their miRNA triggers can complement the efficacy of selective silencing of desired target genes (Zhang 2014). Currently, numerous syn-tasiRNA-mediated multiple virus resistance studies have been successfully implemented against pathogens like, *Turnip mosaic virus* (TuMV) and *Cucumber mosaic virus* (CMV) in *A. thaliana*, *Tomato spotted wilt virus* (TSWV) in *Nicotiana benthamiana* and *Solanum lycopersicum*, and *Potato spindle tuber viroid* (PSTVd) in *N. benthamiana* (Chen et al. 2016; Carbonell and Daròs 2017; Carbonell et al. 2019). However, extended research is still required in the area of syn-tasiRNA-mediated gene silencing approach, in order to employ their application in metabolic engineering of plants, to encounter with the perpetual need for plant-derived secondary metabolites.

7 Conclusions and Future Perspectives

The escalating evidence of noncoding RNAs modulating the biosynthesis and functioning of plant secondary metabolites has made them an engrossing area in phytochemical research. Apart from the well-admitted identification of the structural genes, enzymes, and other proteins, the advancement in the sequencing technologies and computational tools has enabled the detection of diverse regulatory noncoding RNAs involved in phytochemical biosynthesis. Small noncoding RNAs can be credibly considered as a formidable candidate for metabolic engineering that complements phytochemical biosynthesis in plants. The propensity of these sRNAs in mediating precise silencing of their targets, targeting multiple transcripts and their spatiotemporal mode of action proclaims the significance of these tiny regulators as efficient silencing tools (Carbonell 2019). In spite of the regulatory impact of miRNAs and secondary siRNAs on plant development and their response to diverse biotic and abiotic stresses, the role of these tiny regulators in phytochemical biosynthesis and functionality had recently commenced to expand. Deep sequencing technologies and modern bioinformatic tools enhance the prediction and validation of these regulatory sRNAs that can be further analyzed for their targets in each distinct steps in the biosynthetic pathway. Such sRNA-target interactions can be exploited to implement in metabolic engineering for the production of desired secondary metabolites in adequate amount and combinations. Recent studies in eukaryotes expounds the concept of miRNA-epigenetic feedback loop, where despite miRNAs acting as potent epigenetic regulators that regulate gene expression without influencing their nucleotide sequences, they can also be reciprocally regulated by epigenetic modifications like DNA methylation and histone modifications (Yao et al. 2019). Hence, exploiting this miRNA-epigenetic modulator interaction can be confidently considered as an engaging area for future studies concerned with phytochemical biosynthesis. The discovery of miRNA-phytochemical coexistence

in the exosome-like nanoparticles/edible nanoparticles (ENPs), along with other bioactive compounds in plants, unveils the possibility of cross-kingdom regulation mediated by miRNA-phytochemical interactions in humans and other entailed animals (Sundaram 2019). Even so, exhaustive research should be undertaken for the application of the ENPs to consider it as a potent dietary supplement constituting desired phytochemicals and respective regulatory miRNAs. Moreover, the silencing activity of miRNAs are spatially limited when compared to tasiRNAs, which can impart their effect over several cell layers (Schwab et al. 2009). This non-cell autonomous behavior of tasiRNAs, together with their biosynthesis-related factors and initiator miRNAs, demands an in-depth investigation to be performed, in order to expose their potency in regulating diverse biosynthetic pathways.

Artificial sRNAs like artificial miRNAs (amiRNAs) and synthetic tasiRNAs (syn-tasiRNAs) are two eminent emerging tools that promise immense applications in the field of crop improvement (Cisneros and Carbonell 2020). The exceptional potency of these artificial sRNA-mediated gene silencing, over conventional dsRNA silencing approach and their persisting significance in the era of CRISPR/CAS9-mediated gene editing, is complemented by several features like; least off-target silencing that prevents silencing sequences with high degree of similarity, accessibility to highly efficient cloning approaches and automated tools for designing desired sRNA constructs, secure optimization of sRNA cassette with multiple sRNAs, targeting single or more genes belonging to same or different gene family, without interrupting the expression of other genes (Carbonell 2019). Aiming to the ample application of artificial sRNA-based approaches, efficient in vivo and in vitro validation methods are required, so as to pick the finest amiRNA/atasiRNA candidates. One such recently emerged in vitro screening technique is represented by epitope-tagged protein-based amiRNA (ETPamir) screens. An inclusive enhancement is needed for such validation techniques to create a surge in the deployment of artificial sRNA-mediated approaches and to complement their best utilization (Zhang 2014). A summary of amiRNA and atasiRNA functioning is illustrated in Fig. 25.2. Another means to tinker with the regulation of phytochemical biosynthesis is by exploring lncRNAs that interact either in cis or trans manner with the genetic elements associated with plant metabolic pathways. Though there is considerable evidence disclosing the modulatory role of lncRNAs on significant genes of rate-limiting steps in distinct phytochemical production (Zhang et al. 2018), the proper investigation is hindered by their low sequence conservation and narrow expression level (Jha et al. 2020). Nevertheless, these regulatory transcripts with modest expression can perform diverse modalities like epigenetic modifications and alternative splicing, acting as molecular decoys and even scaffolds, making them far-reaching (Jha et al. 2020). Furthermore, elevating evidences that pinpoint the functional interconnections between sRNAs, lncRNAs, and significant genes can be together exploited in metabolic engineering to meet the escalating demand for improved plant varieties with increased phytochemical production.

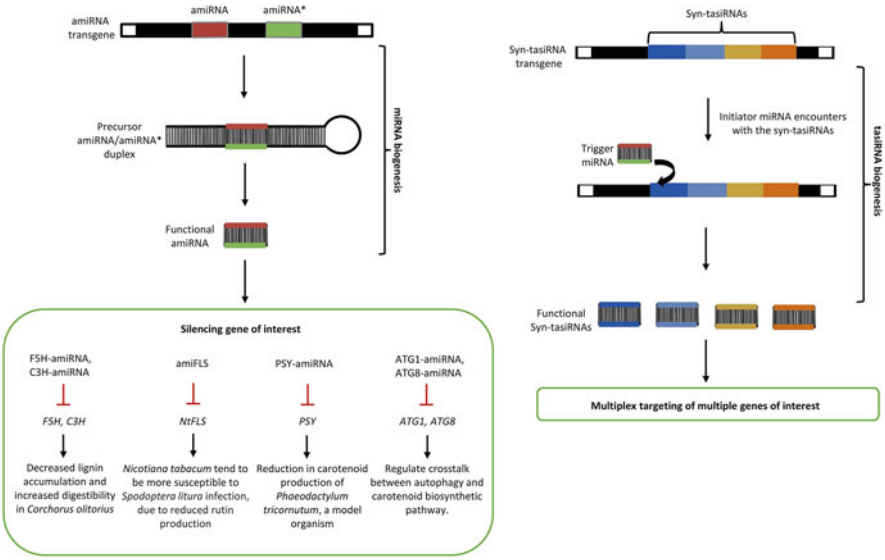

Fig. 25.2 amiRNAs and atasiRNA functioning with some example targets

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Part IV
Applications of Phytochemical Genomics

Chapter 26
Metabolomics and Genomics for Understanding Stress Biology of Plant Metabolites

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1 Introduction

Environmental stresses like salt, drought, cold, high temperature, salinity, oxidative stress, and microbial infection change the plant growth and development and disturb the metabolic homeostasis of plants (Atkinson and Urwin 2012; Ma et al. 2020). To further attend to their metabolic homeostasis, plants secrete certain metabolites that tolerate the stress condition of plants. This process of attaining metabolic homeostasis by plants through secreting metabolites is known as acclimation (Mittler 2006). In the process of acclimation different phases are involved. Firstly, the plant senses the stress condition and activates various signalling pathways that stimulate the production of different compounds and proteins that help in rejuvenating homeostasis. The different types of compounds involved in the rejuvenation of homeostasis are the compounds secreted by plants as protectants such as osmoprotectants and antioxidants, the compounds which are released due to damage of plant parts, and the compounds which are part of signal transduction pathway of stress response. The metabolites which are produced by plants to re-attend homeostasis are dimethylsulfoniopropionate, sorbitol, mannitol, myo-inositol, and sugars such as fructose and sucrose, amino acids, tocopherols, anthocyanin, jasmonic acid, and ascorbic acids (Roychoudhury et al. 2011). The sugars and amino acid molecules act as osmoprotectants and osmolytes during drought and salt stress. The

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M. K. Swamy, A. Kumar (eds.), *Phytochemical Genomics*,
https://doi.org/10.1007/978-981-19-5779-6_26

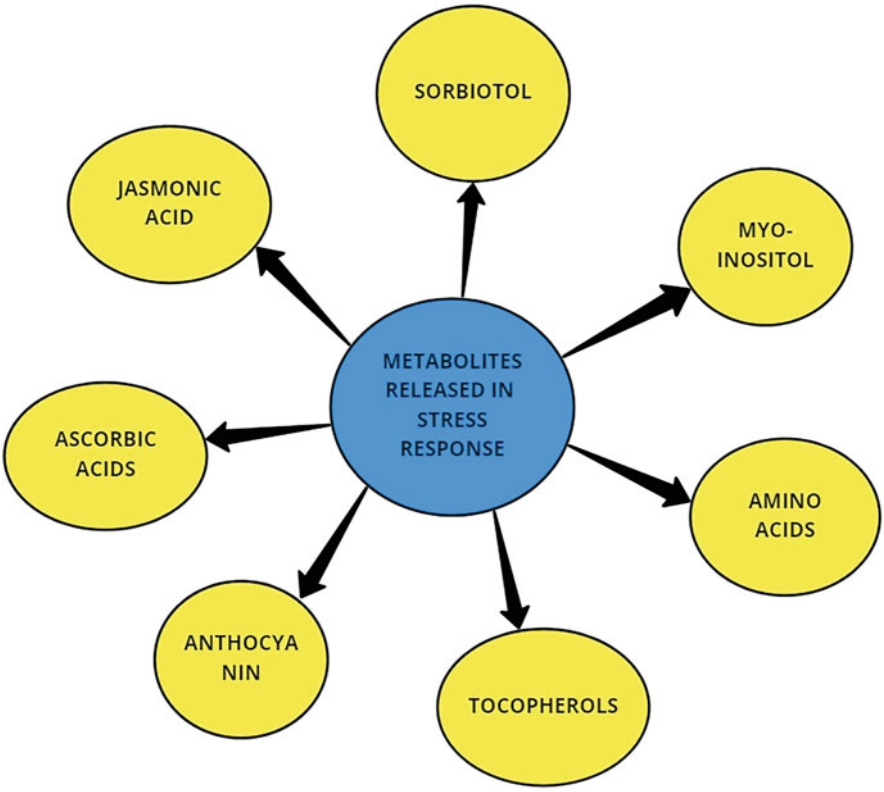

Fig. 26.1 Metabolites released in plants in response to stresses

epicuticular waxes of plants protect them from external pathogens and also prevent water loss during drought (Cameron et al. 2006; Laskoś et al. 2021). The other substances such as tocopherols, glutathione, ascorbic acids, and carotenoids protect plants from oxidative damage by free radical compounds by scavenging them. The methyl jasmonate, methyl salicylate, jasmonic acid, and many other molecules are a part of signalling cascade that activate the defence response of plants. The different metabolites produced in plant stress response are depicted in Fig. 26.1.

Plant- and microbe-produced metabolites play a critical role in the formation of symbiosis (Abdul Rahman et al. 2021; Kawaguchi and Minamisawa 2010; Thajuddin et al. 2015). There are more than 200,000 secondary metabolites known (Erb and Kliebenstein 2020; Teoh 2016). Chemical variety exists both within and between chemical classes. For example, the flavonoid family is thought to have about 7000 members, each of which produces 232 distinct glycosides from a single flavanol (Gaafar et al. 2020). For both the rhizosphere and root systems, several of these metabolites can predict the species richness and ecology of microorganisms (Kumar and Pandey 2013). Some microorganisms are attracted to specific metabolites, whereas others are not. The compound Blumenol is reported as a key factor in understanding plant–microbes interaction. Blumenol C glycoside belongs to a class

of chemical compounds known as acyl glycosides, which have a mono- or disaccharide moiety connected to a fatty alcohol's hydroxyl group (Wang et al. 2018). Its derivatives have a favourable correlation with AMF root colonization and are transferred from roots to leaves during root AMF association formation and more significantly, non-mycorrhizal plants cannot detect these compounds (Wang et al. 2018).

The genome of an organism is the sum total of the information stored in the DNA. The genome is expressed during stress conditions and lead to certain important metabolites in response to the stresses (Madlung and Comai 2004). When a plant encounters stresses, they stimulate the expression of certain genes which try to re-attend the homeostasis of plants; these genes are stress-tolerant genes. Jwa et al. (2006) have reviewed genes responsible for the production of proteins and secondary metabolites in response to different stresses in rice. Further, expression pattern of genes such as OsPR1ab, OsPR2, OsCATC, and OsPAL and OsCHS, responsible for the production of secondary metabolites in response to biotic and abiotic stresses in rice is also reported (Jwa et al. 2006). The advancement in genome sequencing and systems biology has helped in the genome sequencing of plant varieties and the determination of genes involved in the production of metabolites in response to plant stress. For the genomic and proteomic research, two-dimensional gel electrophoresis, liquid chromatography coupled with tandem mass spectroscopy, and MALDI-TOF are performed and the genomic components of stress defence systems are elucidated by qRT-PCR, microarray, and serial analysis of gene expression (Roychoudhury et al. 2011).

So the metabolomic and genomic studies to elucidate the metabolites and the genes responsible for their expression in stress response certainly helps in agronomic development and achievement of food security (Sun et al. 2020). The different metabolomic and genomic approaches involved in understanding stress biology of plant metabolites are shown in Fig. 26.2. Detailed and time-course profiling of metabolites and genome analysis helps identify many compounds and genes responsible for stress tolerance (Yuan et al. 2018). With this knowledge, we can detect the stress condition and we would also be able to advance stress tolerance in many crop varieties through genetic engineering technology. In this chapter, we discuss the metabolomic and genomic aspects of plant metabolites under stress conditions.

2 Metabolomic Approaches

Metabolomic technology includes three different approaches for the research, which are metabolite profiling, metabolic fingerprinting, and targeted analysis (Fiehn 2002; Halket et al. 2005). These approaches are used to identify the metabolome of the plant; as per the requirement they are used separately or in the combination of two or three. Below we are describing the three approaches involved in metabolomics.

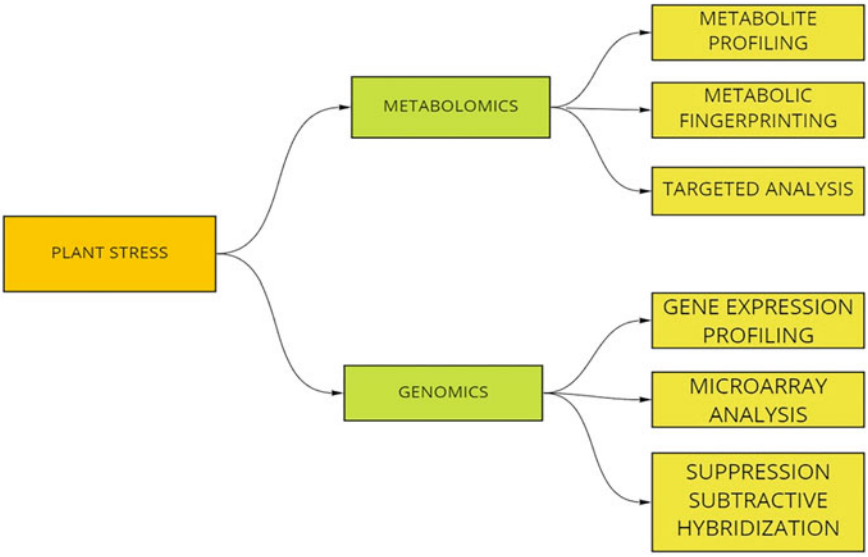

Fig. 26.2 Different metabolomics and genomics approaches

2.1 Metabolite Profiling

The measurement of all the metabolites present in a sample is known as metabolite profiling. The various analytical techniques used in the process of metabolites proofing are GC-MS, liquid chromatography-mass spectrometry (LC-MS), nuclear magnetic resonance (NMR), liquid chromatography-mass spectroscopy (LC-MS), FT-IR spectroscopy, and capillary electrophoresis-mass spectrometry (CE-MS) (Lisec et al. 2006; Shulaev 2006; Theodoridis et al. 2008).

In metabolite profiling analytical methods, gas chromatography-mass spectroscopy (GC-MS) is the most useful and efficient way of metabolic profiling. GC-MS is more reliable and accurate than nuclear magnetic resonance and also more powerful than liquid chromatography-mass spectrometry (LC-MS) (Lisec et al. 2006). The GC-MS is accomplished using time of flight (TOF) mass spectrometry or electron impact (EI) quadrupole (Roessner et al. 2000). The positive point of using GC-MS is that the EI spectral libraries are available both commercially and publically. But metabolite profiling through GC-MS can be performed only for volatile compounds and compounds that can be volatilized. GC-MS can be used for the profiling of many compounds such as organic acids, amino acids, aromatic amines, alcohols, and sugars.

But for the profiling of non-volatile compounds capillary electrophoresis-mass spectrometry (CE-MS) and liquid chromatography-mass spectrometry (LC-MS) are performed. The liquid chromatography-mass spectroscopy is highly efficient in profiling and less time-consuming (Giri et al. 2007). Capillary electrophoresis-mass spectrometry (CE-MS) can separate nearly 1000 charged compounds such as

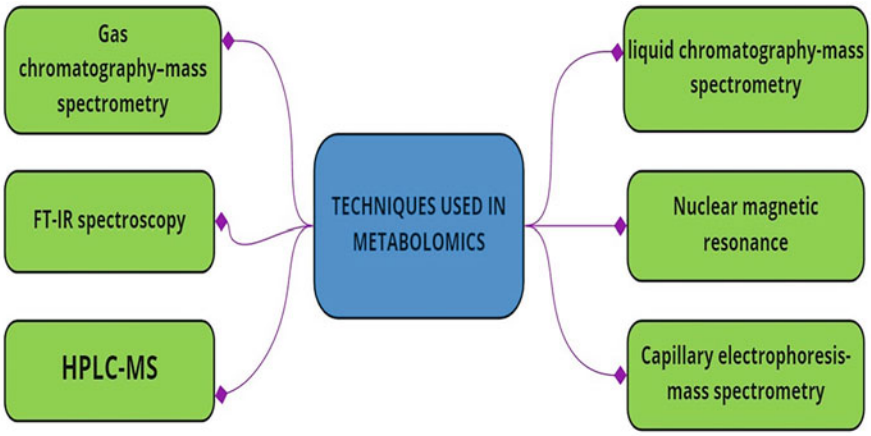

Fig. 26.3 Different analytical techniques used in metabolomics

cations and anions, it requires low sample volume, and has high resolving power (Harada and Fukusaki 2009). Okada et al. (2010) have explained the importance of metabolomics in plants. Several other researchers have also extensively reviewed the metabolomics of medicinal plants and their importance (Mukherjee et al. 2016; Mumtaz et al. 2017; Satheeshkumar et al. 2012; Shyur et al. 2013; Xiao et al. 2022). Gonulalan et al. (2020) have performed the metabolomics of several medicinally important plants such as *Valeriana officinalis* L., *Melissa officinalis* L., *Hypericum perforatum* L., and *Passiflora incarnata* L. and correlated their brain-derived neurotrophic factor (BDNF) activity. The utility of metabolomics in medicinal plants has been extensively proved through the metabolomic profiling of important medicinal plants. Some recent examples of metabolomics in medicinal plants are *Panax quinquefolium* (Di et al. 2022), *Crocus cancellatus* subsp. *damascenus* (Shakeri et al. 2022), *Moringa oleifera* (Abdel Shakour et al. 2022), *Sophora japonica* (Wang et al. 2022a), *Panax ginseng* (Yoon et al. 2022), *Bupleurum chinense* and *Bupleurum scorzonerifolium* (Qu et al. 2022), and *Salvia miltiorrhiza* (Lu et al. 2022). Specialized metabolome data of 337 traditionally important medicinal plants provide important information about the traditionally important plants (Kang et al. 2022). Figure 26.3 shows the different analytical techniques involved in metabolomics.

2.2 Metabolic Fingerprinting

Metabolic fingerprinting is an important approach for comparison of overall metabolic composition of the plant samples, it is also used for the discrimination of the related samples (Krishnan et al. 2004). Using this approach, the metabolic responses associated with a particular stress can be identified (Krishnan et al. 2004). The

analytical techniques used in metabolic fingerprinting are Fourier transform ion cyclotron resonance mass spectrometry, Fourier transform infrared (FT-IR) spectroscopy, nuclear magnetic resonance (NMR), and mass spectrometry (Goodacre 2005).

Different statistic and pattern recognition techniques are employed in metabolic fingerprinting. Supervised, unsupervised, and machine learning methods are utilized in metabolic fingerprinting. Principal component analysis (PCA) and self-organizing maps (SOMs) are unsupervised methods and partial least squares (PLS) and discriminant function analysis (DFA) are supervised methods (Johnson et al. 2003). Supervised algorithms such as DFA and PLS are sometimes used in combination with genetic algorithms, which is a type of evolutionary algorithm (Goodacre 2005). Several studies have reported the metabolic fingerprinting of plants including some of the medicinally important plants such as *Flourensia fiebrigii*, *Berberis laurina and Pleione* spp (Ali et al. 2020, 2021; Choi et al. 2004; Leal et al. 2021a, 2021b; Lim Ah Tock et al. 2021; Sotelo-Silveira et al. 2015; Wang et al. 2022b; Zayed et al. 2022; Zhang et al. 2021a). These studies suggest the role of metabolic fingerprnting in medicinal plants research.

2.3 Targeted and Untargeted Metabolomics

For the determination of the exact amount of the small quantities of known metabolites, targeted metabolomic analysis is performed (Roberts et al. 2012). For precise measurement of metabolite quantities, it is required that the targeted metabolites must be in pure and isotope-labelled form. But it is a great challenge to obtain metabolites in pure form. To resolve this, alternative ways can be used, which involve growing plants in liquid media containing stable isotopes of ^{15}N and ^{13}C (Hegeman et al. 2007). Quantitative metabolic profiling was successfully performed in microorganisms using uniform metabolic labelling combined with mass spectrometry (Wu et al. 2005). Targeted metabolomics is aimed at specific compounds with specific groups whereas untargeted metabolomics involves the comprehensive analysis of the total metabolites including unknown chemical compounds (Roberts et al. 2012). Both these approaches have different experimental objectives. The type of approach directly depends upon the type of plant sample and the objective of the research. Various assays/protocols have been developed for the targeted and untargeted metabolomics of the plants for different plants (Zheng et al. 2021). Some researchers have used both approaches for the understanding of the metabolic responses in plants such as *Camellia sinensis, (*Wu et al. 2021, 2022). On the other hand, several studies have deployed targeted approaches for the metabolomics, and some have used untargeted approaches. Targeted metabolomics of plants such as *Cistanche deserticola* (Ai et al. 2021), *Rehmannia glutinosa* (Zhou et al. 2021) has been reported. Examples of untargeted matabolomics include *Pisum sativum* (Calabrese et al. 2023), *Hibiscus mutabilis, H. schizopetalus*, and *Malvaviscus arboreus* (Abdelhafez et al. 2020) and *Annona muricata* (Cárdenas et al.

2021). Various researchers have reviewed the metabolomics of plants in detail (Beale et al. 2018; Begou et al. 2017; Daskalchuk et al. 2006; Kharbach et al. 2020; Tian et al. 2020).

3 Stresses and Metabolomics

Metabolomics is used to analyze the effects of different stresses in plants like water (Ju et al. 2018; Warren et al. 2012), temperature (Kaplan et al. 2004; Obata and Fernie 2012), oxidative stress (Noctor et al. 2015), sulphur (Ghatak et al. 2018; Nikiforova et al. 2005), phosphorus (Hernández et al. 2007; Zhang et al. 2021b), metal stress (Feng et al. 2020; Zhang et al. 2018), and biotic stress (Balmer et al. 2013). The study of metabolome profiles of medicinal plants under various stress can provide important information about the diverse metabolomic profiles. The metabolomics of plants under a given tissue provides set of metabolites that are expressed in that condition. This information is important for further studies related to understanding of the metabolic regulation of the medicinal plants stress adaptation. Several authors have reviewed the metabolomics of plants under stress conditions (Anzano et al. 2021; Bueno and Lopes 2020; Jorge et al. 2016; Villate et al. 2021). Targeted metabolome analysis of rice under pesticide (Diazinon) stress show variation in metabolite accumulation when compared with control plants (Mahdavi et al. 2015). Osmotic, salinity, and low temperature stress is shown to alter carbohydrate dynamics in medicinal plant *Olea europaea* (Rejšková et al. 2007). These studies are crucial for better understanding of the metabolic dynamics in plants under stressful conditions. We briefly describe some of these studies in the following sections.

3.1 Water and Salinity Stress

Metabolic profiling was performed on the two species of genus *Eucalyptu*s using GC-MS to analyze the water stress effect (Warren et al. 2012). This study found that water deficit decreases the osmotic potential of the plant and amplifify the amount of total active osmolytes in the leaves of plants (Warren et al. 2012). Martinelli et al. (2013) performed metabolic profiling of *Olea europaea* (L.) fruit to analyze the effect of water stress using GC-MS, they analyzed 176 metabolites and identified 57 of them. Similarly, another experiment to detect the consequence of water deficit on the malting of barley grain was performed using metabolite profiling (Wu et al. 2017). Chen et al. (2019) studied metabolic response in Apocyni Veneti folium, staple traditional Chinese medicine extracts due to salt stress using ultra-fast liquid chromatography. Johnson et al. (2003) studied salt stress on tomatoes using the metabolic fingerprinting method. They used Fourier transformed-infrared spectroscopy and a total of 882 variables were present in sample, which were difficult to analyze manually, so machine learning methods were employed. Alhaithloul et al.

(2019) observed changes in secondary metabolites in *Mentha piperita* and *Catharanthus roseus* under drought and heat stress. Salinity-induced changes in the alkaloids contents were observed by Wang et al. (2008) in *Catharanthus roseus*. Lipidomics of *Thymus serpyllum* (tolerant) and *Thymus vulgaris* (sensitive) under drought stress showed changed accumulation of the lipids in both the species suggesting that drought stress alters the lipid profiles of the plants (Moradi et al. 2017). Multiple abiotic stresses in *Artemisia annua* led to alteration in artemisinin content (Vashisth et al. 2018). These studies suggest that abiotic stress particularly salinity and drought affects the metabolic functioning of medicinal plants.

3.2 Temperature Stress

Arabidopsis was analyzed by metabolic profiling, for which gas chromatography-mass spectrometry was used. The profiling was done to analyze metabolite temporal dynamics of Arabidopsis in response to temperature stress i.e. heat shock and cold shock. The 143 metabolites in heat shock and 311 metabolites in cold shock were altered (Kaplan et al. 2004). Cook et al. (2004) examined the effect of low temperature on *Arabidopsis* using gas chromatography-time of flight mass spectrometry (GC-TOF MS) and found that 75% of metabolites were increased to an abnormal level in cold-stressed *Arabidopsis.* Morsy et al. (2007) performed targeted profiling on *Oryza sativa* and found alteration in carbohydrate metabolism under cold stress, salt stress, and osmotic stress. In this study, the quantitative HPLC assay was performed, and soluble carbohydrate level was measured on chilling-tolerant and chilling-sensitive genotype under chilling conditions.

3.3 Phosphorus and Sulphur Stress

A combined metabolomics and transcriptomics study was performed on bean plants cultivated under phosphorus-deprived and phosphorus-sufficient conditions to investigate the metabolomic and transcriptional response of the plant (Hernández et al. 2007). The authors used GC-MS for the metabolite profiling of the bean roots (Hernández et al. 2007). Gao et al. (2020) performed a metabolomic analysis of lettuce leaves under low phosphorus stress and low nitrogen stress. For metabolic analysis, they used ultra-performance liquid chromatography-quadrupole time-of-flight mass spectrometry. The metabolic studies related to sulphur stress were also performed. Nikiforova et al. (2005) studied sulphur deficiency in *Arabidopsis.* They used GC-MS profiling and LC-MS and analyzed the response of metabolites in sulphur-deficiency stress. Untargeted and targeted metabolomic profiling of sulphur deficiency in barley was performed using ultra-high-performance liquid chromatography-quadrupole/time-of-flight mass spectrometry (Ghosson et al. 2018).

3.4 Oxidative Stress

Under low oxygen stress, the metabolic profiling of pears (*Pyrus communis*) was performed using gas chromatography/electrospray ionization-time of flight-mass spectrometry GC-EI-TOF-MS. The researchers found that the pear develops core breakdown if it is stored in low and high oxygen conditions. Through metabolic profiling, they hypothesized that there is a gradual upturn in the level of fumaric acid and gamma-aminobutyric acid and a reduction of mallic acid (Pedreschi et al. 2009). Baxter et al. (2007) elucidated the changing aspects of metabolic changes in *Arabidopsis thaliana* cells under oxidative stress. The GC-MS profiling was used by them and the level of metabolites in stressed cells was measured. They found that due to oxidative stress, TCA cycle and amino acid metabolism were inhibited.

4 Genomic Approaches

Genome is the complete genetic material of an organism. In genomics, the complete genome of an organism is studied. Genomics tools can be used for the study of stress related changes in the metabolomic profiles of the plants. In this chapter, we are more focused to understand the biology of metabolites production during different stresses condition. So we have to focus on genes that are involved in metabolites production. The molecular analysis of expression of genes that are expressed during stress is performed. The results of this molecular analysis are collected as expressed sequence tags (ESTs). Different techniques such as qRT-PCR, gene expression profiling, and microarray analysis are performed to detect the different genes involved in metabolites production (Bohnert et al. 2001). The recent advancement in sequencing technologies, such as next-generation sequencing (NGS), has made it more fast and easy to sequence the genes responsible for the production of metabolites during stress (Kim and Buell 2015). Another technology to identify the genes that are differentially expressed is suppression subtractive hybridization (SSH); it is much more inexpensive than microarray technology. The SSH technology is utilized to identify the genes expressed under stresses such as ozone stress and UV-B radiation (Ban et al. 2007; Peal et al. 2010). Hazen et al. (2003) studied the gene expression profiling of plants during stress condition and elucidated how plant physiology is affected by abiotic stress. By this genomic approach, they found some regulators which can be used in biotechnological methods to improve plant response to stress. Thus genomic approaches are very useful in finding genes and regulators associated with plant stress tolerance. Biosynthesis of plant metabolites undergoes alterations in response to the biotic and abiotic stresses. However, analysis of the metabolomes of the plants alone under stresses is not enough to understand the stress biology of the plants. The genomic approaches must be integrated to the metabolomics data to look into the expression pattern of the key genes that code for the enzymes crucial for the biosynthesis of the corresponding metabolites. Since the stresses alter the

metabolome of a plant, the stresses must be at the gene level as well. Therefore understanding the behaviour of the genes through genomics approaches is important to get an integrated and wholesome view of the abiotic and biotic stress tolerance in medicinal plants. This integrated analysis also provides crucial details about the quantities of the metabolites that are produced under a given stress condition. Accordingly, stresses can be used to induce the production of medicinally important metabolites in plants. Zhang et al. (2021c) have identified several differentially upregulated metabolites between the control and drought stress-induced natural inbred lines of maize. This study further provides important information about the metabolome regulated drought stress adaptation in maize. Targeted metabolome analysis of maize under heat and *Cochliobolus heterostrophus* stress shows that preexposure of maize to heat stress increases its vulnerability to *C. heterostrophus* infection (Christensen et al. 2021). This study found the correlation between hydroxycinnamic acid and p-coumaric acid deficiency and increased susceptibility of maize to *C. heterostrophus* infection. Wu et al. (2003) observed changes in the expression of phosphate deprivation responsive genes in Arabidopsis roots and leaves. Combined application of metabolomics and transcriptomics to *Dendrobium sinense* showed links between the differentially expressed genes and metabolites (Zhang et al. 2021d). So, below we are discussing some stress conditions in which genomic approaches to detect the genes responsible for metabolites production are studied.

4.1 Salinity and Drought Stress

Salinity is an important stress factor that reduces the crop productivity. The effect of salinity stress in various medicinal plants has been studied at genomics as well as metabolomics level. Salinity-induced changes in the metabolites were observed in *Salvia mirzayanii* (Valifard et al. 2019). This study further identified an important gene in *S. mirzayanii* named cineole synthase 1 gene (*SmCin*1). In a study, the *Hibiscus tiliaceus* plant's gene expression profiling was performed using cDNA microarray technology, under salt stress condition. The researchers identified 486 genes that are expressed in salt stress response (Yang et al. 2011). Increased expression of glycyrrhizin biosynthesis related genes under salinity stress was observed in *Glycyrrhiza glabra* (Shirazi et al. 2019). Salinity stress-induced changes in the transcriptomic changes in *G. inflata* show that differential expression of several genes including transcription factors might be crucial for salinity stress tolerance (Xu et al. 2021). Differential expression of several thousand genes under salinity gradients was observed in Prunellae Spica (Liu et al. 2020). Wei et al. (2017) proved the role of *AtDREB1C* gene in imparting drought stress tolerance in *S. miltiorrhiza*.

4.2 Temperature Stress (Cold and Heat Stress)

Chauhan et al. (2011) identified and characterized the genes responsible for high-temperature stress tolerance in the wheat plants (*Triticum aestivum*). They utilized RT-PCR analysis for the confirmation of differential expression of the genes**.** Another study on the expression of genes involved in abscisic acid metabolism and transport under cold and heat stress was performed in *Arabidopsis thaliana* (Baron et al. 2012). In this study, it was found that during stress, expression pattern of the genes involved in the metabolism and transport of abscisic acid is altered (Baron et al. 2012). This study found differential expression of important genes linked to ABA metabolism and transport. In an experiment, the reference genes were selected for standardisation of qRT-PCR in sugarcane buds affected by cold stress (Yang et al. 2016). Transcriptome profiling of *Tetrastigma hemsleyanum* under cold stress helped in the identification of genes such as *PAL*, *4CL*, *CHS*, *ANR*, *FLS*, and *LAR* (Peng et al. 2019). The expression of these genes also correlated with an increased accumulation of the flavonoids in *T. hemsleyanum*.

4.3 Metal Stress

Plants secrete phytochelatins (PCs) in response to heavy metals, which bind to heavy metals (Gupta et al. 2013). The genes responsible for the production of PC synthase were identified using different genomic approaches in Arabidopsis (Cobbett 2000). AtPCS1, a gene responsible for the production of phytochelatins synthase was isolated from *Arabidopsis* and in vitro reconstituted and expressed in the microbial systems against heavy metal stress (Vatamaniuk et al. 1999). The expression of the gene responsible for the production of the plant hormone epibrassinolide was studied in *Oryza sativa* and found that they are capable of tolerating heavy metal stress. Epibrassinolide (EBL), a plant steroidal hormone, is known to regulate heavy metal stress in plants. A study demonstrated that application of EBL in rice helps it to tolerate chromium metal stress and this tolerance is partly achieved through the upregulation of rice antioxidant machinery (Sharma et al. 2016). Effect of chromium stress was studied on radish (*Raphanus sativus* L.) root by Xie et al. (2015). In this study they identified the genes that were differentially expressed due to chromium stress by utilizing RNA sequencing technique. They found that the expression of 2985 genes was altered, expression of some of genes were up-regulated, while of some genes were down-regulated. Several other studies have also reported the metabolomic and transcriptomic profiling of medicinal plants under metal stresses (Li et al. 2021; Wang et al. 2020; Yuan et al. 2022).

4.4 Microbial Stress

Apart from abiotic stresses, plants are prone to various bacterial and fungal pathogens and thus plants secrete metabolites that usually have antimicrobial properties. These secondary metabolites help plants survive the pathogenic attacks. One such metabolite secreted by the plants is phytoalexin. *Fusarium oxysporum* is known to cause root rot disease in *Panax notoginseng* (Ning et al. 2021). Ning et al. (2021) found higher expression of *PnWRKY22* gene in response to *P. oxysporum* infection in the resistant genotype. This study suggested its involvement in conferring root rot resistance against *P. oxysporum* to resistant genotype of *P. notoginseng*. Recently, Wen et al. (2022) showed that WRKY gene family might play an important role in *Akebia trifoliata* against *Colletotrichum acutatum* infection.

4.5 Oxidative Stress

The analysis of gene expression through cDNA array analysis and mRNA differential display was performed under oxidative stress in the plant *Nicotiana tabacum*. The study suggested that the expression of nearly 95 genes was altered and the genes involved in the production of metabolites, antioxidant compounds, and part of signal transduction pathway were also altered (Vranová et al. 2002). In the tobacco plant, expression of gene under oxidative stress due to hydrogen peroxide was analyzed using transcriptome profiling. This study revealed that the differential expression of different genes. Such genes responsible for the biosynthesis of certain metabolites like ethylene and jasmonic acid were differentially regulated (Vandenabeele et al. 2003).

5 Data Analysis

The amount of metabolomic data created by the experiments is enormous, and it must be compiled and thoroughly analyzed in order to produce meaningful results (Cambiaghi et al. 2016). These data are linked to the host plant, resident microorganisms, and environmental stress.

From species to species, the metabolites and metabolic pathways are different (Moghe et al. 2017). The combined analysis of metabolic, transcriptomic and genomic data will aid in the understanding of stress tolerance in plants and the production of metabolites under stress (Castrillón-Arbeláez and Délano Frier 2016; Li et al. 2022). Various approaches and methods are used for the analysis of metabolomics data for comprehending the metabolites and their diversity and their biosynthesis. The metabolomics data analysis is dependent upon the type of method/tool used and the objective of the study. Preprocessing, targeted, nontargeted

metabolomic data, and peak identification are important steps in data preprocessing (Lamichhane et al. 2018). Further analysis includes application of univariate or multivariate approaches for data analysis (Lamichhane et al. 2018). The integration of the metabolomics data with other omics data needs further expertise in other bioinformatics tools (Li et al. 2012). The linking of the metabolites and other omics data with the pathways is also done to understand the connections between the metabolites and their biosynthetic pathways (Lamichhane et al. 2018). Linking of genomic and metabolomic data under various stress conditions is an important aspect in phytochemical genomics because integrated analysis of metabolomics and genomics data can provide an overall picture of the genetic and metabolic regulation of tolerance to stresses. It also provides information about the set of genes that are expressed under a given condition in a particular tissue. The stress-induced changes in metabolite quantity and quality are also an important area. Therefore, integration of the omics data is crucial in medicinal plants research.

6 Conclusions

Plants face continuous abiotic and biotic and stresses throughout their life cycle. Stresses such as salinity, heavy metal drought, and oxidative stresses disturb their natural homeostasis leading to reduced productivity and yield losses. Plants secrete different metabolites to cope with the stresses and to reorient homeostasis. Metabolites such as amino acids, organic acids, sucrose, fructose, and myo-inositol are key compounds that help plants to resist stress. With the help of system biology and 'omics' technology, we can find the metabolites produced by plants and genes associated with the production of these metabolites. For this, we use metabolomics and genomics approaches. In metabolomic approaches, with different techniques such as metabolic profiling, metabolomic fingerprinting, and targeted analysis using GC-MS, NMR, and HPLC a metabolite produced during a particular stress can be deciphered. Similarly, genomic approaches involve genome profiling, microarray technology, and qRT-PCR to determine the genes responsible for the production of metabolites. Knowing all this information about metabolites and their genes may help in developing new stress resistance crops through genetic engineering. Integrated application of metabolomics and genomics tools to medicinal plants is crucial for the understanding of the important genes that code for the enzymes responsible for medicinally important metabolites. The information obtained through combined metabolomics and genomics tools can be meaningfully translated to the medicinally important plants for enhanced production of important metabolites with health benefits.

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Chapter 27
DNA Barcoding for the Substantiation of Herbal Products

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1 Introduction

Treatment and management of diseases in humans and animals have relied upon medicinal plants, since ancient times. In traditional medicine, there is ample use of medicinal plants. In addition, they have been employed adequately in modern drug discovery as well. These have significantly contributed towards treatment of chronic diseases and maintenance of health conditions of the world populace. The World Health Organization (WHO) has reported that about 25% of the modern drugs have originated either directly or indirectly from medicinal plants. A significant proportion of the population both in developed and developing countries is known to rely on traditional medicines as a means of primary healthcare. Around 60% of the anticancer drugs have been derived from natural products. The drugs discovered from medicinal plants have also been applied in treating diseases, such as Alzheimer's, malaria, and HIV/AIDS.

Due to the importance that medicinal plants have gained in recent times, their accurate identification has become extremely essential, and is a prerequisite for their use in drug discovery. Traditional taxonomic plant identification systems require an

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M. K. Swamy, A. Kumar (eds.), *Phytochemical Genomics*,
https://doi.org/10.1007/978-981-19-5779-6_27

accurate identification of a set of morphological characteristics, particularly of the floral parts and fruits, which often involve awaiting the appropriate growing season hence making it time-consuming, and often difficult. Although prevalent in the traditional uses, lack of proper taxonomic identification of medicinal plants have hampered the herbal industry as the problem of substitution and adulteration prevails particularly by inclusion of closely related species.

In 2003, DNA barcoding technology was proposed for accurate species identification. This technology involves using a standard, short DNA sequence as a marker for rapid, accurate, and automatic species identification. Such a marker is called as a DNA barcode. Since then, DNA barcoding has been recognized as a renaissance for taxonomic identification of species and has been widely employed in many applications. Recently, DNA barcoding has emerged as a novel tool for not only species identification but also the study of molecular evolution. Hence, this is a molecular taxonomic, bioinformatics-based tool applied for identification, differentiation, and discovery of new species. DNA barcodes can be amplified by using universal primers, recovered and sequenced routinely for the characterization of a species. Global DNA reference libraries have been developed, which enable comparison and identification of barcode sequences of unknown plant species, and hence enable evaluation, preservation, and sustainable utilization of biodiversity for various purposes (Yu et al. 2021).

DNA barcodes employ short stretches of DNA (300–800) sequence from a quantified region of the genome. Various regions of the DNA act as a marker in case of barcoding but the marker should have low intra- and high interspecific variability (DNA barcoding gap) to depict efficient discriminatory power. The DNA barcode is said to be ideal when it provides discernment between species, contain a single primer pair that is universal with success in amplification and sequencing, is easy to retrieve, cost-effective, and has commendable discriminatory power. Consortium for the Barcode of Life (CBOL) and the International Barcode of Life (iBOL) are the two global ingenuities working to develop DNA barcodes. CBOL came into existence in 2004 to develop DNA barcodes for the identification of flora and fauna globally, while iBOL provides scientific and technical expertise in DNA and meta-barcoding. It maintains Barcode of Life Data systems (BOLD), barcode references and makes it accessible to the public (Mishra et al. 2016).

In vascular plants, chloroplast gene markers have been the primary focus for barcoding and several have been tested. The most commonly used combinations include *rbcL*, *matK*, and *trnH-psbA*, with a nuclear internal transcribed spacer (ITS2) (Yu et al. 2021; Saddhe and Kumar 2018; Pathak et al. 2018). DNA barcoding studies have been done on several medicinal plants. Of the 219 medicinal plant families recorded, 142 (including 832 genera) have characterized DNA barcodes. Overall, DNA barcodes have been applied to about 33.3% and 78.1% respectively of monocot and eudicot medicinal plant species, thus indicating the requirement of DNA barcode characterization for several remaining species (Yu et al. 2021).

Types of DNA barcode markers employed for the identification of medicinal plants include single-locus, multiple-locus, and genome-based DNA barcode markers including DNA sequences of the chloroplast genome and have been applied

to identify several medicinal plants, in the last decade. These contain more genetic information for species identification than any of the commonly used single-locus markers. The chloroplast genome of 3452 plants have been published on NCBI by the end of 2019 and hence is a promising contribution towards development of DNA barcodes present in the chloroplast genomes for the identification of plant species (Yu et al. 2021; Saddhe and Kumar 2018; Pathak et al. 2018).

However, recently consensus is emerging regarding the best technologies and markers applicable to medicinal plants. Cost, efficiency, and convenience are the general considerations of the DNA barcoding technology. It has been observed that single-locus markers are cost-effective, while marker combinations greatly improve efficiency. However, for intraspecific taxa such as different ecotypes, complete chloroplast genome sequences (super barcodes) are required for increased resolution. However, this is not usually required for medicinal plant identification, including most substitutes/adulterants. The development of DNA barcoding has been facilitated by sequencing technologies becoming cheaper and the availability of specific DNA barcodes targeting specific taxa of medicinal plants (Yu et al. 2021; Rajphriyadharshini and Weerasena 2020; Saddhe and Kumar 2018; Pathak et al. 2018; Mohamed et al. 2017; Enan et al. 2017).

In this chapter, various DNA barcode markers are discussed for the analysis of medicinal plants for phylogenetic, phylogeographical patterns, authentication process, inter- and intraspecific diversity, and adulteration detection.

2 Features of Barcoding Sequences in Plants

The universal animal barcode marker is the 5′ end of mitochondrial DNA (mt-DNA) cytochrome C oxidase 1 (CO1) which is 684 base pairs (bp) long. The preference for mitochondrial genes is due to lack of introns, low recombination, and abundant resource. While in the case of plant barcoding, the genome from chloroplast and nucleus are preferred, as the rate of mutation is low in mitochondrial genome (Vijayan and Tsou 2010).

Different kinds of DNA barcode markers used for the authentication of medicinal plants include (i) Single-locus DNA barcode markers, such as the *mat*K and *rbc*L, which are the main DNA sequences of plant DNA barcodes, with internal transcribed spacer (ITS) and *trn*H-*psb*A as complementary sequences. Wide-ranging experimentations and substantiation have established the ITS2 region as the primary DNA barcode and *trn*H-*psb*A as a complementary sequence for identifying medicinal plant species; (ii) Multiple-locus DNA barcode markers including combinations of DNA markers *mat*K, *rbc*L, *trn*H-*psb*A, and ITS sequences. Single-locus markers do not provide adequate information for precise identification in certain cases, and hence the combination of markers has been used for identifying medicinal plant species; (iii) Genome-based DNA barcode markers include DNA sequences of the chloroplast genome. Compared with commonly used single-locus markers, chloroplast genome-based markers provide more genetic information for species

identification. By 2019, NCBI has published chloroplast genomes of 3452 plants thus furthering development of chloroplast genome DNA barcodes identification and characterization of plant species.

MaturaseK gene (*matK*), ribulose-bisphosphate carboxylase gene (*rbcL*), internal transcribed spacer 1 and 2 (*ITS1* and *ITS2*) region of the nuclear ribosomal cistron, *trnH-psbA* intergenic spacer, plastid *trnL-F* and *atpF-atpH* encoding ATP synthase subunit CFOI and CFOIII respectively, *ycf5*, *psbK-1* encoding two polypeptide K and I, *psbM trnD*, ribosomal protein S16 (rps16), NADH dehydrogenase subunit I (*nad1*), DNA-directed RNA polymerase subunit β′(*rpoC1*), 5S-rRNA, and 18S-rRNA are some of the genes identified for DNA barcoding of plants (Techen et al. 2014).

In plant barcoding, amplification of the barcoded regions is hindered by the secondary metabolites, often which can be overcome by modifying the extraction method and use of polymerase. The traditional barcoding system following a single locus approach did not exhibit greater discriminatory power (Mishra et al. 2016). CBOL recommended a multilocus approach to discriminate plant species, for which they evaluated seven chloroplast genome sequences. *matK* and *rbcL* combination was recommended as the suitable plant barcode with 72% discriminatory efficiency. The China Plant BOL Group recommended the addition of *ITS* to the combination for better discrimination in closely related species (Techen et al. 2014). However, it was later found that the use of multiple loci did not make any improvement in differentiating at the species level (CBOL Plant Working Group 2009). Currently, the interest has shifted towards the use of complete chloroplast (cp) genomes as it is a circular and conserved gene content. The entire cp-genome contains the same amount of information as that of CO1 locus in animals, approximately. So, it can be utilized as a super-barcode to identify and differentiate closely related species (Wu et al. 2021). The chloroplast genome sequence in the plant could be considered as an analogue of the mitochondrial genome in animals, and both of them have high copy number per cell, universal primer, and conserved gene order. But, the limitation associated with chloroplast gene sequence is that the rate of evolution is slow (Shen et al. 2018). Some of the widely employed barcodes are described in this section.

matK is about 1550 bp in length, and is embedded within the intron of the lysine *trnK* gene, a chloroplast gene. It encodes maturase enzyme, which catalyzes the splicing of Group II intron from RNA transcript. It is a rapidly evolving plastid genome with a higher substitution rate compared to *rbcL*. Different taxonomic groups require a different set of primers. The CBOL Plant Working Group (2009) tested *matK* gene on 550 plant species and it revealed 90% efficiency in angiosperm DNA amplification using single primer pair, but the success rate was limited despite using multiple primer sets to 83% in gymnosperms and 10% in cryptogams. A 100% amplification success rate has been achieved in 1667 angiosperm plant samples when a specific set of primer was used to amplify matK gene (Lahaye et al. 2008). The discriminatory power of *matK* gene is more than 90% in the *Orchidaceae* species, while it is less than 49% in discriminating the nutmeg family (Newmaster et al. 2006). It can be inferred that *matK* alone is not an appropriate universal

barcode, but when combined with *rbcL* it is considered as a standard multiple-locus for plant barcoding.

rbcL is 1430 bp in length and it encodes the larger subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (RUBISCO, EC 4.1. 1.39), a key regulatory enzyme in carbon dioxide fixation and photosynthesis. *rbcL* gene is highly conserved with a slow rate of evolution, but the discriminatory power at the species level is moderate (Chen et al. 2015). It is one of the most characterized gene sequences widely used for phylogenetic studies with more than 50,000 sequences available in GenBank (Li et al. 2015). *rbcL* alone may not meet all the criteria of barcoding locus, but when combined with other nuclear or plastid loci it acts as a potential candidate for accurate identification.

rpoB and *rpoC1* are two of the genes encoded by plastid-encoded RNA polymerase (PEP). During the plastid gene transcription of photosynthetic higher plants, at least two RNA polymerases are involved. One of the RNA polymerases contains homolog of *Escherichia coli* enzyme, which includes the α (encodes plastid gene *rpoA*), β (encodes plastid gene *rpoB*), β′ (encodes plastid gene *rpoC1*), and β″ (encodes plastid gene *rpoC2*) subunits, and the genes are referred as PEP (Serino and Maliga 1998). *rpoC* was used for the phylogenetic analysis of Dipterocarpaceae, and rpoB formed the core gene for the phylogenetic analysis of bacteria. *rpoB* together with 16S rRNA is used for the identification and refining of bacteria along with demarcating new bacterial species. Despite yielding a good quality sequence and being highly universal, *rpoB* and *rpoC1* were eliminated by CBOL Plant Working Group for barcoding plants owing to their discriminatory power, which is low at the species level (Chase et al. 2007). Currently, *rpoC1* was found to be useful in *mosses* (bryophytes) barcoding (Liu et al. 2010).

With the current advancement in sequencing technology, the *ycf5* gene can be utilized for plant barcoding, although it is a small-single copy region encoding protein containing 313 amino acids. It is reported to yield a higher proportion of polymorphic sites and contains conserved genes in land plants. *ycf5* gene did not gain attention in the past due to problems in aligning the sequence and poor universality. It is absent in bryophytes (Kahlau et al. 2006).

accD (873 bp) is a chloroplast gene encoding the acetyl-CoA carboxylase subunit β (EC:2.1.3.15). Acetyl-CoA carboxylase is a rate-limiting enzyme that catalyzes the first step of fatty acid synthesis i.e. conversion acetyl-CoA to malonyl-CoA. *accD* gene is completely or partially absent in a few monocots (Poales and Acoraceae), wherein nuclear-encoded ACC enzyme replaces the plastid enzyme inside the chloroplasts of these groups (Harris et al. 2013). *accD* is often tested for its suitability to be used as a barcode. In buckwheat (*Fagopyrum* species), 5′ coding region of the *accD* is reported to advance five times faster, when compared to *rbcL* gene (Yasui and Ohnishi 1998).

ndh-11 family of genes (*ndhA*-*ndhK*) are involved in photosynthesis and codes for NADH-specific dehydrogenase. *ndh* genes are homologs of mitochondrial NADH dehydrogenase subunits involved in respiratory electron transport. *ndhJ* (ORF159) codes for NADH dehydrogenase 30 kDa subunits, and is associated with *ndhC* and *ndhK* as a single operon (Nakazono et al. 1996). It was proposed

as a supplementary locus for barcoding due to its low discrimination power and the absence of this gene in many economically important plants (ex: pines), which limited its usage as a suitable barcode. Recently *ycf1-ndhF* genes were reported to be a promising coding plastid DNA barcode in peach (*Prunus persica*) (Amar 2020).

Intergenic spacers and introns form the non-coding sequence, which is widely utilized to study the low taxonomic species. Chloroplast intergenic *psbA-trnH* spacer is one of the non-coding regions with a high rate of insertion/deletion, and exhibits high sequence divergence among species. *psbA-trnH* intergenic spacer is located between the *psbA* gene (encodes protein D1 which forms reaction centre of photosystem II along with protein D2) and histidine transfer RNA (*trnH*) gene. *psbA* region is highly conserved, while *trnH* is variable. It has an approximate length of 450 bp, but according to the available data, it may vary from 290 to 1260 bp. The length of these genes varies due to the presence of pseudogene and duplicate loci; in some cases, it is >1000 bp in some monocots and confers, <300 bp in angiosperm, and <100 bp in bryophytes (Kress and Erickson 2007). *psbA-trnH* gene could categorize close to all species of *Hydrocotyle, Dendrobium,* and *Pteridophytes*. The limitation associated with these intergenic spacers is their alignment because of their variable length, which can be overcome by improvising the the Basic Local Alignment Search Tool (BLASTn) search, and the other being used shorter spacers may not lead to suitable sequence variation in discriminating species (Degtjareva et al. 2012). The CBOL Plant Working Group, in 2009, reported the discriminatory power of *psbA-trnH* intergenic spacer to be 69%, when compared with seven loci, and categorized it into supplementary locus. When non-coding spacers (*psbA-trnH*) were combined with either of the coding regions (*rbcL-a, rpoB2, rpoC1*), there was an increase in polymerase chain reaction (PCR) primer success rate, and also found higher proportion of differentiated species pairs. *psbA-trnH* + *ITS1* and *rbcL* + *matK* locus combination exhibited better-differentiated species pair compared to single-loci (Kress and Erickson 2007).

trnL-trnF locus includes tRNA-Leu ($trnL^{UAA}$) and tRNA-Phe ($trnF^{GAA}$) genes, introns, and intergenic spacers separating *trnL* from *trnF* that are widely used for phylogenetic analysis of intraspecific variations. This locus is unique as it consists of a conserved secondary structure along with a conserved and variable region (Hao et al. 2009). Taberlet et al. (1991) introduced the use of *trnL* intron-*trnF* spacers to study various taxonomic levels. The limitation associated with *trnL* intron is low resolution. As of now, the CBOL Plant Working Group has not tested the suitability of this region for plant barcoding but it is used as a supplementary locus for PCR amplification of DNA from highly degraded tissue, as universal primers are available for these (Vijayan and Tsou 2010).

atpH-atpF genes code for the chloroplast ATP synthase located in thylakoid membranes. ATP synthase is a multi-subunit complex required to generate ATP by utilizing the proton motive force produced by the electron transport chain. It is composed of two trans-membrane domain CF_o ($I_1II_1III_{14}IV_1$) and catalytic domain CF_1 ($\alpha_3\beta_3\gamma_1\varepsilon_1\delta_1$) modules. CF_o subunit I codes for gene *atpF*, II for *atpG*, III for *atpH*, and IV for *atpI* (Zhang et al. 2019). The use of *atpH-atpF* spacer for plant barcoding was proposed in the Second international Barcode of Life Conference in

Taipei, 2007. The CBOL Plant Working Group reported the universality of sequence with diffident discriminatory power and intermediary sequence quality, thereby listing it as supplementary loci apt for combining with two-locus standard barcode (Wang et al. 2010).

Photosystem II is required for the light reaction of photosynthesis, and it consists of 20 diverse polypeptides, out of which 12 encodes chloroplast gene. *psbK* (ORF98) and psbI (ORF52) genes code for low molecular weight polypeptide K and I, which are a part of photosystem II. $trnG^{UCC}$ (691 bp intron in the D-stem) and $trnR^{UCU}$ are present downstream of *psbK–psbI* (Meng et al. 1991). The use of this intergenic spacer as a barcode in plants was proposed in the Second international Barcode of Life Conference in Taipei, 2007, along with *atpH-atpF*, as it displayed lower sequence quality with a high level of discriminatory power and universality. The use of intergenic sequence (*atpH-atpF* and *psbK-psbI*) as potential barcodes were evaluated on trees and shrubs of the flora of Kruger National Park in South Africa. There is a paucity of data for these two markers as they are not utilized widely in phylogenetic studies. It is categorized as a supplementary loci (Lahaye et al. 2008).

For the plants, which have clearly defined barcode gap between inter- and intraspecific *ITS* can be employed as a sequence region. The internal transcribed spacer (ITS) region of the nuclear ribosomal DNA (rDNA) is the only nuclear genome sequence tested for suitability in plant barcoding (Vijayan and Tsou 2010). rDNA cistron encoding the ribosome nucleic acid core belongs to a multigene family, which consists of tandemly repeating units of 18S, 5.8S, and 26S coding regions. The coding regions are separated by *ITS1* and *ITS2*, which adjoin 5.8S. They are in turn bordered by intergenic spacers (IGS). The *ITS2* is a target region of a shorter length (mini barcode), which in comparison to *ITS1* is suitable for amplification and sequencing, while *ITS1* is more variable due to the presence of variable repeats that *ITS2* lacks. The limitation associated with *ITS* is believed to be due to paralogy, which would lead to sample misidentification (Li et al. 2015). However, Hollingsworth et al. (2011) reported that the presence of paralogous copies does not affect its ability to identify the regions.

3 Molecular-Based Approaches to DNA Barcoding

Various types of molecular techniques have been discovered and optimized to evaluate the authentication of plant taxa. Some of the methods being used are restriction fragment length polymorphism (RFLP), PCR, and sequencing-based technologies. Hybridization-based methods (RFLP) were developed by Botstein et al. (1980). The DNA is cleaved by restriction enzymes at a specific restriction site, resulting in variable-length fragments. Upon the electrophoretic separation, an RFLP banding pattern emerges for a given isolate, revealing its genetic diversity.

RFLP markers are single locus and of co-dominant inheritance. The polymorphism can be determined by pattern analysis after the DNA has been cleaved by a single restriction enzyme. The length of fragments determines the difference in DNA sequences. DNA hybridization combined with RFLP has been used for phylogenetic studies of plants such as *Lupinus, Musa,* and *Triticum* species (Heubl 2010).

PCR is a basic molecular technique developed by Kary B. Mullis in the year 1983, for which he was awarded Nobel Prize in Chemistry in 1993. It is one of the widely used amplification techniques with high sensitivity and good reproducibility. It involves isolation of DNA, amplification of target DNA with oligonucleotides primers and thermostable DNA polymerase (Taq polymerase) followed by electrophoresis (Heubl 2010). DNA is amplified here by repeated cycles of strand separation and replication. PCR has been employed to analyze genetic variation and the identification of various plant species (Li et al. 2015).

Random Amplified Polymorphic DNA (RAPD) is employed in the rapid detection of genomic polymorphism. It amplifies the genomic DNA with a single, short synthetic oligonucleotide primer (10 bp in length) of an arbitrary or random sequence which anneals (low annealing temperature 35 °C) randomly at multiple sites of genomic DNA. The amplicons are separated and visualized by agarose or polyacrylamide gel electrophoresis (Uddin and Cheng 2015). The polymorphism can be detected by the presence or absence of bands, which may be due to the length of the amplified region amid primer sites, the appearance of a new primer site, or mismatches at the primer site. Most of the RAPD markers are multilocus and dominant, which makes it difficult to distinguish the amplified DNA between heterozygous or homozygous at a particular locus. RAPD has been widely applied to study the relatedness in many plant groups, such as *Glycyrrhiza, Magnolia*, and *Indigofera* (Heubl 2010).

Amplified Fragment Length Polymorphism (AFLP) employs cleavage of DNA by restriction enzyme followed by amplification of the subset of fragments with oligonucleotide primers corresponding to sequences being ligated. AFLP is a powerful DNA fingerprinting technology developed by Keygene in the early 1990s, where multilocus DNA markers are generated without the need for prior sequence knowledge. AFLP markers are considered to be dominant (Vreugdenhil et al. 2011). DNA fragments of 80–500 bp length are generated after the digestion of genomic DNA by restriction enzymes (Ex: *EcoRI, MseI*), then ligated by oligonucleotide adapters (~20 nucleotides) and selectively amplified by the combination of PCR primers. Agarose gel electrophoresis is used to separate and visualize the amplified DNA fragments. AFLP is very sensitive in detecting polymorphisms between closely related species, but it requires purified high-molecular-weight DNA in a large amount. The banding pattern is due to variations in the restriction sites; however, degraded DNA can mislead the pattern. AFLP has been widely employed to study the genetic diversity of Chinese medicinal plants (Arif et al. 2010).

Simple sequence repeats (SSRs), or microsatellites, are highly informative genetic markers that were discovered and developed by Litt and Luty (1989).

SSRs are short tandem repeats of DNA stretches with di-, tri-, tetra-, or penta-nucleotide motifs. They have been found in most eukaryotic genomes and they undergo spontaneous mutation thereby allowing differentiation between closely related species (Ben-Ari and Lavi 2012). SSRs are highly polymorphic single-locus co-dominant markers with more than ten alleles, and they are considered as the second-generation molecular marker. The loci are amplified by PCR primers homologous to the conserved flanking DNA sequence. The amplification products are separated by acrylamide gel electrophoresis and visualized by silver staining or fluorescent dyes. Each band represents an allele with a specific size. These SSR markers have been used to characterize many medicinal plant families and genera, for example, *Acanthaceae* family, *Artemisia* genus, *Camellia* genus, and Chinese jujube (Heubl 2010).

Inter-Simple Sequence Repeat (ISSR) markers are abundant, highly polymorphic, reproducible, and informative. These markers detect polymorphism in the inter-microsatellite DNA regions without sequence information nor prior genetic studies (Uddin and Cheng 2015). The primers are based on repeat sequence, often with a degenerate 3′ anchor. These primers amplify the sequence between two microsatellites producing a large number of amplicons. They have been employed to authenticate *Cannabis, Dendrobium,* and *Fritillaria* (Heubl 2010). Single nucleotide polymorphism (SNP) is a single-base pair substitution (point mutation) in the genome, which leads to the formation of two alleles with alternative bases at a given position of nucleotide within a locus. They are considered the third-generation molecular marker of co-dominance inheritance that can differentiate between homozygous and heterozygous alleles efficiently (Amir et al. 2020). SNPs are abundant compared to SSRs in both plant and animal genomes with a mutation rate of 10^{-9} per locus per generation, while for SSR it is 10^{-3} to 10^{-4} per locus per generation. SNP was used to determine the population genetics of Castor bean (*Ricinus communis*) (Arif et al. 2010).

Next-generation sequencing (NGS) is a non-Sanger-based technique that enables sequencing of the entire genome at an unprecedented rate. They are very helpful in phylogenetic studies and genome evolution analysis. In NGS, the template DNA is fragmented and immobilized using solid support followed by amplification by PCR and sequencing. Currently, there are three main platforms in practice for NGS, i.e., Roche/454 Life Sciences (Indianapolis, IN), Illumina/Solexa Genome Analyzer (San Diego, CA) and Applied Biosystems/SOLiD System (orange county, CA). Out of these Roche/454 sequencing is commonly used (Sarwat and Yamdagni 2016). Roche/454 sequencing uses pyrosequencing technology, which determines the sequence of DNA strands when pyrophosphate (PPi) is released when a nucleotide is incorporated during DNA synthesis by a polymerase. The chemiluminescent signals are detected in the form of light by the firefly luciferase enzyme (Sucher et al. 2012). The number of cp-genome sequencing has rapidly increased with the advancement in NGS.

4 Applications of DNA Barcodes and Their Availability

The traditional approaches for identification of medicinal plant take account of organoleptic methods (special senses used for the identification, such as, sight, touch, taste, and smell), macroscopic as well as microscopic methods (texture, shape, and colour) and using chemical profiling with the help of analytical instruments (e.g. TLC, column chromatography, GCMS, HPLC-UV, HPLC-MS, etc.). Conversely, these methods are suitable to identify the associated species present in the processed products. The above-mentioned methods require highly trained and knowledgeable personnel for microscopic and macroscopic examinations. Generally, the tools engaged for authentication of herbal-based products are based on physical, chemical, and biochemical analysis, whereas the most and accurate validation could be achieved through the recently established molecular analysis and tools (DNA dependent) (Mishra et al. 2016).

According to the World Health Organization (WHO) reports, around 80% of the world population have started to use medicinal plants to cure and to control various diseases and metabolic disorders. Recently, herbal products demand has increased globally, adulteration in the herbal products also increases day-by-day, either intentional (through adulteration focused at turning a profit) or unintentional, could be due to lack of appropriate quality control measures (Fotiou et al. 2009; Mackey and Liang 2013; Gaudiano et al. 2016). Nearly 10% of herbal products in the developing countries are adulterated, leading to illegal supply chains. The severity of the issue is particularly prominent in Asia, Africa, and Latin America (Medina et al. 2016; Shanmughanandhan et al. 2016).

Increased usage of new herbal products with significant health benefits is one of the important reasons for launching unreliable plant products in the market, which are high in costs, but can cause adverse effects to the consumers. In order to identify the contaminants or adulterants present in the medicinal plant products, development of new technology is the need of the hour (Mukherjee et al. 2010). DNA (responsible for the transfer of genetic information, less degradable, more resistant, and found in all the tissues)-based identification of herbal products will be more reliable than RNA and proteins. Identification and certification of medicinal plant products by specific DNA sequences offers new possibilities to ensure the quality. DNA barcode offers a solution to the problem of identification of medicinal plant products and promises a significant quality check in herbal products market (Zahra 2019). Important features of this method include the usage of short, standard, and unique sequences of the genetic material (DNA) to identify the plant species (Hao et al. 2008, 2009; Gao et al. 2010; Chen et al. 2010; Gu et al. 2013).

DNA barcoding has other intriguing applications, such as herbal product authentication, which supports the development of new techniques for their propagation. Plants mitochondrial genes are evolving slowly, due to slow evolve, which are inappropriate for barcoding. Exploration for barcode for plant material shifted to nuclear and chloroplast genomes with extraordinary replacement rates (Gu et al. 2013).

5 Identification of Medicinal Plants Using DNA Barcode

One of the studies describes the protocol using ITS2 barcode for documentation of medicinal plants. The steps involved to identify species using DNA barcode for medicinal plants are as follows (Fig. 27.1): *DNA Isolation, Amplification, and Sequencing*: Medicinal plant leaf tissues were scrubbed in liquid nitrogen, and total genomic DNA isolated. ITS2 barcode universal primer and PCR were used (Chen et al. 2010). The purified PCR products were subjected to sequencing in both directions (forward and reverse) with the appropriate primers used for PCR amplification. *Sequence Assembly and Quality Control*: The original forward and reverse sequences were gathered to evaluate the quality of the produced sequence traces. Sequence assembly and quality control were performed (Chen et al. 2010). *Sequence Alignment, Genetic Analysis, and Species Identification*: There are two methods for species identification, namely, basic local alignment search tool 1 and the nearest distance method, both the methods are performed (Ross et al. 2008).

6 DNA Barcode Data Availability

Accessibility of documents is needed to all the medicinal plant products used. Presently, numerous barcode libraries are freely accessible (Taylor and Harris 2012). *BOLD (The Barcode of Life Data System)* presently contains more than 370,000 plant barcodes representing 58,510 plant species including vouchers, images, and maps. BOLD offers an appropriate method for researchers to collect,

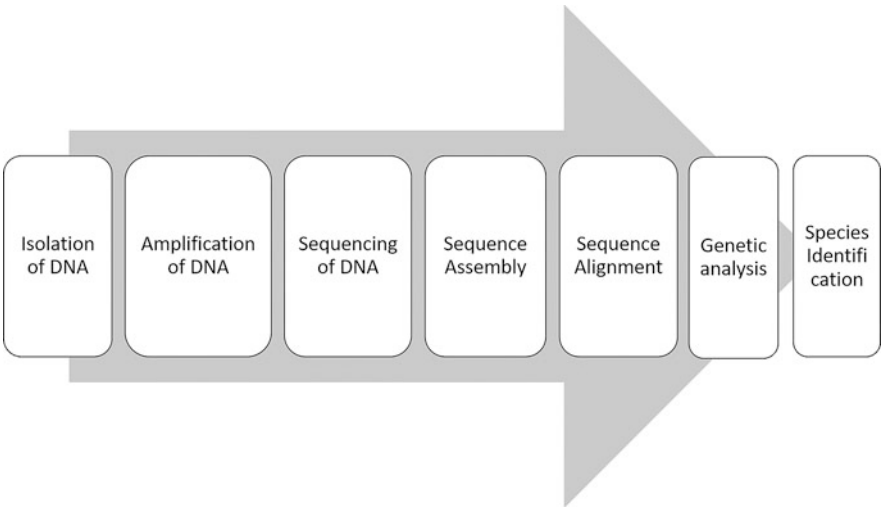

Fig. 27.1 Steps involved in species identification using DNA Barcode

manage, and analyze the data of DNA barcode, which is aimed to offer a barcode for all eukaryotic organisms in another 20 years. *iBOL (International Barcode of Life project)* is focused to build a DNA barcode reference library, which could be the foundation for DNA-based documentation systems for the entire multicellular life. *CBOL (Consortium for the barcode of life),* which is considered as a reference library of species identification, will be helpful to assign unknown samplings to known species (CBOL Plant Working Group 2009).

NCBI GenBank. GenBank is one of the important and biggest online databases built by NCBI, available to the public, and is one of the most frequently used databases (De Boer et al. 2015). It has 108 million records, over 260,000 named organisms, and is the most used database source for genomic validation. *MMDBD (Medicinal Materials DNA Barcode Database)* consists of the DNA sequences of medicinal plants registered in China and US pharmacopeia. Currently, it contains more than 15,000 sequences representing 1660 species of medicinal plants. It also comprises multiple regions, including nuclear (4 regions), mitochondrial (4 regions), and chloroplast (7 regions) (De Boer et al. 2015).

7 DNA Mini-barcode

Diverse drying and processing methods (grinding, extraction, leaching, purification, concentration, dehydrating, and granulation) of medicinal plants and the presence of secondary metabolites result in the extraction of fragmented or degraded form of DNA, thus causing DNA strand breakage (Kazi et al. 2013). If such breakages occur at the region of primer annealing, amplification will be unsuccessful. Fragmented and degraded DNA is not appropriate to determine DNA barcoding (De Boer et al. 2014). DNA mini-barcoding (DNA-MB) uses a lesser length of DNA, which helps overcome the issues associated with DNA barcoding. Using DNA-MB, ≤200 bp can be amplified rapidly due to their smaller size (Srirama et al. 2014). Contrasting traditional DNA barcodes, DNA-MB is also more diversified and can distinguish between limited species. MB can precisely identify targeted species, based on the specifically designed primers. Even though DNA-MB can support categorising processed products, this technique has some limitations due to its length constraint.

8 DNA Barcode Library Worldwide

Over two million species that are recognized are just a fraction of the overall diversity of species globally (Mora et al. 2011). Encountering new species are nowadays technique-dependent. DNA meta barcoding (As shown in the Fig. 27.2) is one amongst the most significant and effective methods for the identification of species, understanding the diversity of species (Chen et al. 2016), observing the dynamics in the composition of microorganism in the environment (Barberán et al.

Fig. 27.2 Structure of a barcode

2015), and evaluating the presence of species in drug substances or processed food (Chin et al. 2016). Hence, DNA barcoding is strongly based on the reference library with the varied species covered globally. For the construction of a reference library with an increasing number of species coverage in a comparatively short span of time, there are several major challenges to overcome. They include increasing costs in the collections of raw data, incomprehension in choosing next-generation sequence platform, complexity of data processing, and difficulties in determining thresholds for data processing (Hebert et al. 2003; Liu et al. 2021).

Broadly, a DNA Barcode library encompasses an nb linked batch and nt target codes. The barcodes in the library, the $N = nb \times nt$, are usually composed of pairwise sequence of the batch linked with the target ones. As a result, in a combined study, nb is associated with the maximum number of different experiments while nt relates to the highest number of target code in a specific experiment. (Lyons et al. 2017).

9 Barcoding Libraries Generation Overview

Generating barcodes requires parameters like number of barcodes N, number of batch codes nb, number of target codes nt, length of barcode length L, length of batch code λb, length of target code t λ, and generation of constraints (least Hamming distance d), maximum length of homopolymer m, GC-content in min/max limits, and a list of prohibited sequences as illuminated in the below given chart (Fig. 27.3).

The generation of the barcode library can be categorised into two phases: the first wherein nb batch codes are produced; and in the second phase, nt target codes that are generated, once fixed to any of the batch codes, shall pass through all the filters. As a result, this shall ensure that all the linked codes passes through filters by enabling the computational process rigorous.

As per the Markov chain model of order, nucleotide sequences with pre-specified length can be generated with maximum number of homopolymer length. Using this model, the number of candidate barcodes that can pass through homopolymer filter can be augmented in comparison to the randomly generated sequences (Lyons et al. 2017).

Over the past decade, DNA barcode libraries have been profoundly used in the chemical compounds and genomes screening and for understanding the diversity of clones. With the technology developed, it has been used in the novel drug discovery and understanding the interactions of proteins. DNA barcode libraries are broadly classified into spontaneously generated and rationally designed libraries.

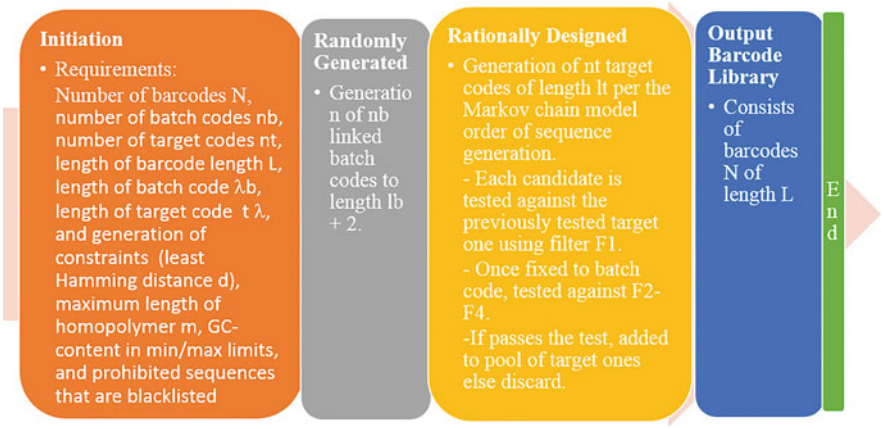

Fig. 27.3 Key steps of a barcode library generation

Spontaneously generated libraries usually involve the assembling of oligos in groups physically, potentially for actual reasons. Rationally designed barcode libraries, i.e., those designed in silico and manufactured, are more advantageous because they are less prone to misinterpretation due to sequencing and generation errors.

Although there were some technical challenges that limit the DNA barcode library's size, with the biggest rationally designed library being comprising of 240,000 codes, advancements in the DNA read and write technologies led to novel applications in large-scale barcode libraries. As a result, an increasing number of short reads are generated by the NGS technology. The reduced cost in the manufacturing of synthetic DNA is an added advantage. Altogether, these developments have led to the performance of high-throughput experiments with synthetic DNA libraries used along with screening experiments and NGS technology.

A study proposed that the gene synthesis cost can be equivalent with oligo's groups (1 USD/103–105 bp), as it can enable DNA barcodes use in practical applications wherein a unique DNA barcode and a designed DNA sequence are designed and developed along with a synthetic DNA. Consequently, it can be advantageous for the barcode and biomolecule sequence to be identified in advance for theidentification as well as for the design of the barcode robustly (Kosuri and Church 2014).

10 Conclusion and Future Prospects

DNA barcoding is a widely accepted technology, and has played an important role in medicinal plant classification, identification of adulterants/substitutes, and regulation of the pharmaceutical market. Most rough-wrought products and plant raw materials in the medicinal plant market can be utilized effectively for identification by the

current technology. However, finely processed products, such as tablets and pills, still lack standardized, rapid, and effective identification methods. Particularly, characterization of complex formulations involving multiple botanical components like those in Chinese patented medicines is quite challenging. Additionally, development of genome sequencing technologies significantly influences identification of specific DNA barcodes, including those of medicinal plants. In spite of the recent advances in the genome sequencing technologies, research on chloroplast/plastid genomics remains inadequate.

Chloroplast genomes of several medicinal plants need to be sequenced, so that new specific DNA barcodes can be developed. Development of DNA barcoding technologies have led to the construction of sequence databases, and most plant barcoding applications employ *rbc*L, *mat*K, *trn*H-*psb*A, and ITS, the standard four markers although there is a lack of consensus among all scientists pertaining to the usage of these as universal plant DNA barcodes. Several DNA barcode databases have been maintained by various countries, such as Canada and China. A continuous improvement in this technology has evolved super-barcode and metabarcoding, thereby expanding the DNA barcode family. Hence, in the field of medicinal plants, DNA barcoding technology has a vast application and will adequately contribute to the identification and authentication of traditional medicines globally, thus enabling their more scientific application for the betterment of human health.

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Chapter 28
Improvements in Taxol Biosynthesis by Metabolic Engineering: Recent Trends

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M. K. Swamy, A. Kumar (eds.), *Phytochemical Genomics*,
https://doi.org/10.1007/978-981-19-5779-6_28

1 Introduction

Taxol, also known as paclitaxel, is recognized for its anti-cancer properties (Sabzehzari and Naghavi 2019). Globally, it is expected that by 2025, the annual growth rate of taxol would be 8.2%. It is also expected to generate $152 million by the end of 2025 (Ning et al. 2020). Taxol has properties to kill cancerous cells via stabilization of microtubules and inhibition of its degradation (Schiff et al. 1979). Generally, for treatment via the use of taxol, a patient suffering from cancer needs 2–3 g of taxol, whereas, only 2 mg of taxol is synthesized within each adult yew plant. Thus, eight adult yew plants would be required for providing a sufficient amount of taxol for one cancer patient (Malik et al. 2011). Therefore, scientists and researchers are trying to improve the biosynthesis and yield of taxol by different engineered mechanisms. Several reports from different investigations have confirmed that taxol can directly induce apoptosis to carcinoma cells, as well as it can regulate many types of immune cells, such as T-cells, dendritic cells, natural killer cells, effector T cells, and macrophages (Zhu and Chen 2019). Taxol has been introduced against AIDS, non-small-cell and small-cell lung cancer, and head and neck cancers (Chen and Shi 2016). Taxol can induce its anti-cancer properties by impeding with dynamics of microtubules, which can further inhibit mitotic spindles assemblage along with separation of chromosome in cell division (Zhu and Chen 2019). This cell cycle arrest contributes to the apoptosis of such cancerous cells via the activity of intrinsic and extrinsic pathways of apoptosis. The apoptotic pathways include properties of caspase 3, 8, and 10 (Mielgo et al. 2009). Along with these properties, taxol also has an oxetane ring in its side chain; this property was reported to be maintained by C30 amide-acyl group in the C13 chain (Kingston 2000).

Therefore, the most common challenge with the use of paclitaxel is recognized to be the availability of its sufficient quantity. So, researchers are continuously trying to implement advanced metabolic-engineered mechanisms to improve the yield and biosynthesis of taxol. In this chapter, we try to explore several such mechanisms by which the biosynthesis of taxol could be improved via the use of engineered processes.

2 Biosynthesis of Taxol

Taxol biosynthesis starts from two isoprene units called IPP and the DMAPP, which are produced from isoprenoid and mevalonate pathways. Initiation of mevalonate pathways happens when acetyl-CoA and acetoacetyl-CoA condense to synthesize 3-hydroxy-3-methylglutaryl-CoA (HMG)-CoA. The HMG-CoA synthase (HMGS) catalyzes the aforementioned step. Further, HMG-CoA reductase reduces the HMG-CoA to mevalonic acid, which is itself the rate-limiting step in the overall process of taxol synthesis (Chappell et al. 1995; Goldstein and Brown 1990). Afterward, phosphorylation of mevalonate takes place with the aid of mevalonate

kinase to form phosphomevalonate, which is further phosphorylated with the help of phosphomevalonate kinase (PMK) and mevalonate pyrophosphate is synthesized. Furthermore, decarboxylation of mevalonate pyrophosphate via the action of mevalonate pyrophosphate decarboxylase (MVPD) gives rise to IPP, which is converted to dimethyl allyl pyrophosphate (DMAPP) by the activity of IPP isomerase. The IPP and DMAPP units are condensed together to form geranyl pyrophosphate (GPP), which is a C10 unit (Ogura 1998; Wriessnegger and Pichler 2013). Afterward, farnesyl pyrophosphate synthase (FPPS) helps in the condensation of GPP and one IPP unit to synthesize farnesyl pyrophosphate (FPP), which is a C15 molecule. Furthermore, geranylgeranyl pyrophosphate synthase (GGPPS) helps in the condensation of IPP and two molecules of GPP or one molecule of FPP that leads to the formation of GGPP.

Biosynthesis of taxol involves a few more steps which are carried out, i.e., enzyme taxadiene synthase (TS) helps in the cyclization of GGPP, which aids in the formation of taxadiene. It is a 75 kDa monomeric protein, which undergoes further oxygenation and hydroxylation forming taxa-4(20),11(12)-dien-5α-ol (Jennewein et al. 2004). In the next steps, oxidation of ketone followed by hydroxylation in side chains of 3/-N-debenzoyl-2′-deoxypaclitaxel leads to the formation of 3′-N-debenzoyl-2′-deoxtaxol-N-benzoyltranserase, which is commonly known as taxol (Kusari et al. 2014; Roberts 2007; Malik et al. 2011). The above-described steps are illustrated in Fig. 28.1.

Taxol has been extensively used in the field of medicine for its anti-cancer properties. It usually helps in the treatment of breast cancer, ovarian cancer, and lung cancer. Furthermore, it is also used as a second-line treatment for AIDS-related Kaposi's sarcoma. Moreover, it has been also utilized against neurodegenerative diseases such as Alzheimer's and Parkinson's (Zhang et al. 2005). The most productive species to produce taxol within itself is *Taxus brevifolia*. It can produce a concentration of 0.001–0.05% of taxol. To produce 1 g of taxol, at least 10 kg of *Taxus* bark is needed, which needs 60 years to grow. Thus, the high demand for taxol cannot be met by depending on the bark of *Taxus*. Alternative methods have been often explored for synthesizing it to meet high demand. Many reports have claimed that endophytic fungi can produce taxol; metabolic engineering to such fungi raised the hope for production of this drug (Wriessnegger and Pichler 2013). Different metabolic-engineered methods within many species to improve taxol yield are represented in Table 28.1.

3 Endophytic Fungi Help in the Biosynthesis of Taxol

According to reports from Li et al. (2009), co-culturing of *Taxus* along with *Fusarium mairei* results in increased production of taxol. Another report confirmed that *Paraconiothyrium* sp., which is an endophytic fungus, helps *Taxus* to increase biosynthesis of taxol and makes the plant more pathogen resistant. Additionally, when genes responsible for taxol production in *Paraconiothyrium* sp., were induced

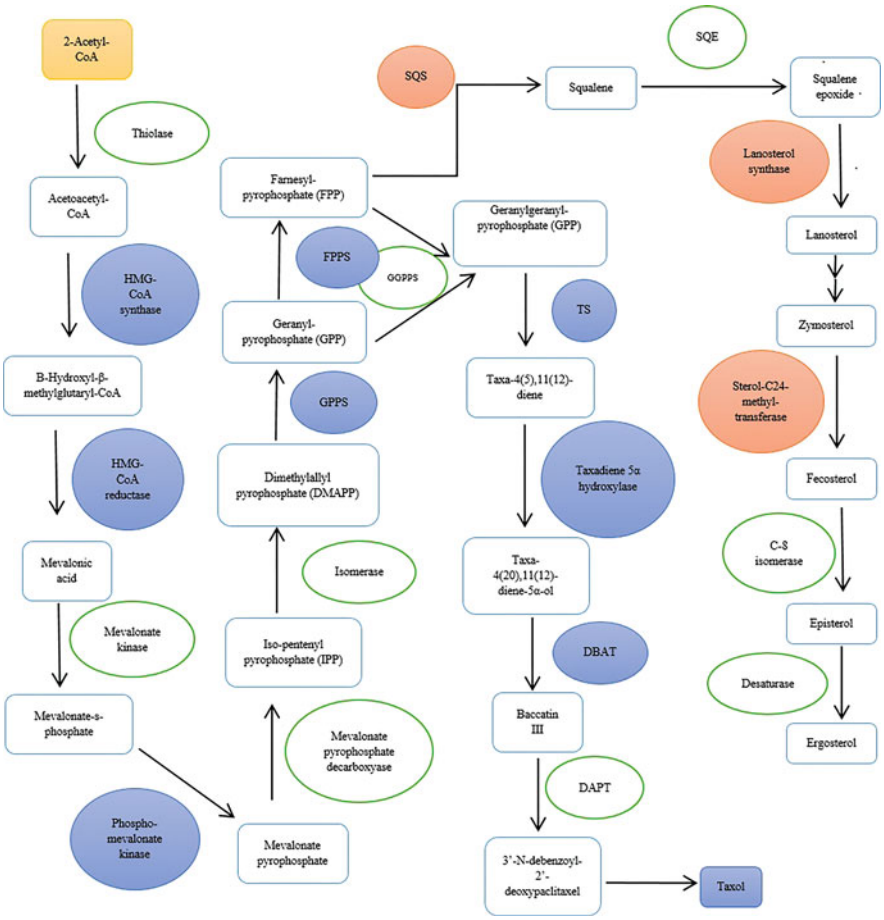

Fig. 28.1 Diagrammatic representation of biosynthesis of Taxol along with bioengineered steps to enhance the yield of taxol. The precursor for taxol enhanced is represent by *Yellow box*, enzymes which are overexpressed are represented by *Blue circles* and competing steps which are blocked are represented by *Red circles*. *GPPS* gernayl pyrophosphate synthase; *FPPS* farnesyl pyrophosphate synthase; *GGPPS* gernaylgeranyl pyrophosphate synthase; *SQS* squalene synthase; *SQE* squalene epoxidase; *TS* taxadiene synthase; *DBAT* 10-deacetylbaccatin III-O-acetyltransferase; *DAPT* DAPT baccatin III-13-O-(3-phenylpropanyoyl) transferase

with non-taxol producing fungi such as *Phomopsis* sp., and *Alternaria* sp., the translation rate increased up to eightfold (Soliman and Raizada 2013). The fermentation process using the bulk fermenter paved the way for producing taxol from *Taxomyces andreanae* (Stierle et al. 1993). Similarly, many fungal specimens belonging to Deuteromycetes and Ascomycetes have been identified as taxol producers. A few examples are *Pestalotiopsis, Trichothecium, Alternaria, Monochaetia, Fusarium, Pestaotia, Sporomia, Tubercularia, Pithomyces, and Penicillium* (Flores-Bustamante et al. 2010).

Table 28.1 Representation of metabolic engineering in different genes and enzymes within several species to improve taxol biosynthesis

Species used for engineering taxol production	Enzymes or genes engineered	Observation	References
Pestalotiopsis microspora	Squalene synthase	Sterol inhibitors viz., tebuconazole and triadimefon are incorporated within the cells of *P. microspora* to increase taxol production sixfold	El-Sayed et al. (2017)
E. coli	Taxadiene-5α-hydroxylase	The engineered mechanism allowed specificity towards the selection of taxa-4(20)-11(12)-diene, which is an alternative cyclization product that increased the yield of taxol biosynthesis within *E. coli* cells 2.4-fold	Edgar et al. (2017)
Aspergillus flavipes; A. terreus	Fluconazole + taxol precursors	The synthesis of taxol within these species was increased 1.2-fold for *A. terreus* and 1.8-fold for *Aspergillus flavipes*, respectively	El-Sayed et al. (2020)
Aspergillus flavipes	Porphyrin conjugated with taxol	After conjugation, the anti-proliferative activity of taxol towards liver carcinoma (HepG2) was enhanced 1.5-fold	El-Sayed et al. (2020)
Bacillus subtilis	Txs + crtE from *Pantoea sp.* + SDFHCEGA	Strain with txs + crtE + SDFHCEGA showed 83 times higher levels of taxadiene within its cells	Abdallah et al. (2019)
Saccharomyces cerevisiae	Taxadiene synthase from *Sulfolobus acidocaldarius*	Taxadiene synthase from *Sulfolobus acidocaldarius* showed a 40-fold increase in the expression level of taxadiene as compared to the CEN9 (*Taxus. chinensis*) strain of yeast	Engels et al. (2008)

Taxadiene synthase has been recognized as a molecular marker for identifying paclitaxel or taxol-producing fungi from *Taxus chinensis.* According to Zhang et al. (2009), phenylpropanoyl side-chain CoA acetyltransferase (BAPT) and 10-deacetylbaccatin III-10-O-acetyl transferase (DBAT) are also used as taxol biosynthesis markers. Siddiqui et al., 2012 reported many metabolic-engineered processes to increase the production of IPP via the mevalonate pathway. If the precursors for taxol, i.e., IPP and DMAPP, are increased via overexpression of the HMG-Co synthase, which is the rate-limiting step, then a positive effect could be observed in the production of taxol. Additionally, if the HMG-CoA reductase is overexpressed in yeast and the squalene synthase is suppressed, it could result in enrichment of cells with farnesyl pyrophosphate (FPP) (Martin et al. 2003). Furthermore, Taxadiene synthase, which is a crucial enzyme for taxol biosynthesis, could be overexpressed along with BAPT; the overexpression is done in control of specific promoters, which leads to increased taxol yield within cells (Engels et al. 2008). However, many other metabolic pathways like sterol biosynthesis overlap with the production of taxol, thus there is a competition for the utilization of FPP. Enzymes,

such as squalene synthase and lanosterol synthase are knocked out using metabolic engineering mechanisms, which inhibit sterol synthesis (Do et al. 2009). Moreover, inhibitors of squalene synthase are also used for inhibiting sterol yield and increasing taxol anabolism (Davidson 2007). Therefore, suppressing the sterol synthesis in cells directs the usage of GGPP pools towards the biosynthesis of taxol. Sterol inhibitors namely, tebuconazole and triadimefon are incorporated within the cells of *P. microspora* to increase taxol production sixfold (El-Sayed et al. 2017). From several investigations, it has been concluded that blocking sterol biosynthetic pathways usually leads to the formation of increased taxol yield. Therefore, knocking out squalene synthase and lanosterol synthase using the action of CRISPR/Cas9 can prove to be a productive mechanism for the increased synthesis of taxol.

4 Engineered Approaches in *E. coli* for Improved Taxol Biosynthesis

Taxol is a chemotherapeutic drug that has been studied extensively because of its medicinal equivalence (Nicolaou et al. 1994; Wilde et al. 2014). It has been reported that taxadiene-5α-hydroxylase (CYP725A4) is a cytochrome P450 enzyme that catalyzes the first oxygenation to the core of taxadiene. Furthermore, when expressed in *E. coli*, this results in the synthesis of the product taxadien-5-α-ol. However, only 10% of taxadien-5α-ol is produced from taxadiene with the help of taxadiene-5α-hydroxylase (CYP725A4). According to reports from Edgar et al. (2017), three probable mechanisms were applied to improve the efficiency in the biosynthesis of taxol within cells of *E. coli*. The selection of a holistic approach to specific improvement based on CYP725A4 carried out this process by catalyzed oxidation. Reports from their investigations confirmed that TS acts upstream of an oxidation step and proves to be an important enzyme that synthesizes precursors (taxadiene-5α-ol) for taxol. Furthermore, they performed mutagenesis to carry out a metabolic engineering pathway for directing the formation of an alternative isomer of taxol precursor. This engineered mechanism also allowed specificity towards the selection of taxa-4(20)-11(12)-diene which is an alternative cyclization product. Moreover, the mutagenesis effort also increased the yield of taxol biosynthesis within *E. coli* cells 2.4-fold (Edgar et al. 2017).

5 Conjugation of Porphyrin with Taxol Extracted from *Aspergillus flavipes*

Taxol is considered to be a major anti-cancer drug around the globe. However, its impact has been challenged by its cytotoxicity. Generally, taxol is extracted from *T. brevifolia* (El-Sayed et al. 2019; Wani et al. 1971). Recently, biosynthesis of taxol

within endophytic fungi has been engineered, and four such fungal species, namely, *Aspergillus flavipes, A. terreus, A. parasiticus, A. flavus*, were selected. According to the study of El-Sayed et al. (2020), *A. flavipes* and *A. terreus* were found to be great reservoirs of taxol. Furthermore, the biosynthesis potential of taxol from both the species was evaluated in the presence of fluconazole and silver nitrate; when fluconazole was added in the culture, the synthesis of taxol within these species was increased 1.2-fold for *A. terreus* and 1.8-fold for *A. flavipes*, respectively. This observation confirmed the suppression of biosynthesis of sterol and diverting the pool of geranyl phosphate towards terpenoids synthesis. Another impact by the addition of fluconazole was observed to be a remarkable suppression of biosynthesis of ergosterol by *A. flavipes*, which contributes to the high yield of taxol biosynthesis. Furthermore, taxol was engineered via chemical conjugation with porphyrin to increase its solubility and decrease its cytotoxicity. UV spectral analysis and thin-layer chromatography were used to detect the degree of conjugation. Thereafter, a comparative study was carried out between anti-proliferative activity of native and engineered taxol conjugated with porphyrin, wherein it was observed that after conjugation with porphyrin, taxol activity towards liver carcinoma (HepG2) was enhanced 1.5-fold, whereas its cytotoxicity towards VERO cells was decreased threefold (El-Sayed et al. 2020).

6 Metabolic Engineering in *Bacillus subtilis* for Taxol Biosynthesis

As described earlier in this review, taxadiene synthase is a crucial enzyme responsible for taxol biosynthesis. Thus, it is metabolically engineered in many biological specimens for increased production of taxol. According to reports from Abdallah et al. (2019), one such biological specimen is *B. subtilis*, in which the taxadiene synthase is overexpressed. Initially, the gene responsible for translating taxadiene synthase was isolated from the genome of the plant *T. baccata* and amplified. The gene encodes an 862 amino acid-based protein. Further, to improve the solubility, catalytic activity, the expression level, and stability of the final protein, 60 amino acids (the target sequence for plastid) were deleted from the pre-protein to produce a pseudomature form of TXS. Therefore, this 60 amino acid removed a truncated protein that was expressed within the cell of *B. subtilis*. Hence, for proper expression of the truncated protein within B. subtilis *mntA* RBS, *txs* gene along with N-terminal 6X His-tag was incorporated into the chromosome of pDR111 plasmid. The flanking regions of the txs gene consist of the *amyE* gene, which aids in the integration of the *txs* gene within the amyE locus of pDR111. Thereafter, IPTG induction in *B. subtilis* helped in the expression of the soluble 89 kDa-TXS protein. This was further confirmed by Western blotting. The IPTG induction was carried out in 20^{o} C. However, the concentration of taxol produced from this mechanism was not up to the mark. To overcome the problem of the lower yield of taxol, the precursor of

taxol i.e., IPP and DMAPP were converted into the GPP pool with the help of GPPS. Then, GPP was further elongated into FPP by the activity of FPPS. Both the FPPS and GPPS enzymes are encoded by a gene called *ispA,* therefore, maintenance of this gene is also very crucial for taxol biosynthesis. Another gene called *crtE* found in *Pantoea* has found its utility in metabolic engineering; it helps in the production of carotenoids in *E. coli*. Therefore, this gene was cloned in the pBS0E vector and then transformed within the txs strain of *B. subtilis*. The reports from this experiment confirmed that a 20 times increase in taxadiene synthase production was observed when txs + crtE strain was engineered. This proves that when GGPPS enzyme is overexpressed, it aids in the improved synthesis of taxadiene synthase. However, IPP isomerase encoding gene *idi* was found to be non-responsible for the increased formation of taxadiene synthase in *B. subtilis* (Takagi et al. 2004). To improve this production yield, p04_SDFHCEGA was controlled using an inducible promoter. Therefore, strain with txs + crtE + SDFHCEGA showed 83 times higher levels of taxadiene within its cells (Abdallah et al. 2019). Additionally, the strain expressing txs + crtE + SDFHCEGA was further engineered with few essential enzymes (cytochrome P450 and acyltransferase) for producing 10-deacetybaccatin III. Moreover, 10-deacetybaccatin III was converted into taxol or paclitaxel (Walker and Croteau 2000). Therefore, this review showcases how *B. subtilis* can be used for a better yield of taxol biosynthesis.

7 Metabolic Engineering in Taxol Biosynthesis within Yeast Cells

A forward step was taken and *Saccharomyces cerevisiae* cells were utilized for the production of taxol via metabolic engineering. The geranylgeranyl pyrophosphate pool is required for taxol biosynthesis within *S. cerevisiae* much like *B. subtilis*. However, due to sterol production, a huge amount of farnesyl pyrophosphate is utilized leaving behind only a small concentration of GGPP. To overcome this challenge, Engels et al. (2008) expressed taxadiene synthases from *Taxus chinensis* in yeast cells but the result was not effective because GPPS from *T. chinensis* competes with squalene synthase for FPP, which is utilized in sterol synthesis. Similarly, when *T. chinensis* taxadiene synthase and GGPP synthase were co-expressed in yeast cells, they failed to increase taxol production due to negative feedback from sterol biosynthesis. Later on, they created a truncated protein version of 3-hydroxyl-3-methylglutaryl-CoA reductase (HMG-CoA) isozyme 1, which is not responsive against such negative feedback inhibition, and this leads to an increase in taxol production by 50%.

Furthermore, improvement was carried out when GGPPS from *T. chinensis* was replaced by GGPPS from *Sulfolobus acidocaldarius*. The GGPPS from *S. acidocaldarius* is non-competitive to the biosynthesis of sterol in yeast cells. Therefore, GGPPS (*S. acidocaldarius*) + TS (*T. chinensis*) + tHMG-CoA, and CEN8

strain showcased an increase in geranylgeraniol concentration 100-fold when compared to CEN7 strain, which consists of only GGPPS from *T. chinensis*. Observations from Northern and Western blots confirmed that the TS gene from *T. chinensis* was poorly translated in *S. cerevisiae* due to the presence of many arginine codons. For improvement of TS expression levels, the *S. cerevisiae* was codon-optimized for the amino acid sequence and two new strains were created using codon-optimization for tHMG-CoA reductase, TS from *T. chinensis*, the transcription factor gene *UPC2-1* and either of GGPPS from *T. chinensis* (CEN9) or its counterpart from *S. acidocaldarius* (CEN10). In the final result, CEN10 i.e., taxadiene synthase from *S. acidocaldarius* showed a 40-fold increase in the expression level of taxadiene as compared to the CEN9 strain of yeast (Engels et al. 2008). Therefore, this review demonstrates that metabolic engineering can be used to enhance the production of taxol in recombinant yeast cells.

8 Conclusion

Several efforts have been made to improve the taxol biosynthesis within many alternative sources other than plant species of *Taxus*. The endophytic fungi, yeast, *E. coli*, and different species of *Aspergillus* have been metabolically engineered by overexpressing enzymes required for the synthesis of different precursors for the production of taxol. Few of such modified enzymes are taxadiene synthase, taxadiene-5α-hydroxylase, etc. In *E. coli crtE* gene from *Pantoea* was also expressed along with the taxadiene synthase enzyme from *T. baccata* (Yew plant) to improve the formation of taxol. Several other mechanisms involving the application of CRISPR/Cas were also implicated for the better synthesis of taxol and its precursor within the cells of endophytic fungi. Inhibition of sterol biosynthesis was also observed to be contributing towards the better production for taxol; this inhibition was carried out by squalene inhibitors such as tebuconazole and triadimefon. In summary, we may conclude that various engineered mechanisms can be implemented within different species for improved biosynthesis of taxol, and a sufficient amount of taxol can be found in its application as anti-cancer drug usage in the field of clinical medicine.

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Chapter 29
Computational Interaction Study of Immunomodulatory Plant Derivatives Against SARS-Cov-2 M^{pro} Target

Abhimanyu and Chakresh Kumar Jain

1 Introduction

Novel corona virus (COVID-19) is a fatal and deadly viral pandemic disease as per WHO (Gorbalenya et al. 2020) and a threat to mankind, which is caused by the new type of human severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) (Lai et al. 2020). It is known that the novel coronavirus has emerged through zoonotic organisms i.e. bats, pigs, etc., and transmitted into the human system (Guan et al. 2003). These viruses are disseminated through penetration and pass various species barriers and cause illness ranging from normal cold to critical diseases as in SARS (Severe Acute Respiratory Syndrome) and MERS (Middle East Respiratory Syndrome) (Payne 2017). Various studies showed that bats are considered to be reservoirs and progenitors of SARS-CoV (coronavirus) and SARS-CoV-2 and a main cause of infection into the human system as COVID19 (Payne 2017). The dynamics and origin of SARS-Cov-2 are unknown but have similarities like other diseases speculations in terms of its origin i.e. from animals. According to the World Health Organization (WHO), viral diseases will continue to grow and will cause serious health problems in the future. In the last 20 years, several viral respiratory diseases such as SARS, H1N1, and MERS-CoV have been detected. In genomics, coronaviruses are single-stranded RNA viruses (+ssRNA) having a crown-like appearance because of the presence of spike glycoprotein on the envelope (Vellingiri et al. 2020; Hassan et al. 2020). The novel SARS-CoV-2 shows similarities ~96% to the whole genome of bat corona (Sun et al. 2020). The outbreaks of disease originated from seafood markets and substantially infected people. The disease can be symptomatically exhibited by fever, breathing difficulties (dyspnea), dry cough,

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M. K. Swamy, A. Kumar (eds.), *Phytochemical Genomics*,
https://doi.org/10.1007/978-981-19-5779-6_29

headache, and pneumonia, and it further causes severe respiratory failure because of alveolar damage which could be observed by chest computerized tomography (Fehr et al. 2017).

The recent outbreak of COVID 19, a major threat, leading to the high morbidity rate all across the world and poses an urgent need for therapeutic intervention (Hassan et al. 2020). So far, no therapeutics i.e. potential drug or vaccine, could be developed due to the high mutation and diversity rate in the viral genome. Coronaviridae Study Group of the International Committee on Taxonomy of Viruses is behind the classification and nomenclature of the Coronaviridae family (Sun et al. 2020; Wang et al. 2020). Medicinal plant-based bioactive phytocompounds have been known to exhibit good potential as antiviral, antibacterial and antimicrobial, and anti-cancer drugs, and have been reported as good therapeutics against several diseases. In pathophysiology, these bioactive phytochemicals invoke and elicit the immunomodulation pathways and inhibit viral propagation and growth (Elfiky 2021; Pant et al. 2021). Recently, the protein structure of the main protease structure (SARS-CoV-2 M^{pro}, PDB ID: 6LU7) of the SARS-CoV-2 virus has been resolved, and it has also been reported to be a potential drug target (Zhou et al. 2020). The chapter explains all the associated steps/execution of programs with a demonstration of the antiviral capacity of the phytocompounds from immunomodulatory plants through the case study.

2 Different Natural Phytocompounds Used in Ayurvedic Kadha

A total of eight major constituents from various immunomodulatory medicinal plants are used in ayurvedic kadha preparation Cinnamaldehyde and Styrene, (*Cinnamomum verum*), Piperine (*P. nigrum*), Rosmarinic and Ursolic acid (*O. tenuiflorum*), Shogaol and Zingerone (*Zingiber officinale*), and Withaferin A (*W. somnifera*) (Ashwagandha) in the experimentation (Tito et al. 2021; Balkrishna et al. 2021; Prasanth et al. 2020). These plants have been taken in ayurvedic kadha preparation by Ayush mantralaya (Gautam et al. 2020). Among these in the Ayurvedic system, ashwagandha is known as Sattvic Kapha Rasayana, which works as a nervine tonic, and is referred to as Rasayana or adaptogen/anti-stress agents or regulators helping to prevent several diseases (Singh et al. 2011).

Many studies support that the bioactive compounds of these plants exhibit different properties like anti-inflammatory, immunomodulators, antiviral and antitumor properties due to which these bioactive compounds might have abundant possibility to be used as good candidates against COVID-19. Based on these properties this research aimed to show the interactions between protein and active compounds and to investigate the insight at microenvironment of ligand–target complex during molecular docking, molecular dynamics and simulation makes a

different scenario for examining the docking score, potency, drug-likeness properties, ADMET and its side effects.

3 Molecular Docking

Molecular docking is one of the advanced methods used in the drug discovery process, helpful in the prediction of the binding site of the given protein target for desired ligands (Meng et al. 2011). Docking can be of two types on the basis of the binding site of the target. (a) Site-specific docking: In this method, the particular active site of a protein on which the one or more ligands can bind is known. (b) Blind docking: In this method, the binding site is not known of the target protein and the docking is performed on the whole structure of the target. There are different softwares avaiable for molecular docking, in which some are commercial and some are free to use. All of the softwares using some algorithm/scoring functions on which they provide results. Some of the examples of free-to-use software are Autodock, Autodock Vina, and Haddock, and some of the commercially available software are Schrodinger GOLD, Glide, and Ligand fit.

Before performing molecular docking there are two main processes: (1) Preparation of the target protein, including removal of HET atom and water molecule and addition of the required hydrogen molecules and desired charges, specific to the type of the docking, blind or site-specific, and (2) the post-docking analysis including prediction of the conformational change of ligand and conformational energy. In molecular docking the finest conformation is the one having the lowest energy level obtained by altering ligand structural conformation according to the coordinate of the active sites.

Generally, the following steps are adopted for molecular docking by most software/pipelines (Autodock vina).

1. Preparation of the protein
   (a) Addition of H atoms
   (b) Addition of charges
   (c) Conversion the PDB into PDBQT file format
2. Preparation of the ligand
   (a) Detection of torsion angle
   (b) Conversion into suitable file format
3. Grid box formation
4. Docking score calculation
5. Interaction study using Discovery studio/ligplot

4 Molecular Dynamics

A very essential and important step is to be followed for the confirmation of the molecular docking process. In the MD simulation, the energy of interaction/robustness and the physical movements of different atoms and molecules from the molecular trajectories is calculated, which is based upon the complex mathematical equation and incurs high computational complexities (Durrant and McCammon 2011). Therefore, a cloud computing/supercomputing, high-end computational platform is needed for molecular simulation purposes. There are many freely available simulation software, i.e., NAMD, VMD, AMBER, GROMACS, and CHARMM, that are commonly used for molecular dynamics and simulation studies. A case study has been outlined below.

5 Materials and Methods

5.1 Preparation of the Ligand

For identifying the nature of the ligands such as its drug likeliness and its activity there are different software present from which different activity of the ligands can be determined.

5.1.1 ADMET Property

ADMET stands for Adsorption, Metabolism, Excretion, and Toxicity, the very essential properties are used for the pharmacokinetics studies of a drug molecule. These are very important to explain the drug suitability in the body system.

The properties of different ligands for analysis of drug-likeness were selected based on ADMET properties. It is the calculation of the pharmacokinetics properties which is extensively used to determine how a drug behave inside the human body. ADMET analysis was done with the help of the software admetSAR (Guan et al. 2019).

5.1.2 PASS Program

Prophecy of different ligands through which we can identify the antiviral activity by using the software PASS (Goel et al. 2011). PASS is a computational method-based program that is used for the prognosis of different types of physiological actions for different compounds of phytoconstituents. It gives the results based on probable activity and probable inactivity i.e. (Pa) and (Pi). Those substances having more Pa then Pi can be used for further medical activity.

5.2 *Preparation of Viral Protein*

The structure of the main protease of COVID-19 in complex with an inhibitor N3 was taken from protein data bank www.rcsb.org (Jin et al. 2020). The structure that we have taken from PDB is based on the method X-ray diffraction with a resolution of 2.16 Å. The structure contains neither carbohydrate polymers nor any breakage of chains. Complexes bound with protein molecules were removed. The preprocessing of the structure was performed using Autodock software. Complexes attached to protein were removed and extra water molecules were also removed which are presented in the PDB file. Polar hydrogen molecules were added and no bond order was selected for PDB molecule. After completion of preprocessing of the protein, the suitable structure in Autodock format has been used in Autodock vina software using, the command-line interface for performing molecular docking (Trott and Olsan 2010).

5.2.1 Ligand Preparation and Analysis of Its Likeliness

For determining the best interaction of ligands with PDB id 6LU7 as shown in Fig. 29.1, we have used different natural compounds taken from the PubChem database. These compounds are Cinnamaldehyde (Pubchem CID: 637511), Piperine (Pubchem CID: 638024), ROSMARINIC (Pubchem CID: 5281792), Shogaol (Pubchem CID: 5281794), Styrene (Pubchem CID: 7501), Ursolic Acid (Pubchem CID: 64945), Zingerone (Pubchem CID: 31211), and Withaferin A (Pubchem CID: 265237) as shown in Fig. 29.2. All of these structures were in sdf format, making them compatible with the Autodock vina format. We have converted the structures into the PDB format. For conversion of the ligands, we have used open Babel software (O'Boyle et al. 2011). For determining the likeliness of the ligands,

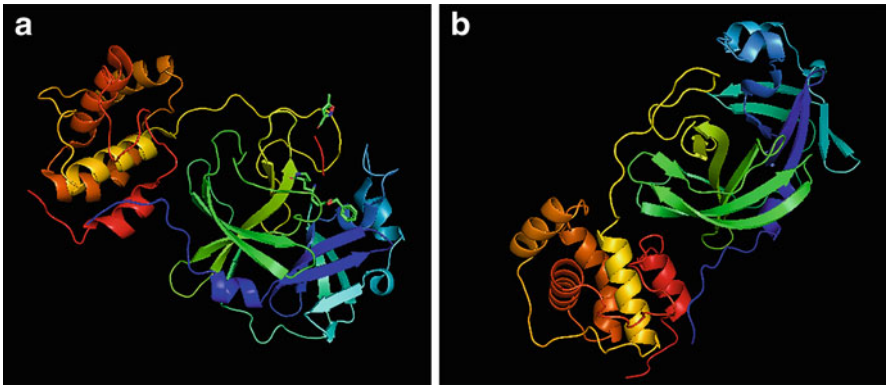

Fig. 29.1 (**a**) Three-dimensional structure of the main protease of 6LU7; (**b**) Crystal structure of the main protease of COVID structure in complex with inhibitor N3

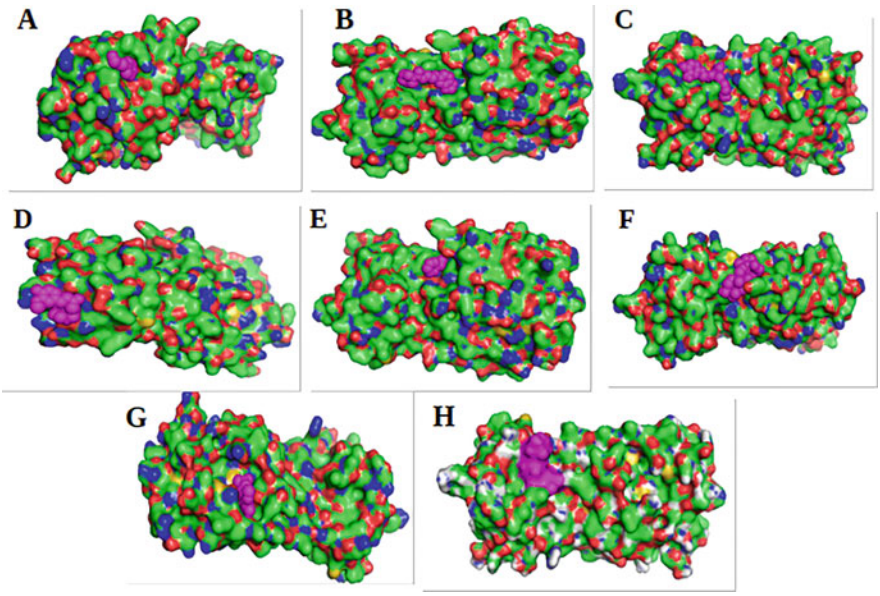

Fig. 29.2 3D visualization of the docked structure of different ligand molecules with the receptor protein (Protein—whole colored structure, ligand—magenta color). (**a**) Cinnamaldehyde; (**b**) Piperine; (**c**) Rosmarinic; (**d**) Shogaol; (**e**) Styrene; (**f**) Ursolic acid; (**g**) Zingerone; (**h**) Withaferin A

we have used a software termed as drulito software (Niper.gov.in 2021) (http://www.niper.gov.in/pi_dev_tools/DruLiToWeb/DruLiTo_index.html).

5.2.2 Docking and Analysis

For docking of protein-ligand the grid box size that we have taken for protein molecule (PDB ID: 6LU7) have center coordinates (x, y, z = $-26.16A^0$, $12.584A^0$, $59.064A^0$) while that of points in dimensions x, y, z are 126, 126, 126, which is large enough to cover the maximum portion of the given PDB structure so that the binding of the ligand can take place whereas the exhaustiveness was set at 8.0. For visualization of the binding between both the structures, we have used PyMol software. In this study, we have used molecular docking of protein with different natural compounds for determining the best affinity (most negative) and identifying the best ligand which has been visualised using pyMol software for analysis. The 2D interactions between the interacting residues are determined with the help of Biovia Drug Discovery Studio 2020 software (https://discover.3ds.com/discovery-studio-visualizer-download). After performing docking, we can conclude that the compound Withaferin A possesses the best affinity with the PDB id 6lu7. As Withaferin A shows great affinity with the target protein which has been forwarded for molecular simulation.

5.3 Molecular Dynamic Simulation and Analysis

The ligand that was identified after performing docking analysis was Withaferin A with which the molecular dynamics can be performed with the crystal structure of the main protease (PDB ID: 6LU7). The docked complexes were subjected to MD simulation using the dynamic software GROMACS 5.1.2. MD simulation of both the protein and ligand Withaferin-A complexes was performed for a time period of 50 ns by using the GROMOS96 43a1 force field with SPCE water model. The molecular topology for the ligand Withaferin A was constructed using the PRODRG web server (http://davapc1.bioch.dundee.ac.uk/programs/prodrg), which is freely available. It is an online server that generates ligands topology files and is freely available. The complex of protein and ligand was maintained with a proper concentration of salt of 0.15 M by the addition of appropriate numbers of Na^+, Cl^- ions. All runs were performed at constant volume and temperature and constant volume and pressure with a time step of 2 s at constant temperature and pressure under certain periodic boundary conditions. The structures we get after performing NPT equilibration were further used for final production and for the collection of data. The production md was run at 50 ns. Trajectory analysis for determining the various graphs of root mean square deviation and root mean square fluctuations were analyzed through which we can understand all the changes that occur at the position of Cα atoms from its backbone of the protein and also for understanding the fluctuations that occur at the position of each amino acid residues of the protein. For understanding the conformational stability between the protein and ligand complex we have also calculated the radius of gyration whereas we have also performed hydrogen-bond analysis through which we can identify the interactions of H-bonds so that we can understand the fitting of ligands at the active site of the protein. All these data were obtained by the inbuilt packages provided by GROMACS through different representative graphs and plots. For plotting the different graphs, we have used the Xmgrace program (http://plasma-gate.weizmann.ac.il/Grace/). To quantify the strength between both the ligand and protein complex the average Coulombic interaction energy and Lennard-Jones energy has been computed which referes the total interaction energy.

6 Results

6.1 Molecular Docking and Analysis of Target Prediction

For management of COVID-19, we performed molecular docking on various prospective candidates. We have executed many candidates from which we have selected eight phytoconstituents acquired from different natural compounds. All of the eight compounds were docked against the target COVID-19 structure, and based on their docking score they were ranked (Table 29.1). Docking was performed with

Table 29.1 Physicochemical properties of the natural compounds by the rule of drug-likeness. Where *MW* molecular weight, *HBA* hydrogen bond acceptor, *HBD* hydrogen bond donor, *TPSA* topological polar surface area, *AMR* atom molar refractivity, *nRB* no. of rotatable bonds

Ligands	MW	Logp	Alogp	HBA	HBD	TPSA	AMR	nRB	No. of violations
Cinnamaldehyde	132.06	1.968	1.376	1	0	17.07	46.27	2	2
Piperine	285.14	2.518	−0.635	4	0	38.77	82.03	4	2
Rosmarinic	360.08	1.578	0.099	8	5	144.52	97.84	7	4
Shogaol	276.17	3.775	0.217	3	1	46.53	82.07	9	2
Styrene	104.6	29.37	1.915	0	0	0.0	39.98	1	0
Ursolic acid	456.36	8.554	1.484	3	2	57.0	132.26	1	1
Zingerone	194.09	0.791	−0.0311	3	1	46.53	38.17	7	0
Withaferin A	470.27	3.987	0.642	6	2	96.36	125.06	3	3

the help of the software Autodock and Autodock Vina. Through Autodock, we get the suitable format files that were used in Autodock vina software from which we get the best affinities values with different ligands. All of the docked 3D structures can be seen in Fig. 29.2. Visualization of the docked structure was done with the PyMol software. As we can see from Fig. 29.3 the structure of the protein molecule 6LU7 was docked with the compound Withaferin A more densely and formed different hydrogen and hydrophobic bonds, which can be seen in 2D visualization of the complexes also from which we can identify the various types of interactions energies (interactions energy (Van der Waals, conventional hydrogen bond, Pi sigma, Pi-Pi stacked, alkyl, Pi-alkyl, Pi-sulfur, Pi cation, carbon hydrogen bond) between complexes.

6.2 *Interaction Studies Between Molecules*

The results we got after performing docking were determined in the software Biovia discovery studio from which we can understand their interactions. Physicochemical properties and the affinities and interactions between protein and ligands can be seen in Tables 29.1 and 29.2. The main protease shows the best docking score of −7.6 with Withaferin A, in comparison with other compounds, while other ligand compounds Cinnamaldehyde, Piperine, Rosmarinic, Shogaol, Styrene, Ursolic acid, and Zingerone show binding energies of −5.2 kcal/mol, −6.8 kcal/mol, −6.6 kcal/mol, −5.1 kcal/mol, −4.3 kcal/mol, −6.9 kcal/mol, −4.4 kcal/mol, respectively (Table 29.2, Figs. 29.1 and 29.2). This in silico analysis shows that all of the different phytoconstituents interacted with the main protease, showing that these ligands probably have inhibitory actions against COVID-19. Withaferin A with the main protease of COVID structure formed four hydrogen bonds, i.e., ASN A:238 (2.88), THR A:199 (2.95), ARG A:131(3.15), ASP A:289 (2.79), and another three amino acids form hydrophobic bonds, i.e., TYR A:239 (5.00), LEU A:286 (4.32), LUE A:287 (4.81).

6.3 *Prediction of Drug-Likeness Properties*

The prediction of the different physicochemical properties of various selected compounds was detected by druLito software. As all of the compounds were taken from natural sources, many of these did not obey the Lipinski rule (Table.29.1). Rosmarinic and Withaferin A show higher TPSA (144.52, 96.36) and AMR (97.84, 125.06) Table 29.1 and Fig. 29.3). AMR and TPSA are those properties that involve absorption of the drug, penetration of the drug, and transport mechanism (Ertl et al. 2000).

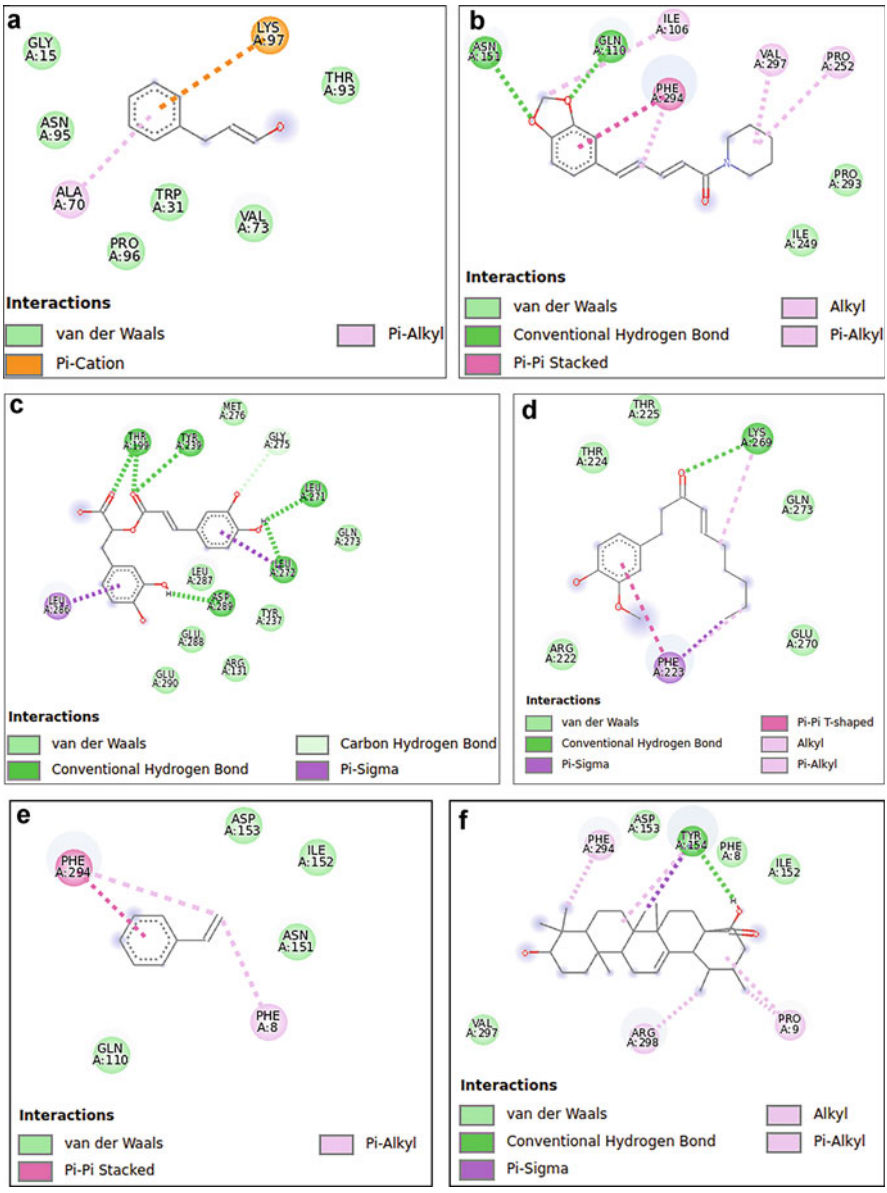

Fig. 29.3 Various two-dimensional visualizations of the docked structure of different ligand molecules with the receptor protein showing different interactions energies (**a**–**h**)

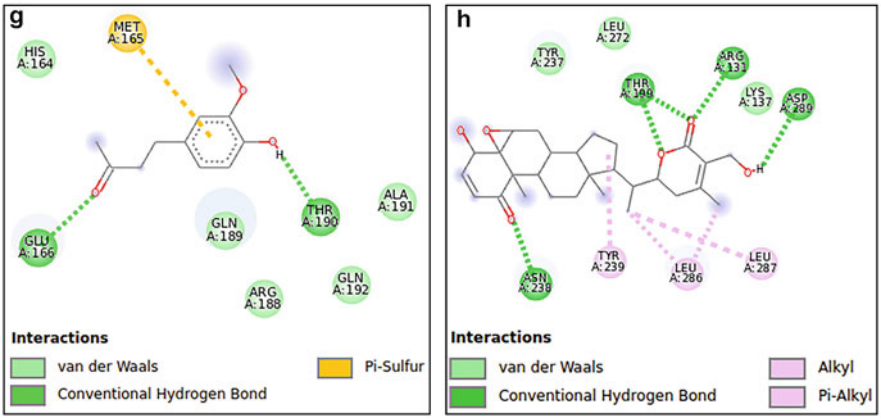

Fig. 29.3 (continued)

Table 29.2 Interaction of amino acid residues between main protease and ligands

Ligands	Affinity (kcal/mol)	Involved amino acids and their distances		
		Hydrogen binding interaction	Hydrophobic interaction	Electrostatic interaction
Cinnamaldehyde	−5.2	–	ALA A:70(4.23)	LYS A:97 (4.74)
Piperine	−6.8	ASN A:151(2.93), GLN A:110(2.81)	ILE A:106(4.92), PHE A:294(5.27), VAL A:297(4.73), PRO A:252(4.85)	–
ROSMARINIC	−6.6	THR A:199(2.30), TYR A:239(3.33), LEU A:271(2.46), LEU A:272(1.95), ASP A:289(2.13), GLY A:275(3.69)	LEU A:286(3.56)	–
Shogaol	−5.1	LYS A:269(3.10)	PHE A:223(3.56)	–
Styrene	−4.3	–	PHE A:294(3.70), PHE A:8 (4.85)	–
Ursolic Acid	−6.9	TYR A:154(2.59)	PHE A:294(4.87), ARG A:298(4.80), PRO A:9(4.39)	–
Zingerone	−4.4	GLU:166(3.14), THR A:190(1.99)	–	–
Withaferin A	−7.6	ASN A:238(2.88), THR A:199(2.95), ARG A:131(3.15), ASP A:289(2.79)	TYR A:239(5.00), LEU A:286(4.32), LUE A:287(4.81)	–

6.4 Evaluation of ADMET Properties

ADMET properties of different ligands were evaluated with the help of the online platform admetSAR. ADMET properties used in this research study were determined by admetSAR. All of the ligands showed excellent intestinal absorption and blood-brain barrier permeability. Almost all of these ligands showed negative value for carcinogenicity and also showed negative AMES toxicity. The results of HIA, BBB, and LD50 values are listed in Table 29.3.

6.5 Prediction of Antiviral Activity by Pass Calculation

PASS is defined as Prediction of Activity Spectra for Substances. From PASS we can identify the previously identified biologically active spectra of phytoconstituents. These predictions were calculated and demonstrated in Table 29.4.

7 Molecular Dynamics Simulation and Analysis

The molecular simulation was carried out in GROMACS software where as a first step of the process topology files were generated for both the ligand and protein. A cubic box of 0.3 nm with a distance between the protein and the edges of the box has

Table 29.3 Different ligands with their ADMET properties where HIA stands for human intestinal absorption, BBB stands for blood-brain barrier

Ligands	HIAvalue	BBB value	AMES test for toxicity	Carcinogenicity	LD50 Rat acute toxicity (kg/mol)
Cinnamaldehyde	0.9746	1.0000	No toxicity	No carcinogenicity	1.485
Piperine	0.9639	0.9921	No toxicity	No carcinogenicity	2.201
Rosmarinic	0.9666	0.3334	No toxicity	No carcinogenicity	1.668
Shogaol	0.9904	0.9362	No toxicity	No carcinogenicity	2.267
Styrene	0.9814	1.0000	Toxicity	Carcinogenicity	1.8
Ursolic acid	0.9853	0.6782	No toxicity	No carcinogenicity	2.72
Zingerone	0.9928	0.9321	No toxicity	No carcinogenicity	1.905
Withaferin A	0.9729	0.9537	No toxicity	No carcinogenicity	3.392

Table 29.4 The given table shows the results generated through calculation performed by PASS prediction method for different predicting activities of different phytoconstituents

	Cinnamaldehyde		Piperine		Rosmarinic		Shogaol		Styrene		Ursolic acid		Zingerone		Withaferin A	
Predicting activity	Pa	Pi	Pa	Pi	Pa	Pi	Pa	Pi	Pa	Pi	Pa	Pi	Pa	Pi	Pa	Pi
Antiviral (Rhinovirus)					0.399	0.092	0.477	0.034	0.319	0.214	0.492	0.027	0.350	0.159	0.383	0.111
Antiviral (HIV)					0.270	0.012	0.208	0.027					0.163	0.052		
Antiviral (Influenza)	0.403	0.046	0.221	0.167	0.412	0.043	0.424	0.039	0.384	0.052	0.761	0.004	0.384	0.052	0.431	0.037
Antiviral (Herpes)	0.321	0.077			0.339	0.066	0.352	0.059	0.427	0.025	0.437	0.022	0.342	0.065	0.261	0.121
Antiviral (Hepatitis B)	–	–			0.264	0.045			0.427	0.012	0.176	0.122	0.162	0.153		

been generated to solvate the system containing 16,783 molecules, and 54 Na^+ ions and 50 Cl^- ions were added so that the charges on the system neutralize. Energy minimization takes place at maximum force <10.0 kJ/mol, whereas the maximum number of steps required to complete minimization was 50,000. Energy minimization of the steepest descent method for 1000 steps took place out of which it gets completed at 475 steps whereas the potential energy generated was −9.2150575e +05 and the maximum force was 9.7059442e+02 on atom 2160. Equilibration for the system was completed under constant pressure and temperature.

The molecular dynamics simulations were determined through various analysis methods like root mean square deviation, root mean square fluctuation, and radius of gyration values. All the variations in the structure were calculated based on the values of RMSD of the complex between 0 and 50 ns. Through RMSD analysis we can understand the insights and variations that occur in confirmation of structure during dynamics simulation, including both the stability of protein and equilibration of the system. The value of RMSD increases rapidly from 0 to 5 ns, reaches stability after 10 ns, and remains stable throughout the simulation. The average RMSD value of the complex found was 0.366 of the 50 ns simulation (Fig. 29.4a). Through RMSF analysis we can understand how the fluctuation of each atom occurs during simulation. The binding site and Interactions are directly dependent on the values of RMSF (Fig. 29.4b). The average RMSF value was considerably good. After analyzing these

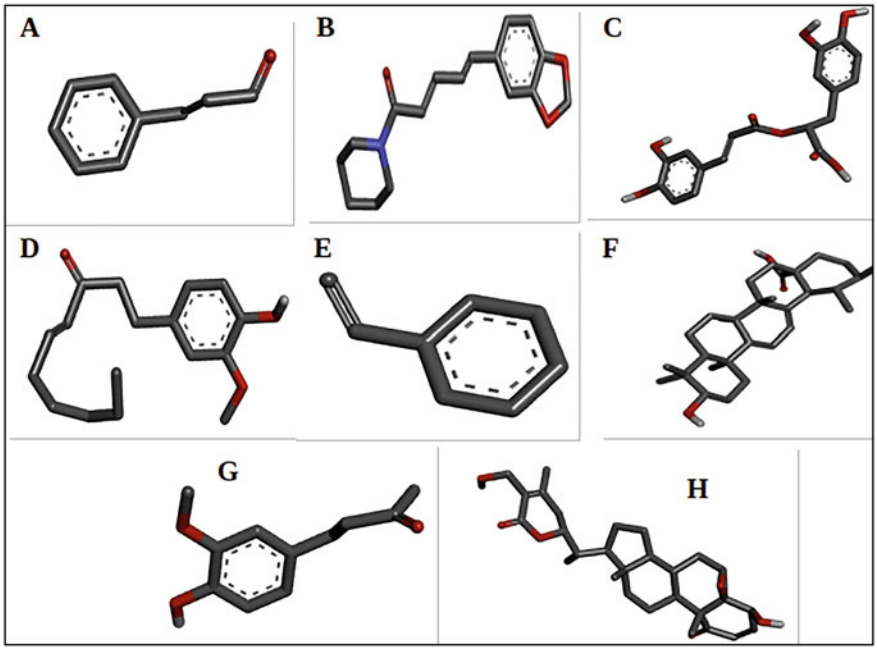

Fig. 29.4 Three-dimensional structure of active in silico ligands. (**a**) Cinnamaldehyde; (**b**) Piperine; (**c**) Rosmarinic; (**d**) Shogaol; (**e**) Styrene; (**f**): Ursolic acid; (**g**) Zingerone; (**h**) Withaferin A

values we can state that the residues present at the binding side showed fewer fluctuations, which concluded that the complex structure remains stable throughout the 50 ns simulation. The value of radius of gyration between main protease and Withaferin A decreased initially and after some time became stabilized, which was an indicator that the binding was stable (Fig. 29.4c). For hydrogen-bond analysis we found the propensity of a maximum number of six H-bonds between the complexes; however, two of the hydrogen bonds remain stably bound with the active site throughout the 50 ns simulation (Fig. 29.4d). The molecular dynamics simulation of the selected ligand with the protein complex confirmed the overall stability of the protein-ligand complex.

To determine the interaction strength between both the main protease and Withaferin A we have identified the two interaction energies from which can identify the interaction between both structures. The average Columbic short-range interaction energy found was -64.27 ± 8.5 kJ mol^{-1}, whereas the average Lennard-Jones short-range interaction energy found was -116.937 ± 5.2 kJ mol^{-1}. The total interaction energy according to the standard formula after propagating errors was 181.187 ± 13.7.

8 Discussion

Cases of coronavirus have been tremendously increasing day by day and its ratio of infecting humans and animals are uncontrollable, which causes respiratory diseases leading to many millions of deaths (To et al. 2013). Due to the non-availability of efficient therapeutics, the disease is very deadly. Hence, there is an urgent need to combat natural metabolites as therapeutics agents. Reports suggest that natural products are generally less toxic and possess various antiviral effects (Gorbalenya et al. 2020) and could be a possible hope towards COVID 19 treatments as well. We have collected eight compounds with which we performed molecular docking to determine the best binding affinity, out of which many of them show good results. Withaferin A exhibited the best docking score with least binding energy −7.6 kcal/mol and was found to make four hydrogen bonds, ASN A:238(2.88), THR A:199 (2.95), ARG A:131(3.15), ASP A:289(2.79), with four amino acids while three amino acids were involved in hydrophobic bonds, i.e., TYR A:239 (5.00), LEU A:286(4.32), LUE A:287(4.81) as mentioned in Fig. 29.3 and Table 29.2. We can see that all of these have less than 5A° bond length in all the hydrogen bonds, which indicates that the bond between protein and ligands is very strong and complex forms are also stable. In a current research study, we have used different software like Autodock, Autodock-Vina, PyMol, and GROMACS for analyzing the potential of the selected phytoconstituents against the main protease. The final selected ligand was subjected to molecular simulation with energy calculations to determine the stability of the bonded protein-ligand complex with a 50 ns time scale where the interaction based on root mean square deviation (RMSD) and root mean square fluctuation (RMSF) was observed and found stable as shown in Fig. 29.5.

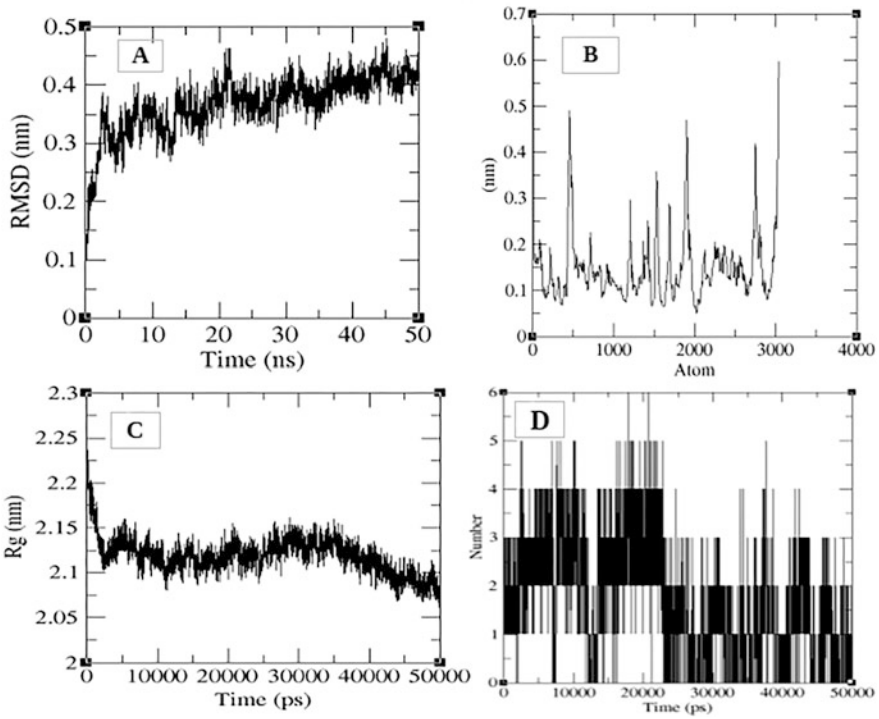

Fig. 29.5 Various plots of the complex formed after performing dynamics simulation (**a**) Root mean square deviation (**b**) Root mean square fluctuation and (**c**) radius of gyration (**d**) Hydrogen bond analysis

Natural sources can be a potential candidate for drug discovery, and can also help in the management and treatment of various diseases. As these compounds are bulky and have high molecular weight, they require further optimization. However, in vitro and in vivo study are required to prove their efficiency against COVID-19.

9 Conclusion

The computational interaction study of some medicinal plant bioactive compounds, which are reported with demonstrated high antiviral and immunomodulatory properties have been performed, and for the stability of intersection, MD simulations procedure have been followed. Out of 8 compounds from various medicinal plants Withaferin A, a steroidal lactone, which are a major constituents extracted from ashwagandha, has shown the best binding capacity on target protein while performing blind docking i.e. 7.4 Kcal mol^{-1}. Further, the RMSD analysis and trajectories analysis of MD simulation conducted for 50 ns on GROMACS package

has supported the interaction. This reveals the stability of docked structure and is helpful to elaborate the list of associated amino acids with their chemical nature i.e., hydrophobic within active site pocket thus shedding light to understand the binding mode and mechanism of interaction. The current investigation explains the comparative importance of ashwagandha plant compounds, though these compounds are already known as Rasayana, and are known as rejuvenators and potent immunomodulators (Krammer et al. 2018) and signifying it potential in the present context. This suggests the comparative significance of ashwagandha dosage as possible immunomodulator-therapeutics towards COVID 19 infections. The work can be extended with wet lab-based experiments further.

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