Munir Ozturk · Khalid Rehman Hakeem
Editors

Plant and Human Health, Volume 2

Phytochemistry and Molecular Aspects

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Dedicated to Our Ancient Herbalists

"Medicine from Honey"—a 1224 Arabic translation of the manuscript De Materia Medica, written by the ancient Greek physician, Dioscorides (40–90 AD)

Foreword

Medicinal plants have been a rich source of medications since the very birth of man. Traditional Chinese medicine has been extensively documented for many thousands of years. The Chinese pharmacopoeia, *Shennong Ben Cao Jing*, records plant medicines such as ephedra and hemp. Egyptian medicine employing plant-based drugs dates back to 2900 BC, but preserved records in the form of *Ebers Papyrus* containing about 700 drugs mainly of plant origin go back to around 1550 BC. There is also evidence of the use of plants for healing purposes date back to 2600 BC in Mesopotamia indicating the existence of a plant-based system of treatment in which about 1000 plant-based medicines were used. Ancient Ayurvedic medicine, as documented in the *Atharva Veda*, the *Rig Veda*, and the *Sushruta Samhita*, employed hundreds of pharmacologically active herbs and spices. The medicinal applications of plants became known to the Western world through Greek and Roman practitioners, particularly through the treatises contributed by the Greek physician Dioscorides (1st century AD), and the Roman physicians Pliny the Elder (1st century AD) and Galen (2nd century AD). Later came the Islamic contributions to herbal medicine with the advent of physicians such as Abu Ali Ibn Sina (980–1037), better known in the West as Avicenna, whose book, *Al-Qanun fi al-Tibb*, was used

as a standard textbook of medicine in Europe for over 700 years. Abu Bakr Muhammad ibn Zakariya al-Razi (865–925 AD) wrote over 200 books and categorized substances as vegetable, animal, or mineral, whereas other earlier alchemists had divided them into "bodies," "souls," and "spirits." He was the first to use opium for anesthesia. Al-Idrisi, born in Cordova, during the Islamic era in Spain in 1099, wrote many books on medicinal plants including *Kitab al-Jami-li-Sifat Ashtat al-Nabatat.* Another major contribution from Spain came from Abu Muhammad Ibn al-Baitar (1197–1248), who composed the encyclopedia on medicinal plants entitled *Kitab al-Jami al-Adiwaya al-Mufrada* that presented the work of 150 authors. Abu-Rayhan Biruni, Ibn Zuhr, Peter of Spain, and John of St. Amand also contributed pharmacopoeias describing the use of medicinal plants. The most comprehensive encyclopedic set of volumes on medicinal plants in recent times has been the 57 volume series entitled *Studies in Natural Product Chemistry* (Elsevier Science, Ed. Atta-ur-Rahman) that describes thousands of bioactive constituents discovered from the most important medicinal plants.

It was at the beginning of the nineteenth century when rational drug discovery from plants commenced with the isolation of the analgesic and sleep-inducing agent morphine from opium by the German scientist Serturner in 1817. Other medicinal herbs were then examined for active principles leading to the isolation of a host of important compounds, including quinine, caffeine, nicotine, codeine, atropine, colchicine, cocaine, and capsaicin, from various plant sources. Following the discovery of penicillin in 1928, attention was also turned to the bioactive substances in microbes. The developments of synthetic drugs led to a certain decrease in interest in natural materials as sources of drugs because of the challenges associated with large-scale availability. However, a significant proportion of new drugs approved by FDA are still derived directly or indirectly from medicinal plants. For instance, out of the 1073 new chemical entities (belonging to the group of small molecules) approved between 1981 and 2010, only 36% were purely synthetic, while the remainder were either natural products or their analogues. Similarly during the period 1940–2014, out of the 175 small molecules approved against cancer, 85 were natural products or their derivatives. These include paclitaxel and its derivatives from yew (*Taxus*) species, vincristine and vinblastine from periwinkle (*Catharanthus roseus* (L.) G. Don), and camptothecin and its analogs initially discovered in the Chinese tree *Camptotheca acuminate* Decne. Other important natural products include the cholinesterase inhibitor galanthamine approved for the treatment of Alzheimer's disease from *Galanthus nivalis* L. and the important antimalarial agent artemisinin originally derived from the traditional Chinese herb *Artemisia annua* L. In spite of the advent of combinatorial chemistry, the actual number of new drugs reaching the market through purely synthetic efforts has diminished. This has resulted in the revival of interest in natural products and triggered the use of multidisciplinary approaches to drug discovery.

The present second volume of *Plants and Human Health*, edited by Khalid Rehman Hakeem and Munir Ozturk and entitled *Phytochemistry and Molecular Aspects*, presents a wealth of information on bioactive compounds isolated from various medicinal plants and their utility in tackling many diseases. The discussions

range from bioactive substances found in terrestrial medicinal plants and freshwater aquatic plants to edible materials and fungi with antioxidant, anti-inflammatory, antiseptic, antidiabetic, anticataleptic, antiarthritic, sedative, calming, antidiuretic, antimicrobial, antifungal, herbicidal, insecticidal, anticancer, and other activities in various classes of flavonoids, terpenoids, alkaloids, and other classes of natural products. The molecular technologies to identify the function of the genes and the effect of the bioactive compound(s) in medicinal plant(s) to treat patients with various chronic diseases are also presented. Transgenic plants produced through bioengineering represent another exciting area that is comprehensively reviewed.

I would like to congratulate the editors for accumulating such a wealth of useful information in this volume. My compliments also go to the eminent authors who produced an excellent overview of the present exciting frontiers of natural product chemistry.

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Preface

According to Huxley (1881), it is easy to sneer at our ancestors, but it is much more profitable to try to discover why they, who were really not one with less sensible persons than our excellent selves, were led to entertain views which look to us strange. For a better look at our future, we need to understand and look deeply at our past. Ethnobotanist Mark Plotkin says that every time a medicine man dies, it is like a library burning down. We are running a race against time. The information held by our medicine men needs to be pooled up fast for further evaluation by researchers.

The answers to the health problems for humans living during 2000 BC were that for an ear ache eat this root, yet with time the notion changed, and in 1000 AD, the same root was regarded as heathen, and was replaced by prayers. Yet in 1850 AD, people started saying that prayer is superstitious and instead advised the drinking of portions. However, in 1940 AD, that portion was regarded as snake oil, and the trend shifted toward the swallowing of pills. Around 1985 AD, the pills were regarded as ineffective, and people were advised to take antibiotics; ultimately in 2000 AD, the antibiotics were accepted as artificial, and the advice was to eat this root. So *we* started with the root in 2000 BC and ended up with the same root in 2000 AD.

Early anthropological evidence for plant use as medicine is 60,000 years old, as is reported from the Neanderthal grave in Iraq. There are clay tablets in cuneiform dated 2600 BC with plant remedies from the Sumerians, Assyrians, and Akkadians as well as Hittites. The Sumero-Akkadian clay tablets show a collection of ≈40 plants with vegetal formula pharmacopoeia. The importance of plants as medicine is further supported from Asia (3500 BCE) and Egypt (1500 BCE). Egyptian medicines report on the use of bishop's weeds (*Ammi majus*) to treat vitiligo, a skin condition characterized by a loss of pigments. More recently, a drug (b-methoxypsoralen) has been produced from this plant to treat psoriasis and other skin disorders as well as T-cell lymphoma.

Our second volume deals with phytochemistry and molecular aspects. It describes several secondary metabolic compounds found in plants, many of which provide protection against diseases. High-throughput robotic screens have been developed by industry, and it is possible to carry out 50,000 tests per day in the search for compounds which have action against a key enzyme or a subset of receptors.

Medicinal plant drug discovery continues to provide new and important leads against various pharmacological targets including cancer, HIV/AIDS, Alzheimer's, malaria, and pain. Numerous compounds from tropical rainforest plant species with potential anticancer activity have been identified. Although drug discovery from medicinal plants continues to provide an important source of new drug leads, numerous challenges are encountered including the procurement of plant materials, the selection and implementation of appropriate high-throughput screening bioassays, and the scale-up of active compounds.

Izmir, Turkey; Amann, Jordan — Munir Ozturk
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Free Radicals, Diabetes, and Its Complexities

F. Taghavi and Ali A. Moosavi-Movahedi

Introduction

Homeostasis

Life in every organism relies on keeping a stable set of interacting chemical reactions and internal processes to preserve the condition of reactions correctly (Torday 2015). It means that organism maintenance is provided by cell cooperative activities which need similarity in developed organization and metabolic requirements. This matter creates a stable and vital internal environment from point of oxygen, glucose, mineral ions, and waste contents (Marieb and Hoehn 2007).

The condition which relies on the stability, balance, or equilibrium of internal environment within a cell or total body is considered as homeostasis (homeo as similar and stasis as stable) (Yadav et al. 2016; Rodova et al. 2016; Andrey and Vladimir 2016).

Homeostasis is the most important concept in the body and has been defined through some developed approach: Claude Bernard, a French scientist, stated the concept of homeostasis beautifully as "all the vital process follow one aim as keeping the constant conditions of life in the internal environment" (Goldberger and Breznitz 1993). Another physiologist Walter Cannon, author of *The Wisdom of the Body* (1932), documented this concept as a self-regulating mechanism with admirable autonomic stabilizers which nature uses in imbalanced conditions (Vander et al. 2001; Siegel 2008). Another physiologist Moore-Ede (1986) stated that

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homeostasis includes reactive and effective responses to spontaneity and timed challenges (Siegel 2008; Moore-Ede 1986).

Homeostatic processes or allostasis (process of achieving stability) is observed in all order of cells, tissues, and organs (Torday and Rehan 2009). This process acts as a resistance to fluctuations of organism's internal environment against external environmental conditions (Torday and Rehan 2009). Osmoregulation, thermoregulation, and chemical regulation are considered as three mechanisms for homeostasis process (Chowański et al. 2017).

All homeostatic control mechanisms act based on three basis which have tightly interdependent relationship: (a) The monitoring receptors for sensing components, which detect the changes in the condition to be regulated and respond to environmental alterations; (b) the nucleus which receives the stimuli from receptors, which adjusts the scope of the changes and appropriate response; and (c) the effectors (cells, tissues, organs, or other structures), which receive the signal from the nucleus and apply homeostasis. These levels lead to correct deviation by negative feedback (depressing or damping action) (Torday and Rehan 2009).

Deficiency in each of homeostatic mechanism or toxicity (cell poisoning) influenced by internal and external factors (life style (nutrition, toxins, psychological or physical events) and environmental exposure) causes homeostatic imbalance, cellular malfunction, and disease. Many metabolism disorders and diseases like diabetes and its complexities are caused by homeostatic imbalance and its consequent internal toxin formation. These phenomena can be repaired by medical treatments (Marieb and Hoehn 2007; Vander et al. 2001; Torday and Rehan 2009).

Redox regulation is an important kind of homeostatic mechanism, which balances the beneficial and harmful effects of reactive species. This process maintains redox homeostasis by controlling the redox status in vivo (Fig. 1), and by this way, living

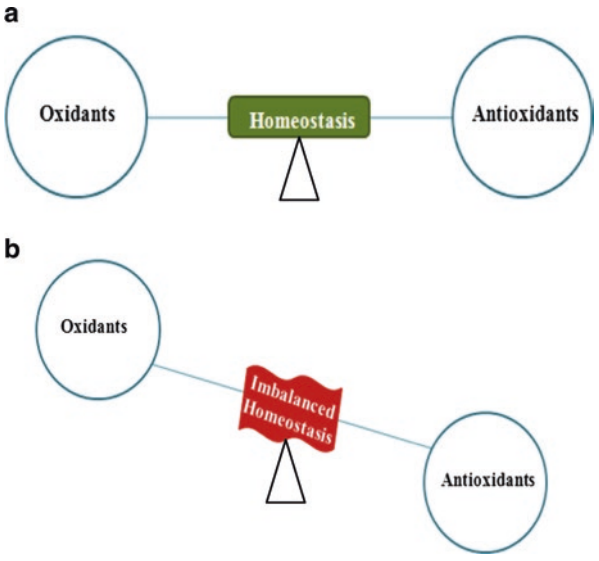

Fig. 1 The homeostasis create the balance between oxidants and antioxidants (**a**), extra formation of oxidants compared with antioxidants lead to imbalanced homeostasis (**b**)

organisms were protected against oxidative stresses. Cellular oxidants and antioxidants have an important role in the maintenance of "redox homeostasis" and redox regulation which is involved in normal physiological functions and pathogenesis of various diseases like neurodegenerative disorders, cancer, diabetes mellitus, inflammatory diseases, and aging (Valko et al. 2007).

It is worth mentioning that cell redox state is limited within a narrow range under normal conditions. This situation is controlled by redox signaling as an adoptive process, which triggers protective responses against oxidative stress. By this way, the original state of "redox homeostasis" after temporary exposure to ROS/RNS is restored (Valko et al. 2007).

Oxidative Stress

Over 90% of oxygen consumed by living organisms in healthy situation is used in mitochondrial electron transport chain which is coupled with nutrient oxidation and results in production of energy, carbon dioxide, and water (Abele et al. 2011).

Reactive Species

The energy which conducts life is released energy during landing of high-energy transmitted electron to low-energy level orbital or outer shell. Indeed, the electron storing and extracting and movement ability of higher-order biological structures form the essential forces for keeping life systems (Singh 2006; Szent-Györgyi 1976). These processes lead to reactive species formation. Interestingly life is the result of reactive species interaction because these species have been selected for some important roles as in evolution, metabolism, aging, and cell events (like apoptosis, mutation, and death). In other words, despite free radicals' destructive nature, surprisingly life with its unique organization can be sustained by these elements and a group of chemical interactions (Rahbar and Figarola 2003). Reactive species are intrinsically unstable and have various degrees of activities (Kikuchi et al. 2003; Betteridge 2000).

Free Radicals

Molecules with arranged pairs of electrons and opposite spin in their outer orbitals are very stable, while any species (atom and molecules) with independent existence and unpaired electrons in their outer orbitals/valence shell become highly reactive and unstable. These compounds are eager to interact with neighboring molecules and repair their outer shell and stability. These kinds of molecules are called free radicals (Singh 2006; Kelly 2003; Rahman 2007).

It is worth mentioning that free radicals with an odd electron usually are formed by splitting weak bonds in a molecule (each fragment keeps one electron) (Kelly 2003) and radical cleavage to form another radical or redox reactions (Ray et al. 2012). These kinds of reactive species attempt to gain their stability by capturing an electron, which leads to starting chain reaction in nanoseconds. This electron stealing makes molecule oxidation and runs a cascade reaction in uncontrolled condition, which leads to living cell disruption and inactivation (Pham-Huy et al. 2008). These compounds generally have been divided into two categories (Singh 2006; Phaniendra et al. 2015): (a) free radicals, species or molecules with an independent existence and one or more unpaired electrons, and (b) non-radicals, the compounds with strong oxidizing potential, which produce strong oxidants, like transition metals.

Reactive species have different longevity (from nanoseconds like hydroxyl radical to minutes as hydrogen peroxide or organic hydrogen peroxides).

It is worth mentioning that long-lived radicals include (1) stable radicals with kinetic and thermodynamic stability (organic radicals); (2) persistent radicals, compounds with persistent radicals which make them physically difficult to react with another molecule; and (3) diradicals, molecules with two radical centers (Pham-Huy et al. 2008). In addition, reactive species can be classified into the following groups:

(a) Reactive oxygen species (ROS): Molecular oxygen (dioxygen) with a unique electronic configuration is a radical. In living systems oxygen-derived radicals are the most important class of radical species.
(b) Superoxide anion radicals: The superoxide anion radicals ($O_2^{\bullet -}$) as "primary" ROS are created by adding one electron to molecular oxygen via metabolic processes (within the cell mitochondria) or physical irradiation. This product can interact with other molecules and produce "secondary" ROS, directly or indirectly by enzyme- or metal-catalyzed processes. The main source of ATP in the mammalian cell is mitochondrial electron transport chain, which is essential for life. During this process, superoxide as an intermediate reactive species is formed and involved in pathophysiology of a variety of diseases.
(c) The hydroxyl radical, $^{\bullet}OH$: The neutral form of hydroxide ion, with high reactivity and very short in vivo half-life (10^{-9} s) at 37 °C, is very dangerous. This kind of radical can react with neighboring molecules (especially nuclear and mitochondrial DNA, membrane lipids, and carbohydrates) very fast, immediately after formation. Its interaction with lipid membrane can trigger a chain reaction and propagates lipid peroxidation. An extra amount of superoxide lead to free ion release from molecules with iron contents (Valko et al. 2007; Gadoth and Göbel 2011; Aprioku 2013).
(d) Peroxyl radicals ($ROO^{\bullet}$): These radicals are another reactive radicals derived from oxygen which usually form in living systems by cellular membrane lipid breaking down. These radicals can be self-propagating, very dangerous, and highly destructive. It is worth mentioning that superoxide anions, hydroxyl radical, singlet oxygen, and peroxynitrite can trigger lipid oxidative decomposition and produce lipid peroxyl radicals and hydroperoxides. These radicals can attack polyunsaturated fatty acids and propagate the chain reaction

(Lushchak and Semchyshyn 2012). Also, the protonated form of superoxide ($O_2^{\bullet -}$) creates the simplest form of peroxyl radical ($HOO^{\bullet}$) termed as either hydroperoxyl radical or perhydroxyl radical (Valko et al. 2007).

(e) Singlet oxygen: Under photooxidative conditions and the presence of photoexcited sensitizers, energy transfer to O_2 and singlet oxygen ($1O_2$) is performed. It is highly reactive and can trigger lipid peroxidation. It has no diradical activity like molecular oxygen (Gadoth and Göbel 2011).

(f) Peroxide: High concentration of hydrogen peroxide (H_2O_2) is toxic within cells. It can diffuse through cellular membranes and interact with distant molecules. Hydroxyl radicals can be formed from hydrogen peroxide in the presence of iron and copper (Gadoth and Göbel 2011).

(g) Reactive nitrogen species (RNS): Nitric oxide ($NO^{\bullet}$) acts as a double-edged sword (Gadoth and Göbel 2011; Aprioku 2013). $NO^{\bullet}$ is an abundant reactive radical and has an important role in biological signaling especially in diverse physiological processes, like neurotransmission, blood pressure, immunoregulation, defense mechanisms, and smooth muscle relaxation. It is a small molecule which is formed in biological tissues by specific nitric oxide synthases (NOSs). This kind of radicals has a short half-life (few seconds) in aqueous system. The presence of extra reactive nitrogen species create nitrosative stress which can nitrosylate biomacromolecule structures and disturb their function. The reaction of nitric oxide and superoxide anion during the immune system activities leads to the formation of very potent oxidative radicals as peroxynitrite anion ($ONOO^-$). This radical can attack DNA and cause its fragmentation; also it can produce lipid oxidation (Valko et al. 2007). The peroxynitrite anion has the same reactivity of $^{\bullet}OH$ and can directly hydroxylate and nitrate the aromatic rings of amino acid residues. Its reaction with sulfhydryls, zinc-thiolate moieties, lipids, and proteins makes it a very toxic radical (Gadoth and Göbel 2011).

The Role of ROS

As mentioned before, however, ROS can be considered as essential intermediates in natural and normal biological processes, but they can produce homeostatic imbalance and pathogenic events (Pham-Huy et al. 2008; Gadoth and Göbel 2011).

Signaling, defense against infections, modification of molecules, neurotransmission, and damage of impaired cellular constituents are some of the various processes which can be impaired by ROS. ROS radicals do their roles via interaction with biomacromolecules based on ROS type and their concentrations. Intra- and intercellular communication (cellular signaling, cellular proliferation, cellular pathway) (Gadoth and Göbel 2011) with ROS takes place at low ROS concentration in specific pathway, but higher levels of ROS are involved in cellular component-specific damages (Lushchak and Semchyshyn 2012).

DNA and protein can be considered as primary targets for the destructive effects of ROS (Kelly 2003). It is noteworthy that the aggressive nature of free radicals and

other non-radicals' reactive derivatives caused them to be considered as oxidants. Non-radical species are more stable than radicals, but radicals are more active than non-radicals. Also, hydrogen peroxide (H_2O_2), ozone (O_3), singlet oxygen ($1O_2$), hypochlorous acid (HOCl), nitrous acid (HNO_2), peroxynitrite ($ONOO^-$), dinitrogen trioxide (N2 O_3), and lipid peroxide (LOOH), as non-free radicals, can easily lead to free radical (hydroxyl ($OH^\bullet$), superoxide ($O_2^{\bullet-}$), nitric oxide ($NO^\bullet$), nitrogen dioxide ($NO_2^\bullet$), peroxyl ($ROO^\bullet$), and lipid peroxyl ($LOO^\bullet$)) reactions in living organisms (Ray et al. 2012; Brennan and Kantorow 2009).

As mentioned before, the reactivity of reactive species is very different, and some of them are more specific and harmful for biological molecules than the others. The most important oxygen free radicals in biological context are superoxide, hydroxyl radical, and nitric oxide (Finkel and Holbrook 2000) which are very unstable and trigger cascade oxidative reaction. Hydrogen peroxide, hypochlorous acid, and singlet oxygen are the most common non-radicals with higher stability and risks for biomolecules (Surai 2002).

ROS Involve in Lipid Peroxidation

Lipid is an important cellular biomacromolecule which can be the prominent target of oxidative stress. Oxidants (reactive species and free radicals) have the main role in lipid peroxidation and increase ROS steady-state concentrations especially as peroxides. This phenomenon modified other biomacromolecules. The products of lipid peroxidation can be categorized based on their instability as primary (with short half-life and free radical nature), secondary (lipid peroxides, conjugated dienes, and ketodienes), and end products (malondialdehyde). Both of the last products have more stability than the first (Lushchak 2011).

The Source of Reactive Species

The reactive species generally include reactive oxygen species (ROS) and reactive nitrogen species (RNS) and are the advantages of cellular redox process. Because of their high biological instability and their electron availability, they can react with various organic substrates such as lipids, proteins, and DNA. In the cells, free radicals and its derivative formation occur in two ways: enzymatic (respiratory and energy chain, the phagocytosis, the prostaglandin synthesis, and the cytochrome P450 system) and nonenzymatic reactions (with organic compounds and ionizing radiations) (Ray et al. 2012).

It is important to note that ROS and RNS just at low or moderate levels show beneficial effects on cellular activities. While the consequence of high concentrations is oxidative stress, deleterious process and biomacromolecule structural damage lead to chronic and degenerative diseases like cancer, aging, and autoimmune disorders.

Also reactive species generation depends on endogenous or exogenous sources. Immune cell activation, inflammation, psychological and mental stress, excessive exercise, ischemia, infection, cancer, aging, cellular abnormalities, malnutrition, and various diseases generate endogenous reactive species. The endogenous ROS are the greatest threat to organisms (Kikuchi et al. 2003; Betteridge 2000).

Exogenous sources arise from air and water pollution (asbestos; benzene; carbon monoxide; chlorine; formaldehyde; MTBE; ozone; tobacco smoke; toluene; chemical solvents such as cleaning products, glue, paints, and paint thinners; prescribed medications; perfumes; pesticides; cigarette smoke; alcohol; heavy or transition metals (Cd, Hg, Pb, Fe, As)), certain drugs (cyclosporine, tacrolimus, gentamicin, bleomycin), industrial solvents, cooking (smoked meat, used oil, fat), and radiation (Ray et al. 2012). These exogenous compounds penetrate into the body by different routes and decompose or metabolize into free radicals (Kelly 2003; Ray et al. 2012). Some of the exogenous sources for reactive species formation will be described in the next sections.

Oxidative stress is defined in many ways and improved during the years (Lushchak and Semchyshyn 2012). Oxidative stress is the acute state of imbalance between generation of active intermediates and the system's ability to neutralize and eliminate them (Rahman et al. 2012).Oxidative stress happens by passing favoring prooxidants and/or disfavoring antioxidants from normal situation and alters the redox condition of internal environment and damaging of macromolecules (Singh 2006). This phenomenon is a condition in which the balance between oxidative activities and antioxidant systems is disrupted and generation of active oxygen species or free radicals becomes excessive, in an unsuitable way (Rahman 2007). Oxidative stress is the harmful condition for the body when oxidative reaction (ROS/RNS production) overcomes antioxidant defense system and body's internal balance is lost (Rahman 2007; Lushchak and Semchyshyn 2012). Indeed, oxidative stress is a disturbed dynamic equilibrium which led to an enhancement in ROS steady-state transiently or chronically. This situation disrupts cellular components, metabolism, regulation, and signaling processes by oxidized cellular constituents via ROS up to the consequent deleterious effects (Lushchak and Semchyshyn 2012; Lushchak 2011). It is noteworthy that three ways provided oxidative stress: ROS production increment, ROS elimination decrement, and appropriate combination of these two ways. All of these processes directly lead to diseases (Lushchak and Semchyshyn 2012).

Oxidative stress can be considered as a biological modulator and signal inducer which modulate many messengers with vital role in living systems. Oxidative signaling functions are important as adaptive strategies and coordinating effect in diverse basic biological processes like differentiation and apoptosis. This phenomenon influences intracellular redox status, protein kinases activities, and cellular responses (like activation, proliferation, differentiation, and other activities) which is considered as a chain between OS and diseases (Rahman 2007; Lushchak and Semchyshyn 2012).

Oxidative Stress-Inducing Agents

Air Pollution

Environmental pollution is a major concern of scientists and contributes to many diseases and death. The harmful or undesirable alteration in the quality of air, water, or soil, physically, chemically, or biologically, is considered as environmental pollution. This approach includes all anthropogenic and natural pollutants and direct or indirect human involvement in environmental pollution (Yanga and Omayeb 2009).

A range of pollutants with different combinations from one microenvironment to another are in the ambient air. The big portion of this mixture belongs to free radicals (like nitrogen dioxide) or compound with the ability of free radical production (like particulate matter and ozone). Exposure to a wide range of air pollutants leads to oxidative stress and disease formation in human organs especially the lungs. Ozone as a relatively insoluble gas with high reactivity is a major constituent of photochemical smog which leads to lung function decrement and pulmonary inflammation. Its reaction with biological environment is based on its concentration and the type of biological molecules which takes part in the reaction (Kelly 2003).

Traffic in urban areas and cigarette smoke are the main sources of nitrogen dioxide. This compound produces cellular destruction, increment cell permeability, and increment tissue inflammation (Kelly 2003).

Our researches also showed that methyl *tert*-butyl ether (MTBE) can be very harmful for human beings. MTBE is a worldwide gasoline modifier which improves fuel oxygen contents and its combustion. It is a toxic component which produces adverse biological effect for human health and diverse environmental concerns (Valipour et al. 2015). MTBE's biodegradation is slow in groundwater spreads in the air widely, pollutes the environment, and enters the blood stream easily. Our previous research revealed that MTBE as a gasoline modifier had an influence on the structure and function of hemoglobin (Hb). This compound can disturb Hb-oxygen affinity, its oxygen transport, and cause metHb formation more than normal condition. The high levels of ROS production were demonstrated due to degradation of hemoglobin's heme by MTBE via chemiluminescence technique. It seems that ROS production has the main role in heme degradation and Hb nonfunctionality (Valipour et al. 2017).

Our results also showed that MTBE induced a molten globule (MG)-like structure in insulin due to reactive oxygen species (ROS) formation which leads to protein oxidation and protein aggregation (Valipour et al. 2015).

Dust

Environmental dusts contain toxic particulates and oxidants which stimulate inappropriate chemical reactions and produce the large amounts of free radicals. This character depends on their micromorphology at the atomic level; mechanical, thermal, and chemical properties; as well as frequency of surface contaminants' presence

(Fubini 1998). Dusts have harmful potential effects on biological membrane integrity because of the generation of free radical on the dust surface, their interaction with many chemical sites on the cell membrane, and stimulating lipid peroxidation (Vallyathan et al. 1988).

It is worth mentioning that the bulk of dust can be chemically intact in biological systems after many years (Dalal et al. 1989).

According to research, coal dust contains carbon-centered free radicals, and its concentration on the dust surface depends on the rank of coal, dust freshness, and its particle size (Dalal et al. 1989). Also it has been reported that all coal dusts generate OH radicals and had lipid peroxidation abilities. This kind of dust has an important role in the development of coal workers' pneumoconiosis (CWP) in different coal mining areas. OH radicals may play an important role in the development of CWP in different coal mining areas (Vallyathan et al. 1988).

The toxic particulates (asbestos, crystalline silica, coal, etc.), chemicals, gases, fume, and man-made dust can be present in the air through industrial operation, automobile emission, and traffic congestion. These conditions lead to chronic or oxidative injury in different organs specially the lung and other chronic and serious diseases. Inhalation of polluted ambient air includes different particulates; chemical and physical agents are the main reason for toxic entrance to the body and occupational or environmental disease formation (Dalal et al. 1989).

Dust also includes particulate matter. Particulate matter is the suspended material and heterogeneous mixture with varying size and chemical composition as minute solid particles or liquid droplets in the air which originate from both human and natural activities. These components comprise aerosols, smoke, metallic, dust, pollen, and oxides (Araújo et al. 2014; Du et al. 2016).

Particulate matter (PM) consists of inert carbonaceous cores and adsorbed multiple layers of various molecules (organic pollutants, metals, acid salts, biological elements, pollen fragments, allergens, endotoxins) (Araújo et al. 2014). PM is observed as the total suspended particulates (TSP) with sizes up to about 50 μm. The biggest size in this group cannot enter the human lungs and originates from wind-blown dust and soiling of buildings and clothes. This group in terms of their potential influence on health and their diameters is divided into three subgroups (Araújo et al. 2014; Du et al. 2016): PM10, PM2.5, and ultrafine particles (UFPs) subgroups according to their diameter (Du et al. 2016). The PM10 particles (coarse particles with the aerodynamic diameter (AD) from 2.5 to 10 μm) related to natural activity (wildfires and windblown dust) and human activities (mining operations, demolition and construction activities, agricultural and road dust, tire wear emission (Du et al. 2016).

The source of PM with size less than 2.5 μm is related to traffic and industries, fuel combustion or mobile brake emissions, power plants, industrial processes, residential wood burning motor vehicles, forest fires, and agricultural burning (Araújo et al. 2014).

These particulate matters have the main role in death due to the adverse cardiovascular effects of air pollution on human health (Du et al. 2016). Particles with diameters less than 0.1 μm called UFPs originate from tail pipe emissions from mobile sources. There are different deposit location for these particulate matters: PM10 particles (upper airways), both PM2.5 and UFP particles (reach the smallest

airways and alveoli), but UFPs can be very toxic and penetrate and spread deeper into the systemic circulation and remote organs (Siegel 2008).

Atmospheric aerosol particles can be emitted as primary particles (directly emitted into the atmosphere) or formed by secondary processes (transformation of emitted precursor gases) (George et al. 2015; Fuzzi et al. 2015).

These matters are the source of atmospheric aerosols (Fuzzi et al. 2015): marine aerosol (mineral dust, biological aerosols, and volcanic ash), mineral dust (Sahara), primary biological aerosol particles (PBAPs) (different biological components like microorganisms as bacteria, archaea, algae and fungi and various materials such as fungal spores, pollen, viruses, and biological fragments), transport-related aerosol in densely populated regions, and wood combustion (responsible for half of the production of organic carbon (OC) in the cold season). Secondary aerosol precursors are released by natural and anthropogenic sources (SO_2, NOx, NH3 and volatility organic compounds (VOCs) and intermediate-volatility organic compounds (IVOCs)) and preindustrial aerosol (PM in the absence of anthropogenic emissions). Also, ambient aerosols as secondary particular matters can interact with atmospheric gases (carbon monoxide, nitric oxides sulfur, ozone). All of these PM can have synergistic or antagonistic effects with each other (Du et al. 2016). Air quality, human and ecosystem well-being, and Earth's climate system can be affected by atmospheric aerosols (Fuzzi et al. 2015). Respiratory and cardiovascular diseases are the main reason of death and its increment due to short- and long-term exposure to air pollution. Sustained oxidative stress and inflammation are induced by exposure to PM10 and PM2.5. PM2.5 increases the activation of autonomic nervous system (Fiordelisi et al. 2017; Brook and Rajagopalan 2010).

Water pollution by chloroform and other trihalomethanes, water chlorination, heavy metals, etc. can be a great hazard for environmental health. Oxidative stress and ROS production can be induced by metal ions due to their interference with metal-related processes or via ion with changeable transition metals or both of them.

Many researchers reported abundant generation of oxidative stress in hydrobionts by transition metals. They are also capable to induce oxidative stress in aquatic animals by reversible oxidation. These effects have been reported about ions of copper, mercury, arsenic, chromium, cobalt, titanium, and vanadium and their complexes. Also it is reported that these metals decrease the activities of catalase, glutathione peroxidase, glutathione-S-transferase, and glutathione of fishes and produce lipid peroxidation and protein carbonyl contents. Arsenic is involved in several ROS and RNS production. It was observed that H_2O_2 formation was happened due to oxidation of As^{3+} to As^{5+} under physiological conditions (Lushchak 2011).

Changes in salinity in aquatic systems can influence the levels of oxidative stress and improve ROS production. Co-exposure of salinity with metals can produce the synergism effect on their toxicity and their effect on oxidative stress (Baysoy et al. 2012; Loro et al. 2012).

Heavy Metals

Oxidative stress and ROS generation can be induced by environmental trace metals (Cd, As, Cu, Fe, Hg, Cr, Pb, Se, Ni, Zn, and V) and organic pollutants. This character arises from their ability to lose electrons, their catalytic activity in Haber-Weiss and Fenton reactions (redox active metals), and their oxidation state alteration which led to ROS formation (Simic et al. 1988). This kind of metals can act as redox inactive agents, suppress antioxidant systems, and improve oxidative reaction (Abele et al. 2012). Organic pollutants especially induce cytochrome P450 and cause intracellular ROS production (Valko et al. 2007).

Moreover, metal can induce reactive species formation due to their interaction with cellular components especially polyunsaturated fatty acid residues of phospholipids as sensitive compartment to oxidation. Malondialdehyde (MDA) and 4-hydroxy-2-nonenal (HNE) are two important toxic and mutagenic products of lipid peroxidation which their formation is triggered by rearrangement of peroxyl radicals ($ROO^{\bullet}$) (Valko et al. 2007).

Temperature

Oxidative stress can be induced by lower and higher temperatures both in endo- and exothermic animals due to their metabolic rate increment, ROS production (Sahin and Gumuslu 2007; Selman et al. 2000; Bagnyukova et al. 2007), and reduction of antioxidant systems (Beamonte-Barrientos and Verhulst 2013; Malek et al. 2004). Cold temperature in aquatic systems increases the solubility of oxygen, decreases conductance, and increases the ROS production per mg of protein. This matter is similar to temperate and warm areas for animals living (Abele and Puntarulo 2004).

Electromagnetic Fields

There are many reports about amyotrophic lateral sclerosis (ALS), childhood leukemia, adult brain cancer, and miscarriage due to EMF exposure of the power line radiation, ELF-EMFs. However the EMFs are non-ionized radiation, but its influence on mainly oxidative stress and metabolic processes which generate oxidants, decrease antioxidants, and alter cellular process like alteration in cell cycle and protein expression and induction of cell death has been proposed. Increased exposure with EMFs can alter the cellular homeostasis by producing reactive oxygen species (ROS) and suppressing enzymatic and/or nonenzymatic antioxidants which result in cellular component damage (lipid membrane, nucleic acids, and protein).

ELF-EMF exposure can increase the lifetime of free radicals and their dispersion which lead to increment of their activity and concentration of the free radicals. ELF-EMFs also have inhibitory effect on some radical scavenger antioxidant hormones like melatonin. This kind of electromagnetic wave enhances the release

of free radicals by inducing cells "activated state." These processes can promote two distinct interaction mechanisms as (a) thermal effects (by rotation of the polar molecules and generation of live cell dielectric heat (Terzi et al. 2016)) and (b) nonthermal effects which are involved in biological response and intracellular or extracellular oxidative stress induction by HF-EMFs (Gaestel 2010). Our previous research also showed the formation of ROS due to cell exposure by mobile phone with frequency electromagnetic field (RF-EMF, 940 MHz).Also, we reported the alteration of HbA structure and its oxygen affinity by mobile phone EMFs based on its time of exposure and intensity (Sefidbakht et al. 2014, 2013; Mousavy et al. 2009). Oxidative stress and free radical formation is the link between living organism and ELF-EMFs. The character of biological pathways as dynamic and nonlinear system and the biochemical effects of nonsignificant alteration in free radical concentrations change EMF as a harmful agent for human health (Terzi et al. 2016). EMFs increase ROS by involving in the Fenton reaction in the cells and enhance hydroxyl free radical formation. It is important to know that any free radical can activate a plausible biological mechanism which leads to diseases. Ionized radiation produces a mixture of hydroxyl/superoxide radicals (Aydina and Akarb 2011).

Alcohol

Alcohol metabolization produces some toxic and reactive compounds: firstly acetaldehyde produced by alcohol dehydrogenase which then changes to acetate by aldehyde dehydrogenase. Nicotinamide adenine dinucleotide which is formed by each of the reactions impairs the redox balance and enhances electron flow and the activity of the respiratory chain, O_2 consumption, and ROS generation. The alcohol induces oxidative stress and reactive species production. Moreover, the morphology of mitochondria membranes can be changed by chronic alcohol (Ignatowicz et al. 2013).

Alcohol activates liver Kupffer cells, with ability of pro-inflammatory cytokine induction and ROS and reactive nitrogen species (RNS) productions. This abnormality is directly related to NAFLD (Ignatowicz et al. 2013). It is important to mention that most of the signal proteins like JNK, PKC, p38, Cdc42, AP-1, EGFR, PI3K, Akt, and cyclins A and D are oxidative stress-responsive proteins. So their activities could be regulated by oxidative stress which is induced by alcohol and leads to alcohol-induced diseases (Ignatowicz et al. 2013; Wu et al. 2006).

Herbicides

This kind of compounds induce oxidative stress through directly entering redox cycle and enhancing free radical generation or inhibit antioxidant activities (Lushchak 2011).

Pesticide

Pesticides include insecticides, herbicides, and fungicides which are physical, chemical, or biological agents for killing unwanted organisms. Pesticide can involve in redox cycles and enhance reactive species formation, decrease or inactivate antioxidant potential, interfere with energy creating process or disrupt genetic process (through impaired transcription and translation), and especially enhance steady-state ROS level indirectly (Lushchak 2011).

Fungicides

Some organic fungicides can stimulate ROS production by binding to cytochrome, uncoupling the electron-transport chain from mono-oxygenase activity, and produce reactive species. The metabolism of these compounds can lead to ROS production by itself (Lushchak 2011). Glyphosate fungicides have the ability to alter humans' microbiome of intestinal flora and form dysbiosis (Samsel and Seneff 2013) which produce allergy and sensitivity, autoimmune diseases, and systemic inflammation as the cause of many modern diseases (Myles 2014).

Cigarette Smoke

The smoke of tobacco is a pool of oxidative organic compounds and free radicals (superoxide and nitric oxide). Also, the cell activation (lung epithelium, fibroblasts inflammatory cells, macrophages and neutrophils) via inhalation of tobacco smoke particles leads to reactive species generation (ROS and RNS) (Aprioku 2013). So, tobacco can damage biomacromolecules like DNA, proteins, lipids, and polysaccharides through their oxidization and cause pro-inflammatory reactions, aging, and other disease formation (Ignatowicz et al. 2013).

Industrial Foods

The presence of industrial agriculture and food has converted people's habit from using low-shelf life natural food (grains, fruits, and vegetables) to long-shelf life processed food. Nowadays, considering food as a product and more production are the real attitude of industries. So, easier feeding and more productivity are the basis of industrial programs. These kinds of foods can involve many diseases like metabolic disorder (obesity, nonalcoholic fatty liver disease (NAFLD), diabetes) and chronic disease (Yach et al. 2010). There are many reports about the contribution of chemical-laden food products in diseases that affect people's quality and length of life. Industrial foods are processed based on using additives, hormones, antibiotics, and genetic engineering (Brownell and Warner 2009; Igumbor et al. 2012).

Oxidative Stress Is the Main Reason of Diseases

Oxidative stress as a consequence of homeostatic imbalance is a destructive process where its products change cell structures, process, and components (proteins, lipoproteins, lipids, nucleic acids).

Excess hydroxyl radical and peroxynitrite are responsible for a chain reaction called lipid peroxidation which destroys cell membranes and lipoproteins. The products of this process are malondialdehyde (MDA) and diene compounds as cytotoxic and mutagenic agents. The attack of ROS/RNS to protein and DNA leads to their structural and functional changes, toxic products, and DNA lesions which lead to mutations and pathologic effect. This condition changes redox regulation and results in acute disease (Valko et al. 2007).

It is worth mentioning that chronic and degenerative diseases, aging process, and some acute pathologies (trauma, stroke) are the consequent events of abnormalities in regulatory and repairing systems like repair enzymes and antioxidants. Also, residues in protein side chains (specially Cys, His, Arg, Lys) are another target for aggressive attack of reactive species. This kind of oxidation leads to protein aggregation and protein backbone breaking which result in their inactivities or toxic activities (Valko et al. 2007).

The oxidative-borne diseases are divided into two groups (Valko et al. 2007): (a) shifting in the thiol/disulfide redox state and impairing glucose tolerance by prooxidants entitled "mitochondrial oxidative stress" conditions (cancer and diabetes mellitus) (Valko et al. 2007) and (b) diseases arose from "inflammatory oxidative conditions" which improve activity of NAD(P)H oxidase (atherosclerosis and chronic inflammation) or xanthine oxidase-induced formation of ROS (ischemia and reperfusion injury). Free radical action on biomacromolecules (lipid peroxidation, DNA damage, protein oxidation) and its consequent damage lead to aging (Valko et al. 2007; Babbs 1988).

Cancer

Endogenous and exogenous stimuli-induced cellular and biomacromolecules changes result in the development of cancer. It is well established that DNA oxidative damage is responsible for cancer development. It has been shown that chromosomal defects by base hydroxylation, base and sugar lesions, strand breaks, DNA-protein cross-links and base-free sites, oncogene activation, altering normal gene transcription, and cancer initiation are induced by free radicals. Tobacco smoking, chronic inflammation, and high consumption of fats are the main reasons for cancer disease due to lipid peroxidation (Horton 2003). The initial mutagenic event in carcinogenesis usually involves ROS or some oxidative chemicals produced by the cytochrome P-450 system. Tissue with a concomitant oxidation process is a target for metastasis (Florence 1995). The reaction of hydroxyl radical with DNA components like purine and pyrimidine bases and the deoxyribose backbone damages them seriously. The formation of genetic

material permanent modification as 8-OH-G implies the first step of mutagenesis, carcinogenesis, and aging.

Cardiovascular Disease

Hypercholesterolemia, hypertension, smoking, diabetes, poor diet, stress, and physical inactivities are the risk factors which provide the conditions of cardiovascular disease (CVD). Many reports showed the role of oxidative stress in atherosclerosis, ischemia, hypertension, cardiomyopathy, cardiac hypertrophy, and congestive heart failure (Ray et al. 2012).

Fatty deposits on blood vessels result in atherosclerosis. At first endothelium was damaged by released ROS from white blood cells, and then lipid peroxide is the main reason for atheroma. Cholesterol epoxides, lipid peroxides, and antibodies are the main contents of atheroma deposition (Florence 1995).

Neurological Disease

Oxidative stress and reactive species have a key role in neuron dysfunction, and its consequent events lead to neurodegenerative diseases like Alzheimer's disease, Parkinson's disease, multiple sclerosis, amyotrophic lateral sclerosis (ALS), memory loss, and depression. Reactive species (especially toxic superoxide radical) and antioxidant deficiency induce the formation of toxic amyloid structures in intra- or extra-neuronal cell which is involved in neurodegenerative diseases (Lushchak 2011). It is worth mentioning that the brain because of its high oxygen utilization, oxidizable polyunsaturated fatty acids, and the presence of redox active metals (Cu, Fe) is sensitive a lot about oxidative stress (Valko et al. 2007).

Pulmonary Disease

Oxidative stress induces the activities of different redox transcription factors (NF-kappa B and AP-1) and kinases which lead to systemic and local chronic inflammation in the lung and pulmonary diseases. Research revealed that systemic and local chronic inflammation and oxidative stress are involved in inflammatory lung diseases.

Rheumatoid Arthritis

Chronic inflammation of the joints and joints around the tissue with penetration of activated T cells and macrophages characterized an autoimmune disease as rheumatoid arthritis. The presence of reactive species in the disease location produces pathogenesis of rheumatoid arthritis.

Nephropathy

Oxidative stress and transition (Cr, Cu, Co, Fe) and heavy metals (Pb, Hg, Cd, As) as potent free radical inducers via lipid peroxidation produce renal diseases which include uremia, proteinuria, chronic renal failure, tubulointerstitial nephritis, and glomerulonephritis (Ray et al. 2012).

Ocular Disease

Oxidative stress and reactive species degenerate cells, change eye's various cell types, aggregate lens protein (cristalin), and promote eye diseases. Likewise, oxidative stress is believed to be involved in retinopathy and macular degeneration. High amount of unsaturated lipids in eye made this organ as a prominent target for oxidative damage (Ray et al. 2012).

Aging

Aging can be considered as a progressive destruction in organism's physiological functions after life's fertility period. Indeed oxygen begins the process of aging because of the leaking electrons from mitochondrial electron transport chain. These leaked electrons can interact with oxygen and produce superoxide radicals. These processes take place during aerobic metabolism and destroy biomacromolecules by reactive species gradually. The accumulations of toxic products lead to aging (Valko et al. 2007).

Defensive Systems Against Free Radicals

Organisms have to maintain their life against various free radicals by a series of defense mechanisms: (1) preventative mechanisms, (2) repair mechanisms, (3) physical defenses, and (4) antioxidant defenses (Valko et al. 2007).

There are an elaborated anti-free radical defense systems in all aerobic forms of life: (a) enzymes (superoxide dismutase (SOD) and glutathione system) for changing free radicals to a much less reactive form; (b) uric acid and ceruloplasmin react with free radicals in the intercellular spaces and bloodstream; (c) self-repair proteins which used and damaged protein scavenger enzymes and break them into their component for reuse by the cell; and (d) nutrients(the oxy radicals are neutralized by vitamins (vitamins C and E, beta-carotene, and bioflavonoids) and other nutrients. All of these systems are considered as antioxidants (Pham-Huy et al. 2008).

Oxygen avoiding is the simplest defense against its toxicity. This matter has been accomplished by (a) packing redox constituents together in electron transport chains and (b) lowering the amount of O_2 in parts of the organism. Designing resistant structural defense against oxidative stress is not enough for encountering with reactive species toxicities. So, specific nonenzymatic and enzymatic antioxidants have produced to react with harmful agents (Halliwell and Gutteridge 2007).

Antioxidants

To cope with extra amounts of reactive species, the antioxidant defense system has been designed by the body (Singh 2006). There is a common definition about antioxidants: A stable and safe substance with high inhibiting influence on oxidant especially at low concentration (Singh 2006).

In other words, homeostasis maintenance needs an endogenous system for free radical scavenging and maintaining the effective balance between reactive species production and their removal. Actually, antioxidant compounds regulate the redox status of living systems and protect them against oxidative stress (Vendemiale et al. 1999). It is important to note that antioxidants suppress uncontrolled formation of reactive species (Chaudieáre and Ferrari-Iliou 1999). Also the exposure of antioxidant at low concentration to oxidized substances caused delay, prevention or removal of counteract with biological structures and biomacromolecules oxidative destruction (Halliwell and Gutteridge 2007; Cadenas and Packer 2002). This inhibitory effect can counteract with body's internal destructive effects of reactive species, prevent the consequent damages, and reduce disease symptoms (Pham-Huy et al. 2008; Lushchak and Semchyshyn 2012). The antioxidants can be categorized as follows: (a) direct antioxidants with scavenging effect of free radicals (SOD/$O_2^{-\bullet}$) or non-radicals (Catalase/H_2O_2) (Rahbar and Figarola 2003). The major antioxidant enzymes participate in ROS neutralization and act directly (Birben et al. 2012).

It is worth to note that scavenging antioxidants with fast kinetic act as chemical traps of oxidizing free radicals and activated ROS. They also can be quenched by excited species physically such as singlet oxygen and triplet states of photosensitizers. The free radical by-products of this scavenging reaction quench by dismutation and secondary scavengers and never come back to chain reactions again. Peroxyl radicals ROO, hypervalent iron species, singlet oxygen, and halogenating oxidants such as hypochlorous acid HOCl are continuously "scavenged" within our cells (Chaudieáre and Ferrari-Iliou 1999); and (b) indirect antioxidants with inhibitory effect on cellular sources of oxidants (chelators/metals, apocynin/Nox) or inducing effect on cellular antioxidants (sulforaphane/Nrf2 targets-GSH) (Singh 2006).

Chelating metals via metal-chelating proteins like metallothionein can decrease metal-induced ROS generation. There are other antioxidants which are derived from the diet (vitamins and carotenoids).

Nonenzymatic antioxidants with low molecular weight (vitamins C and E, β-carotene, uric acid, and GSH) have ROS scavenging properties as indirect antioxidants (Florence 1995; Birben et al. 2012).

Vitamin E has an important role in inhibition of lipid peroxidation (Florence 1995). Under normal conditions, there is a balance between both the activities of intracellular levels of these antioxidants. This balance is essential for the survival of organisms and their health (Valko et al. 2007).

Antioxidants with New Conversation

Based on our research, antioxidant is any factor which produces free radical scavenger elements and removes reactive species. Based on this definition, antioxidants can be considered as follows.

Sleeping

Sleep is the loss or absence of consciousness, relative suspension of sensory perception, and inactivity of almost all skeletal muscle during rest. So, the activity of the mind and body changes, and the actual images are replaced by the visual illusions. Sleep is a behavior which is important for the health and regeneration of nervous, immune, and musculoskeletal systems of humans and animals. Sleep is a balanced and restorative function which seems to be essential for regulating temperature and energy conservation. Sleep also restores body strength by improving the synthesis of ribonucleic acid and proteins (Buysse 2005; Smith et al. 2008).

It is important to note that sleeping is the primary mechanism for removing brain toxic products. Brain cells shrink during sleep and decrease by 60% of their total volume. This matter increases the intercellular space, and by this way, the brain tissue can be washed by cerebrospinal fluid easily. During 24 h, various detoxification events happen in the body which some of them are related to good sleep. One of the sleep benefits is the secretion of the melatonin hormone which is called sleep's hormone. Melatonin regulates the body's circadian rhythm, protects the genetic material, and keeps the body from age-dependent diseases. This hormone also plays an important role as an antioxidant in neuroprotection, inflammatory defense, and immune system.

Melatonin suppresses oxidative stress and cell death by activating a series of antioxidant enzymes (superoxide dismutase, glutathione peroxidase, and glutathione reductase) by indirect or direct interaction with free radicals (hydroxyl) and oxidative stress (Reiter et al. 2000).

Fasting

Fasting as a periodic voluntarily avoidance from eating, drinking, and smoking acts as a therapy which has many molecular and cellular advantages for the human body and one's mental activities. This great blessing and health-promoting behavior can improve neurotrophic factors and neuroendocrine systems, reduce oxidative stress and aging-related signal association, and specially promote autophagy (as a waste collection and recycling mechanism).

Autophagy is an intracellular process for self-destruction of impaired cellular contents (like lysozymes). The complex phenomena are crucial for physiological processes of energy balancing and responding to food stress. Also, autophagy has an important role in cellular protection by eliminating abnormal proteins and cleansing of damaged organelles. Fasting induced ketogenesis which changes cellular processes and metabolic pathways specially stress resistance and chaperon-mediated autophagy. It also regulates the glucose and lipid homeostasis by ketone bodies (3-β-hydroxybutyrate, acetone, and acetoacetate) as liver metabolites which released during fasting. These metabolites are involved in reduction of neurodegenerative disease, cell apoptosis inhibition, lipid peroxidation, and adipocyte lipolysis. Our research showed the antiglycation effect of 3-β-hydroxybutyrate which inhibits the binding of sugar to protein, inhibits the initiation of Maillard reaction and glycotoxin formation specially AGEs, and suppresses diabetes complexities (Bohlooli et al. 2016).

Mountains' Clean Air Have Bracing Effect

Positive and negative ions and neutral molecules are atmospheric components in normal condition which are normally produced by solar radiation, naturally occurring (soil and atmosphere) terrestrial radiation, and also natural phenomena such as thunderstorms, snowstorms, waterfalls, water spray on the shoreline, winds, etc. Atmosphere and weather phenomena are involved in ion production. In city air, the ratio of negative to positive ion is about 4:5 and can be reduced to 1:2 and further in certain situations (Perez et al. 2013).

Atmospheric pollution, air conditioning, television or computer screens, central heating, machinery, artificial fiber materials (clothing, carpets, curtains, etc.), and cigarette smoke are involved in negative ion depletion. The negative and positive ion balance has a critical role for physical and psychological health. It is proposed that negative ion facilitate cognitive functioning through their antiserotonergic effect which leads to reduction of serotonin irritation anxiety and improvement of cognitive performance.

The effects of negative ions on physiological (alertness to circadian rhythms, allergies to migraine decrement, relaxation, calmness, and stimulation increased) and psychological state (irritability, depression, and tenseness decreased, mood improvement and reduced depression severity (Perez et al. 2013; Buckalew and Rizzuto 1982), less anxiety, lower psychological stress, enhanced well-being,

memory increment, and aggression decrement) have been reported previously. In contrary, exposure to positive air ions may induce irritability, heightened anxiety, and unpleasantness. Several studies examined the impact of negative and positive air ionization on relaxation and sleepiness (Perez et al. 2013).

Negative ions can remove environmental pollutants by connecting to contaminants and positively charged particles. In regard to the presence of high number of negative ions in the mountains, this is a desirable factor of mountain's air. So, using mountain weather can regulate biological activities and homeostasis; improving the level of consciousness, removing allergies, and other useful benefits can be due to the effect of mountain air inhalation (Perez et al. 2013).

Lifestyle Alteration Eliminates Oxidative Stressors

Lifestyle changing in recent years has clearly affected people's health (Wheeler et al. 2012; Behnam-Rad et al. 2014). The rapid expansion of urbanization, increasing age, a dramatic change in dietary habits, and physical activities (lack of movement) and its consequent obesity have caused an important increase in the rate of metabolic diseases' growth especially diabetes. All of these suggest that in the coming decades, a high population of metabolic disorder and diabetes mellitus patients will be a major challenge to the health system (Hu and Manson 2001).

With regard to the growth rate of diabetes in developing countries, especially in the Middle East, recognition of the destructive effect of this disease on the body and sole health of the individual and the communities and its causative and aggravating factors is very crucial. Studies on diabetes emphasized on the effect of undesirable lifestyle changes in disease formation (changes in conventional dietary habits, high consumption of fast food, high fat intake and reduced consumption of vegetables and fruits, the replacement of industrial food and drinks (such as various kinds of canned food, artificial juices, carbonated soft drinks, fried potatoes, and processed meat, rich in artificial preservatives and sweeteners) (Buckalew and Rizzuto 1982), and the movement of society toward idle industrial life, tireless, nervous and lack of spirituality along with consuming unhealthy food, and without enough relaxation and sleep) (Golem et al. 2014; Spiegel et al. 1999; Ayas 2003).

These unsuitable alterations have been able to produce intense oxidative stress in the body by disturbing the internal homeostasis and balance between the production of oxidative substances and antioxidants (Dav'I et al. 2010; Alp et al. 2010; Brindley and Rolland 1989). The products of this imbalance formation are destructive, and non-inhibited reactive species with their derivatives will be the cause of the emergence and exacerbation of many degenerative diseases, especially diabetes and its complexities. Obviously, the treatment and prevention of this chronic fatal metabolic diseases can be possible by lifestyle changes based on return to healthy life, healthy food (using high-fiber diets and unsaturated fats), strengthening the body's defenses with external antioxidants, and doing regular exercise which lead to desirable psychological, physiological, and biochemical changes (improvement of

metabolic capacity and blood nutrient function, improving glucose tolerance and weight loss, reducing blood pressure and decreasing triglycerides (Fentem 1994; Helmrich et al. 1991).

It is worth mentioning that improvement of personal and social consciousness, proper planning for treatment management with personal empowerment (Innes and Vincent 2007; Alexander et al. 2008), doing meditational exercises, increasing individual spirituality (Candace 2003; Chopra 1993; Bogousslavsky and Inglin 2007; Lind-Albrecht 2006), and using traditional medicine along with modern medicine (Pathak 2014; Advanced Life Support Group 2001; World Health Organization 2002; Rezaeizadeh et al. 2009; Yuan et al. 2016; Perera and Li 2011; Mohamed 2014) can be very useful for disease prevention.

Diabetes

Diabetes is a group of chronic and destructive metabolic disorders which is formed by blood sugar increment or stressors. This disease can be categorized into type 1 (insulin-dependent diabetes) (Valko et al. 2007), which is caused by blood glucose increment as a metabolic disorder due to the destruction of the pancreatic beta cells and lack of insulin secretion. About 5–10% of the total diabetic patients belong to this type, and type 2 (non-insulin-dependent) (Valko et al. 2007), which is caused by low uptake of glucose into adipose tissue and muscle, increased blood glucose as a metabolic disorder due to decreased levels of insulin secretion from pancreatic beta cells, or body's inability to use insulin. Tissue damage and pathophysiological complications occur by extracellular hyperglycemia. 90–95% of the total diabetic patients belong to this type (Valko et al. 2007; Limón-Pacheco and Gonsebatt 2009). It is noteworthy that Alzheimer's disease is considered as type 3 diabetes (de la Monte and Wands 2008).

The motor of diabetes formation is glycation process as an unplanned and cascade process which destroys biomacromolecules' structure and function. This process can provide a massive change in cells, tissues, and organs which lead to many diseases (diabetes mellitus, cardiac dysfunction, visual impairment, nephropathy, vascular disorders, atherosclerosis, and early aging) (Miranda and Outeiro 2010). The basis of glycation process is Maillard reaction which is a collection of heterogeneous and non-specific complex reactions (Sattarahmady et al. 2007) between carbonyl compounds (reducing sugars such as glucose, fructose, and triose and their derivatives) with free amine groups in protein side chains. The nature of this cascade reaction and its components are similar to those of free radical chain reactions (Wu et al. 2011) and generally include the following steps (Sattarahmady et al. 2007):

(a) Reversible interactions lead to the production of unstable aldehyde as Schiff base.
(b) Irreversible rearrangements and stable ketimine production as Amadori products. These compounds are capable to cross-link with proteins and make chemical changes in protein structure (Brownlee et al. 1984). It is worth to note that in each of these three stages, a large volume of free radicals are also produced (Singh et al. 2001).

(c) Middle phase is associated with the decomposition of preparatory compounds (Thornalley et al. 1999) and formation of several small intermediate products called active carbonyl species (RCSs) (Turk 2010).

These components produce carbonyl stress which is important precursors for the AGE compounds and intra- or extracellular protein aggregation. In fact, carbonyl stress is the result of a defective balance between the production of carbonyl intermediates and the efficiency of their scavenging. It is worth noting that except for the Maillard reaction and glycated protein degradation, there are other oxidative pathways which produce dicarbonyl compounds (glycolytic intermediates, lipid peroxidation, ultraviolet radiation) (Turk 2010). AGEs with high thermal resistance are the final products of Maillard reaction. These products, also known as glycotoxins, have highly oxidizing activities and potentially harmful effects on diabetes and its complexities. AGEs are the link between the complications of diabetes and oxidative stress (Brownlee 2005).

Increasing blood glucose with the accumulation of triose phosphates, ketone bodies, lipid peroxidation, and oxidative stress may contribute to the formation of AGE. These compounds are divided into two groups based on the fluorescence properties and the formation of cross-linking structures (Wu et al. 2011).

According to recent research, sugars, carbonyl content derivatives, and some metabolic pathways are involved in AGE formation (Chowański et al. 2017). Each of glycation products can participate in the formation of other products (Turk 2010). The AGE circulating contents reflect the balance between endogenous formation and exogenous entry (oral absorption) of AGE and its catabolism (including renal excretion). Exogenous AGE (about 10%) is absorbed in the gastrointestinal tract and delivered to the liver and other tissues. The rest (one third) is also excreted in the urine, and the rest of the AGE are involved in diabetes and its complexities.

At tissue levels, macrophages and other endocytosis and AGE degradation systems produce the formation of AGE peptides with low molecular weight through receptor or non-receptor pathways. These peptides are absorbed and catabolized in varying degrees in the proximal nephrons, and the rest are secreted in the urine. Therefore, the AGE effective elimination depends on normal renal function. At the cellular level, there are intracellular protective systems that limit the accumulation of active AGE derivatives. However, in high AGE formation such as diabetes and renal impairment and in particular in increased food AGE absorption, AGE hemostasis can be broken down (Valko et al. 2007). Tissue destruction can be formed by AGEs in two following main paths: (a) intra- and extra-cross-link formation between protein monomers with short or long half-life and their structural disruption. By this way they create the structural alterations of tissues and change the content of extracellular matrix (Abele et al. 2011); (b) interaction with specific and non-specific AGEs receptors which are located on the cell surface. The response causes intracellular phenomena alteration and stress induction and inflammation (Peppa and Vlassar 2005).

Biomacromolecules targeting by all mentioned toxic compounds leads to protein structural changes and protein fibrillar formation, losing their main activities and disease formation. These changes are the origination of many degenerative diseases.

It is worth mentioning that increased uptake of glucose into the muscles and adipose tissue results in extracellular hyperglycemia, tissue damage, and pathophysiological complications like neurodegenerative diseases (Alzheimer's

disease, Parkinson's disease), atherosclerosis, heart disease, retinopathy, NAFLD, cancer, and others (Ray et al. 2012; Cai et al. 2012).

Oxidative stress has a key role in hyperglycemia and diabetic complications. Hyperglycemia induced reactive species in different ways like oxidative phosphorylation, glucose autoxidation, NAD(P)H oxidase, lipoxygenase, cytochrome P450 monooxygenases, and nitric oxide synthase (NOS) (Levine and Stadtman 2001).

Glucose Auto-oxidation

The enediol rearrangements of sugars as ketoaldehydes and α-hydroxyaldehydes let them be oxidized easily and generate H_2O_2 and reactive intermediates including hydroxyl radicals (Kelly 2003). There are many pathways for reactive species formation (especially H_2O_2); this process in glycation and fructation seems to be common.

Reactive species like (RCS) and ROS are produced by Wolff pathway (via monosaccharide autoxidation), Hodge pathway in fructation (via autoxidation of the Heyns compounds), and common Namiki pathway (via Schiff base oxidative fragmentation) (Hunt et al. 1988; Thornalley et al. 1984; Peng et al. 2011).

In our hemoglobin fructation research, it was found that autoxidation of fructose causes ROS production which can affect heme degradation in ROS concentration-dependent manner. Also there was a difference between these heme degradation products and heme enzymatic degradation products (Goodarzi et al. 2014).

Synergism Between Oxidative Stress and Glycation

Oxidative stress and its derivatives (reactive species) have been considered as a component of all pathogenic processes (Dalle-Donne et al. 2003a). Some researches revealed the signs of synergism effect between oxidative stress and protein glycation and their toxic products' development (Levine and Stadtman 2001). It is worth mentioning that in many nondiabetic diseases (like polycystic ovary), oxidized proteins and reactive dicarbonyl substances were detected due to reactive oxygen species (ROS) (Pham-Huy et al. 2008). Research demonstrated that the concentration of protein carbonyl (common product with glycation) can be considered as protein oxidation biomarker (Kikuchi et al. 2003; Dalle-Donne et al. 2003b). Furthermore, protein carbonyl formation is intersection products of some pathological process like lipid peroxidation, neurodegenerative diseases, and NAFLD (Thanan et al. 2014).

Oxidative stress products (free radicals) can interact with proteins directly (via amino acid direct oxidation, oxidative cleavage of protein's backbone, reaction with protein side chain residues) or indirectly by interaction with lipids and carbohydrates as target molecules which leads to formation of new oxidative by-products. These kinds of products can rearrange protein attack. Also, various types of protein oxidative changes can be created directly with reactive species specially ROS or indirectly by interacting with secondary oxidative products which both of them result in carbonyl contents (Kikuchi et al. 2003; Dalle-Donne et al. 2003b).

Cysteine and methionine residues are two residues which are often attacked by ROS compounds. ROS oxidative attack to lysine, arginine, proline, and threonine directly or secondary interaction of cysteine, histidine, or lysine with reactive carbonyl compounds (RCS) produces carbonylated protein derivatives (protein carbonyl), such as aldehyde or ketone derivatives (Dalle-Donne et al. 2003b).

ROS overproduction during glycoxidation is considered as important factor in protein damage due to strengthen Maillard reaction and its products. These products prepare oxidative attack to protein again and again (Rondeau et al. 2008).

All of these interactions lead to changes in protein physicochemical (accessible surface area, binding activities, hydrophobicity) and functional properties that are involved in aging and other human diseases (Levine and Stadtman 2001). So, amino acid direct oxidation, oxidative cleavage of protein's backbone, and reaction with protein side chain residues produce some product which can be observed in both protein glycation and oxidative stress (Dalle-Donne et al. 2003b).

The protein surface-located amino acids (cysteine, tryptophan, histidine, lysine, arginine, tyrosine, and methionine) are the primary targets for reactive species. Interestingly, the interaction between most of these amino acids with free radicals create the persistent carbonyl-ene compounds (PCO) (Dalle-Donne et al. 2003b).

These products are common with glycation product and can strengthen Maillard reaction. It means that deformed amino acids and reactive products can enter into early, middle, and late stage of Maillard reaction and improve this process (Levine and Stadtman 2001).

Oxidized protein (protein carbonyl) is also an important biomarker for identifying oxidative stress.

So, glycation and oxidative stress have the common ancestor and roots, and their common oxidative products can promote both of them simultaneously. It means that a synergism effect is between protein glycation process and protein oxidation. Later, this hypothesis was the fundamental of our next researches.

On the other hand, glycation and oxidative stress make a common fate as amyloid aggregation and fibril formation. Both of these events are involved in degenerative diseases (Taghavi et al. 2017). Protein aggregation can be formed by glycation due to oligomerization of soluble aggregates by interfering of carbonyl content and AGE adduction and fibrillation of insoluble aggregates via formation of cross beta structures with involvement of AGE cross-linking (Taghavi et al. 2017).

Interestingly, protein misfolding and aggregation by itself improve free radical formation and oxidative stress, and because of this, they are considered as toxic elements (Bross and Gregersen 2016).

Preservatives

With regard to vast usage of additives specially preservatives in different fields (alimentary, cosmetic, and pharmaceutical industries), its interfering effects as small molecules on human health can be the main concern of scientists (Taghavi

et al. 2014). The industrial products (food, pharmaceuticals, paints, biological samples, personal care products) include preservatives. Preservatives are the natural or synthetic components for chemical change prevention and contaminant inhibition (Yim et al. 2014; Carbajo et al. 2015). Food preservatives as a subgroup of food additives (coloring, flavoring, etc.) are substances without nutritional value due to their nature but are added to food and considered as part of it. This action is used for elongation of food's shelf life (Gould 1996; Zengin et al. 2011). Preservative, coloring, and sweetening are the main purposes of additive usage. So far, no group of industrial additives is economically more valuable than antimicrobials. Oxidizing preservatives are currently widely used in the food, cosmetic, pharmaceutics, and health products like borax or formalin for the production of edible filaments; sulfites in the fried food industries; nitrites for the preservation of bulk processed and canned meat; benzoates and sorbate especially to increase the durability of beverages, sauces, juices, dairy products, and pharmaceutical and health products; and probiotics as a baking agent for cheese (Lamas et al. 2016; Belz et al. 2012; Darzi et al. 2012). There are many researches which report the effect of food coloring, flavoring, and preservatives on children's hyperactivities and behavioral problems in childhood (Buka et al. 2011).

The increment of food-borne disease and its effect on morbidity and mortality worldwide in developing countries were reported in researches (Scott 2003).

Based on research, preservatives also have the main role in oxidative stress formation and trigger the oxidative damages. Quaternium-15 is a worldwide preservative and xenobiotic agent which is used in cosmetic products. It is a formaldehyde releaser and causes dermatitis and developmental effect. It is shown that this preservative can induce oxidative stress and reactive species formation which is correlated with reduction in GSH activities and increment in lipid peroxidation. An important antioxidant in different kinds of organisms (animals, plants, some bacteria, fungi) is glutathione (GSH). Researchers showed that quaternium-15 because of its toxic effect is a main danger for aquatic animals (Faggio et al. 2016).

Xenobiotic metabolism can induce oxidative stress via increment in ROS production and decreasing of antioxidant potential (Abele et al. 2012).

Researches about the cytotoxicity of most common preservatives which are used in ophthalmic solutions demonstrated the histological effect and morphologic disruption of pharmacological usage of preservatives. The reason of this matter is related to the lipophilic nature of some preservatives which leads them to interact with tissues. Also it has shown that the mechanism of this cytotoxic effect is related to inflammation, free radical formation, and apoptosis by these kinds of preservatives (Debbasch et al. 2001). Some food preservatives like monocarboxylic are weak organic acids (WOAs) which are widely used as microorganism and seem to be mutagenic (Semchyshyn et al. 2011).

In our previous researches, we used some worldwide preservatives (European Commission 1999) as oxidative agents and studied glycation process of human serum albumin in presence and absence of glucose with or without antioxidant (Taghavi et al. 2013, 2016a, b).

It is outlined in our previous research that potassium sorbate with similar structure with unsaturated fatty acid and GRAS character can be very harmful and is seriously involved in diabetes formation and its complexities.

This preservative present in various products (toothpaste, cosmetic, pharmaceutics products, and industrial foods), can access biological stream and interact with biological macromolecules especially proteins. Our results showed that potassium sorbate (PS) can trigger Maillard reaction and amyloid formation in the absence of glucose and can strengthen these reactions in the presence of glucose. This preservative is highly involved in glycotoxin production (Amadori products, AGEs, and reactive species) in higher levels especially in the presence of glucose. All of these toxins particularly AGEs can interact with human serum albumin's structures and alter its secondary structure (by beta-sheet inducing effect) and third structure (increasing hydrophobic patch formation) specially in the presence of glucose. These alterations produce protein abnormal structures and some new hydrophobic contact sites in the surface of protein which improve protein aggregation as toxic structures. The final state of these abnormalities was refolding of protein abnormal structures for achieving thermodynamic stability which leads to protein fibrillation. These changes are followed by HSA physiological defects. Our study revealed that PS induces amyloid structures and fibril formation in the absence of glucose in different shapes (spherulites) compared with the presence of glucose (dense texture) (Fig. 2).

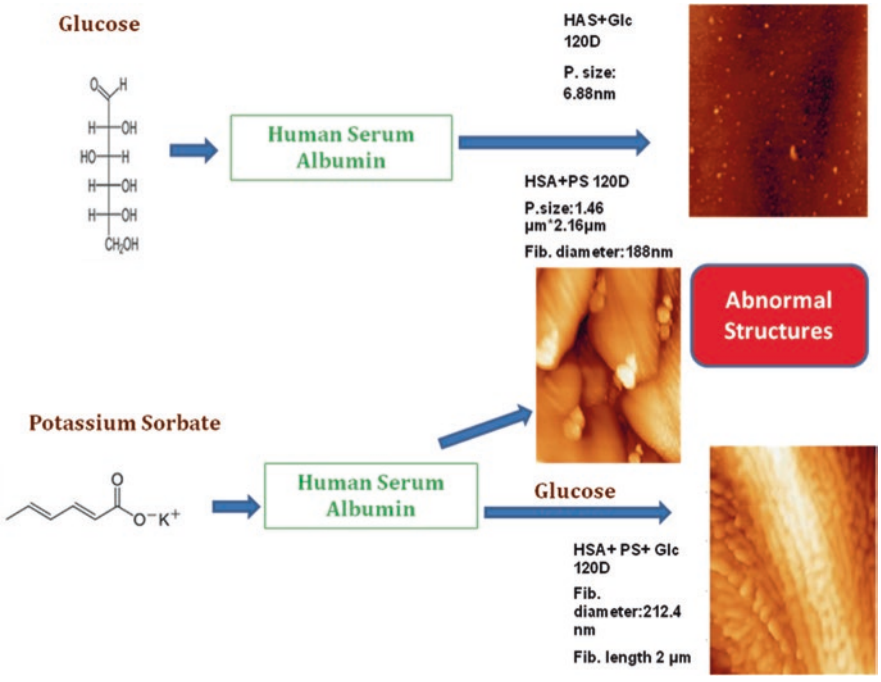

Fig. 2 The formation of HSA amyloidal fibrils due to treatment with PS in the presence and the absence of glucose. The various sizes and shapes of amyloid fibril formation are the consequence of this treatment (Taghavi et al. 2013)

Because of these various shapes, the refolded structures of HSA glycation with PS in the presence and absence of glucose can interfere with many cellular process and molecular mechanisms separately. So, in the absence of glucose, PS as an oxidant can easily promote Maillard reactions and diabetes and its complexities (Taghavi et al. 2013).

In our another investigation, we found that sodium benzoate as a small molecule preservative also with vast presence in food and cosmetic and pharmaceutical products can stimulate the HSA conformational changes. This preservative alters HSA secondary structure through alpha helix formation (which is different from PS) and forms new different energetic domains in the presence and absence of glucose. Sodium benzoate (SB) can induce three different intermediates in HSA conformational changes. These various intermediates can promote protein aggregation in different pathways which leads to different protein fibrillation (Taghavi et al. 2014).

Our research also revealed that both of the mentioned preservatives can induce protein glycation without the presence of glucose, but there are some difference in their manner (Fig. 3). PS is a potent inducer for glycotoxin formation than SB, and this difference leads to complete different amyloid fibril formation with different roles in consequent diseases (Taghavi et al. 2014, 2016a).

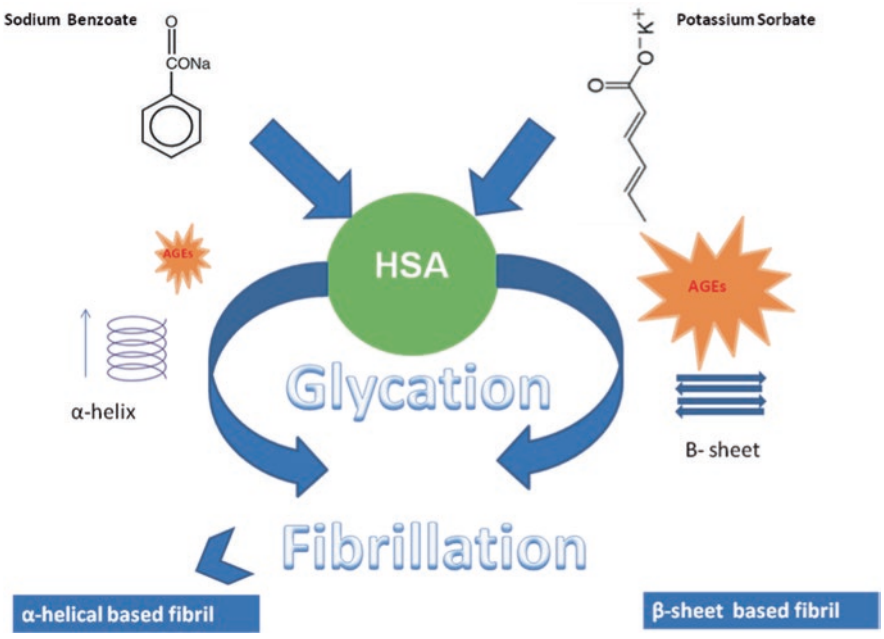

Fig. 3 Different mechanisms for HSA amyloid formation by PS and SB (Taghavi et al. 2013, 2014, 2016a)

New Conversation

Diabetes is not just a glucose-related disease, but it is an oxidative stress disease. It means that growth of diabetes does not depend solely on the high consumption of sugars. So, protein's glycation without high sugar levels can be produced by oxidative substances and free radicals. The result of this reaction is the production of toxic substances which lead to formation of undesirable fibers in the body and causes diabetes and complications.

Molecular Oxidative Stress

The translations of any external stress to the body's internal system produce oxidative stress, disrupt body balances, and lead to various diseases. In molecular approach, oxidative stress means the translation of any external or internal factors to fake body chemical language and produce an acute homeostatic imbalance between the production of oxidative factors and antioxidant defense mechanism.

In another research (Taghavi et al. 2016b), we focused on the pomegranate as the source of antioxidants. Pomegranate with the scientific name *Punica granatum* is a member of Punicaceae family (Seeram et al. 2006). This fruit is one of the most important flavonoid sources, which is considered as ancient, mystical, and highly distinctive fruit. Its main origin is belonging to Iran (European Commission 1999). This fruit has always been admired in ancient times, and it has been mentioned in the sacred scripture of the Old Testament which brings the power of glory, abundance, and success (Jurenka 2008).

The pomegranate has had a long life span about 2000 years. In old medicine, pomegranate is considered as a drugstore (Brook and Rajagopalan 2010). This fruit is potentially treating a wide range of diseases, such as various cancers (prostate, breast, lung, colon), heart disease, diabetes, Alzheimer's disease, infertility, vascular disease and obesity, various types of ulcers, digestive distress, and wound healing (Jurenka 2008; Zarfeshany et al. 2014). In the past decade, significant progress has been made in recognizing the effects of pomegranate extracts and its constituents. Extract and all parts of the pomegranate fruit and trees have medicinal properties. According to research, the most peculiarities of the treatment of pomegranate components are related to ellagic acid and its ellagitannin. Ellagic acid is a natural phenolic antioxidant found in pomegranate and some fruits. After the production, these compounds are converted to a kind of tannin called the ellagitannin. These glycosylated compounds are rapidly hydrolyzed by water and produce reagent acid (Jurenka 2008; Kim et al. 2007; Bana et al. 2007). We added ellagic acid to HSA treatment with PS or glucose (Fig. 4). Our results showed that ellagic acid suppresses the carbonyl content-derived materials as the middle products of Maillard reaction. Also, the presence of ellagic acid interestingly demonstrated the potent inhibitory effect of this antioxidant on HSA amyloid fibril formation due to potassium sorbate in both HSA treatments with glucose or without it. The size decrement of amyloid particles in HSA treated with potassium sorbate and the elimination of particles in HSA

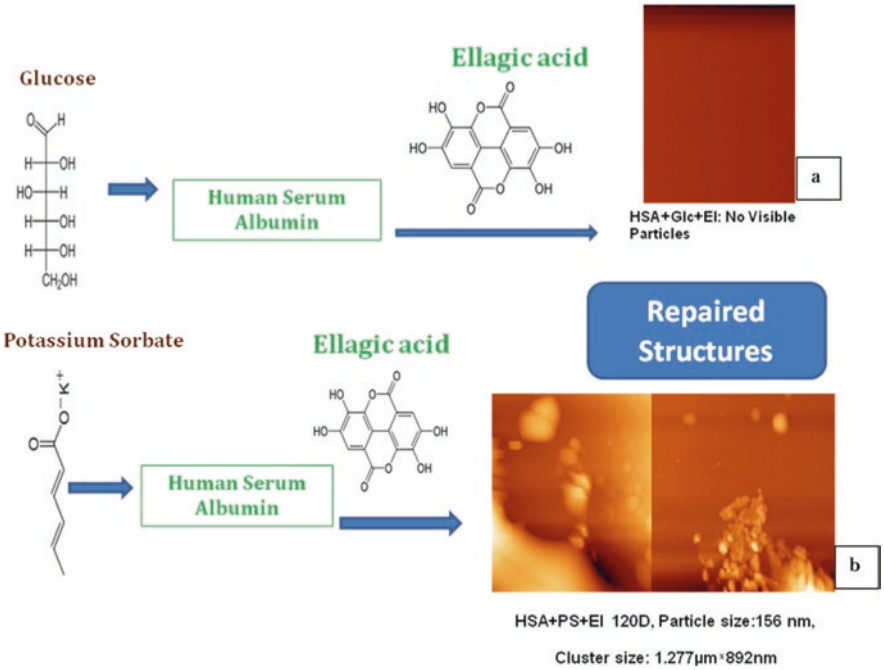

Fig. 4 The effect of ellagic acid on amyloid fibril elimination (Taghavi et al. 2016b). (**a**) The elimination of amyloid particles in HSA treated with glucose. (**b**) The decrement of spherulites in treated HSA with potassium sorbate

treated with glucose were the obvious benefit of using ellagic acid in HSA treated with oxidants. Based on the surface tension results, ellagic acid can repair the destructive effect of oxidants (PS and glucose) on HSA structural changes. So, ellagic acid can remove abnormal structures in treated HSA with glucose and reduce the size of spherulites in treated HSA with PS. Also this antioxidant suppresses other HSA-derived intermediates due to oxidation by glucose or PS.

Another research also revealed that curcumin suppressed the ROS production due to catalase glycation and also activated this enzyme (Mofidi-Najjar et al. 2017).

Diabetes Complications

The diabetes complications include a wide range of diseases (Fig. 5). The wide range of diabetic complications can be due to three important parameters: hemodynamic (changes in fluid balance and blood pressure), metabolic (glycemic and lipid control), and genetic (susceptibility and gene expression) changes. The chronic alteration of these parameters leads to cellular changes (gene modulation and modification, energetic, protein expression) which lead to cellular dysfunction and death (Forbes and Cooper 2013).

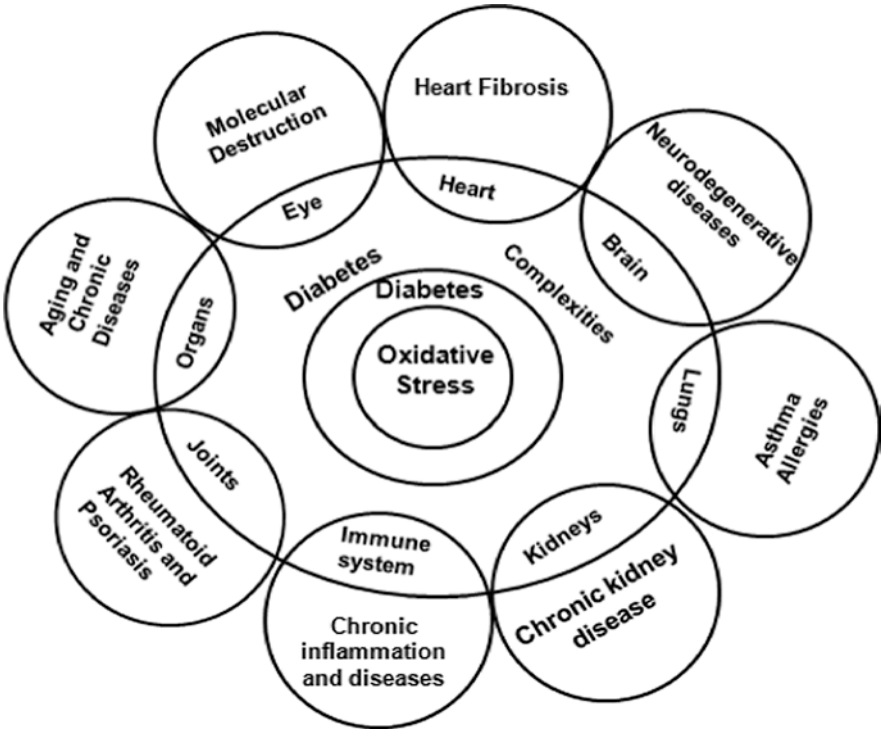

Fig. 5 Oxidative stress and diabetes are the *mother* of many human *diseases* (Forbes and Cooper 2013)

Persistent adverse effects of hyperglycemia on the progression of diabetes complications called "metabolic memory" can be affected by three important pathogenic mediators: oxidative stress, advanced glycation end products, and epigenetic changes. All of these conditions are the basis of two major complications which are observed in diabetic patients (Zhang et al. 2012; Tripathi and Srivastava 2006; Yamagishi et al. 2003).

(a) Microvascular disease or microangiopathy includes dysfunctional changes in microvascular beds, small blood vessels, and a wide range of tissue destruction (Forbes and Cooper 2013).

Retinopathy includes lesions within the retina, dysfunctional neural retina, skin disease, vascular permeability alteration, capillary microaneurysms and cell degeneration, neovascularization, cell death, inability to discriminate between colors, and final blindness (Yamagishi et al. 2005).

Nephropathy is another diabetes complexity which is considered as development of proteinuria increment with a glomerular filtration rate, decrement, and final renal failure which is accompanied with vascular abnormalities' development (Forbes and Cooper 2013).

Glycotoxins especially AGEs have an important role in diabetes complexities. The cross-linking of important matrix proteins (collagen) with AGE

produces structural and functional changes for matrix proteins. Also the interactions of AGE with rennin angiotensin system trigger renal disease. The creation of oxidant stress, adhesion molecules, cytokines, and growth factors which are involved in the pathogenesis of diabetic nephropathy are the consequence of AGEs formation (Yamagishi et al. 2005; Ahmed 2005).

The syndrome of neuropathy is involved in both somatic and autonomic divisions of the peripheral nervous system. In advanced step lead to nerve fiber deterioration, sensitivities alteration to vibrations and thermal thresholds, development of vascular abnormalities like capillary basement, membrane thickening, and endothelial (Yamagishi et al. 2005).

In this diabetes complication, AGEs are involved in glycation of cytoskeletal proteins which change structure and function of nerve fibers and reduce velocities of sensory motor conduction and nerve action potentials and accumulation of AGEs around sciatic nerve. Furthermore, AGE accumulation in cytoskeletal and myelin protein has been distributed in the cytoplasm of endothelial cells and pericytes, Schwann interstitial collagens, and basement membranes of the perineurium cells in both myelinated and unmyelinated fibers and causes vascular abnormalities. Diabetes is also involved in dementia (Forbes and Cooper 2013).

Dermopathy: Skin accumulations of various AGE products alters its physicochemical structure and create skin disorders and even delay in wound healing (Forbes and Cooper 2013).

(b) "Macrovascular disease" includes cerebrovascular and cardiovascular disease which is affected by interplay of various factors including AGE. The kinds of diseases also contain the artery demolition (Forbes and Cooper 2013), atherosclerosis formation, arterial stiffness increment with AGE involvement, vessel rigidity by intra- and intermolecular cross-linking with matrix proteins, and clearance disrupting of arterial wall by trapping lipoproteins. The interaction of AGE with endothelial cell receptors causes vascular permeability increment, migration of macrophages and T-lymphocytes into the intima and impairment of endothelium, procoagulant activity, stiffness, and aortic atherosclerotic lesions via tissue AGE accumulation. AGEs are involved in protein aging and diabetes mellitus (DM) pathological complications (Forbes and Cooper 2013). Nonalcoholic fatty liver disease is the most common liver disease linked to diabetes and obesity deeply (Takeuchi et al. 2015; Yamagishi and Matsui 2010). Advanced glycation end products (AGEs) are a cross-link between the mechanism of nonalcoholic steatohepatitis (NASH) and diabetes mellitus (DM) (Zhang et al. 2012). In NAFLD, advanced glycation end products (AGEs) have been provided by endogenous (like diabetic condition) and exogenous sources (high processed foods, dried food at high temperatures, and high AGE content foods).

The receptor for advanced glycation end products (RAGE) belongs to an immunoglobulin superfamily of cell surface molecules. Interaction between RAGE and AGEs induces oxidative stress and inflammation response which are considered as pathogenic effect of AGEs (Zhang et al. 2012; Tripathi and Srivastava 2006). AGEs are involved in protein aging and DM pathological

complications (Singh et al. 2014). All of macrophages (including Kupffer cells), peripheral blood mononuclear cells, endothelial cells, and vascular smooth muscle cells, have this receptor (Singh et al. 2014).

Researchers showed that AGEs have the key role in transformation of NAFLD as simple steatosis to NASH and liver fibrosis. This interaction also produces acute liver injury. RAGE hepatic expression is enhanced significantly in liver chronic injury. There is a correlation between AGE levels and severity of fibrosis in NAFLD patients because of the AGEs' role in oxidative stress formation as the main hallmark of NAFLD. The increased amounts of AGE products, RAGE expression, and oxidative stress in diabetic patients improve the liver oxidative damage, NASH, and fibrosis in human liver diseases (Leung et al. 2016).

Diabetes chronic complications also involve depression and sexual dysfunction (Forbes and Cooper 2013).

Diabetes and Climate Changes

With regard to human health, negative effects of climate factors induced many changes in some physiological systems and form their consequent problems. Climate change accompanied with inappropriate nutrition and extreme weather events increases the risk of chronic diseases. By this way, large population of human being encounter with abnormal water situations and are exposed to heat stress, water and food shortages, infectious diseases, and mental stress which increase the risk of noninfectious diseases. However climate change is a global health problem, but a little attention has been paid to the importance of its effects on chronic noninfectious diseases (Santer et al. 2007; Wigley et al. 1998).

Direct Effects

Thermal Stress

Chronic noninfectious diseases are known as the main cause of death in the world, except in downstream lands of the African great desert (World Health Organization 2011). The most common chronic noninfectious diseases considered are heart disease, diabetes type 2, cancer, and respiratory diseases, which are the reason of 60% of global annual mortality. These illnesses also account for 46% of total cost of disabilities caused by disease.

Most of vulnerable people by heat and its related shock have chronic illnesses, such as diabetes. The condition of a diabetic patient makes them susceptible to water loss, fatigue, thermal erosion, and ultimately thermal shock. Heat shock is the most important direct cause of heat death (Easterling et al. 1997).

Spiritual Consequences

Migration to major cities has many psychological consequences: stress, occupational worries, crowding increment in areas with high population density, and noise pollution. All of these conditions increase the production of reactive oxygen species (ROS) in the body and weaken the biological defense mechanisms (Kinney et al. 2008).

The results showed the increment risk of developing type 2 diabetes due to nervous stress in diabetic patients, the conversion of adrenaline to methylamine, and the alteration of acetamide to methyl glyoxal as the potent glycotoxin products by amine oxidase 2. This conversion improves the diabetes situation. Noteworthy, the amount of amine oxidase 2 in the blood of diabetic patients is higher than that of healthy people (Gosling et al. 2009).

Indirect Effects

Suburbs

Nowadays, the consequence of modern and industrial life is the people influx to cities and suburbs and slum formation with high population without any urban and sanitary facilities. These conditions lead to air pollution, high population density, and the lack of infrastructure and urban transport facilities. Inappropriate nutrition; lack of urban infrastructure such as adequate green space, public transportation systems, and sanitation facilities; and septic or inappropriate water and food have potential conditions for diabetes formation. Also, the consequences of urban lifestyle are the sedentary and insufficient physical movement, nervous discomfort, and stress. These factors cause obesity, which has a direct relationship with diabetes (Easterling et al. 1997; Lim et al. 2005).

Obese people are less likely to get used to heat and their body temperature is higher than lean ones. These people will be less tolerant to climate change and warming than other people in the community.

Food Quality and Food Habits

Migration of villagers to cities reduced the natural production of fruits and vegetables which leads to consumption high amount of sugar and fatty foods (in both shapes: fast foods or industrial foods). These kinds of nutrients include lots of free radicals and increase the risk of diabetes (Taghavi et al. 2014, 2013; Lim et al. 2005). On the other hand, increment of the livestock production to meet people's needs will lead to further increase in greenhouse gas emissions. High levels of greenhouse gas emissions, such as methane and carbon dioxide, are significant at various stages of breeding livestock. So, their low efficiency and the costs of high energy consumption for meat products need to receive more attention. Brazil, as a supplier of 25% of the world's meat, is the world's fourth largest producer of CO_2.

The livestock holding sector of Brazil is responsible for producing more than 75% of its CO_2 production. Every year, humans use more than 140 billion animals, which require huge quantities of water, food, and land (Oberhuber et al. 1998).

Reduction in Food Security and Increasing the Risk of Agricultural Production

Drought and severe flooding will reduce the agricultural labor privilege for many farmers. By this way, farmers abandoned rural areas and sheltered to urban suburbs. So, urban management and expenditures will be very crucial (Patz et al. 2005). Also the prices of new agricultural products will be raised. Because of this, people will be eager to use unsuitable and cheaper diets that are more likely to cause diabetes (Epstein 2001). So, climate change causes air, water, and food pollution and urban population increment. Also, the lack of mobility, inappropriate nutrition, and especially mental stress in modern urban lifestyle increase the likelihood of diabetes.

Conclusion

Human body is a well-organized system which works based on a defined chemical and biological language. All internal reactions are modulated by the exact reaction and interaction and can be affected by external stimuli. Oxidative stress is a biochemical disturbance between the production of reactive species and antioxidant defenses in the human body. The products of this homeostatic imbalance are highly reactive, and unstable species include free radicals (species with unpaired valence electrons) and non-radicals which both have various quick reactions in a short time. Biomacromolecules, cells, and tissues are the main targets for these aggressive species which lead to a variety of diseases. The formation of diabetes and its complications is related to glycation process as a destructive reaction. Both glycation process and oxidative stress have a common ancestor (oxidative reaction) and common fate (amyloid formation). Reactive oxygen species (ROS) are known as the most risky free radicals in the body and involved in diabetes formation and its complication seriously.

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Secondary Metabolites from Turkish *Astragalus* Species

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Introduction

The genus *Astragalus* belonging to the Leguminosae family is a widely distributed plant throughout the temperate regions of the world, located principally in Europe, Asia, and North America. It is represented in the flora of Turkey by 447 species, of which 224 are endemic (Davis 1970; Aytaç 2000). The roots of various *Astragalus* species represent very old and well-known drugs in traditional medicine for the treatment of nephritis, diabetes, and uterine cancer and as antiperspirant, diuretic, and tonic (Tang and Eisenbrand 1992). In Turkish folk medicine, the aqueous extracts of some *Astragalus* species are used to treat leukemia as well as for wound healing (Çalış et al. 1997; Bedir et al. 2000a).

Previous phytochemical studies on Turkish *Astragalus* species resulted in the isolation of a series of oleanane- and cycloartane-type triterpene saponins (Bedir et al. 1998a, b, 1999a, b, 2001a, b; Çalış et al. 1997, 2008a, b; Denizli et al. 2014; Djimtombaye et al. 2013; Gülcemal et al. 2011, 2012, 2013; Horo et al. 2010, 2012; Polat et al. 2009, 2010; Savran et al. 2012). Cycloartane- and oleanane-type

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glycosides from *Astragalus* species have shown interesting biological properties, including immunostimulating (Çalış et al. 1997; Bedir et al. 2000a; Yeşilada et al. 2005), antiprotozoal (Özipek et al. 2005), antiviral (Gariboldi et al. 1995), and cytotoxic activities (Tian et al. 2005).

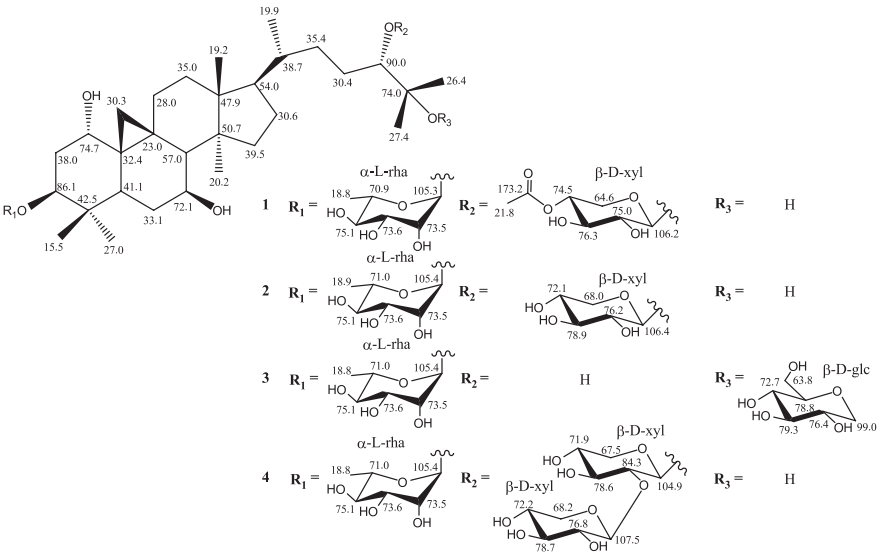
Turkish *Astragalus* species have been studied extensively from phytochemistry and biological activity perspectives for the last 25 years. The following is a summary of these 25 years.

Phytochemistry and Biological Activity

Until now, 31 out of 447 Turkish *Astragalus* species, from 14 different sections, have been investigated for their secondary metabolite contents, and structures of 104 new triterpene saponins, 5 new phenolic glycosides, a new tryptophan derivative, and a new maltol glucoside were identified besides 63 known compounds.

Çalış et al. (1996) reported on the isolation and structural elucidation of four novel cycloartane-type triterpene glycosides, macrophyllosaponins A–D (**1–4**), from the roots of *Astragalus oleifolius* (Sect. Macrophyllium). By means of chemical (acetylation, alkaline hydrolysis) and spectroscopic methods (IR, 1D- and 2D-NMR, FABMS), their structures were established as 3-*O*-*α*-L-rhamnopyranosyl-24-*O*-(4″-*O*-acetyl)-*β*-D-xylopyranosyl-1*α*,3*β*,7*β*,24(*S*),-25-pentahydroxycycloartane (**1**), 3-*O*-*α*-L-rhamnopyranosyl-24-*O*-*β*-D-xylopyranosyl-1*α*,3*β*,7*β*,-24(*S*),25-pentahydroxycycloartane (**2**), 3-*O*-*α*-L-rhamnopyranosyl-25-*O*-*β*-D-glucoyranosyl-1*α*,3*β*,7*β*,24(*S*),25-pentahydroxycycloartane (**3**), and 3-*O*-*α*-L-rhamnopyranosyl-24-*O*-(2-*O*-*β*-D-xylopyranosyl)-*β*-D-xylopyranosyl-1*α*,3*β*,7*β*,24(*S*),25-pentahydroxycycloartane (**4**). According to the authors, the sapogenol moiety of these saponins was encountered for the first time in this study. The presence of hydroxyl groups at C-1 and C-7 positions of the sapogenol moiety are rare in *Astragalus* cycloartane chemistry as well as the absence of a hydroxyl group in ring D (Çalış et al. 1996).

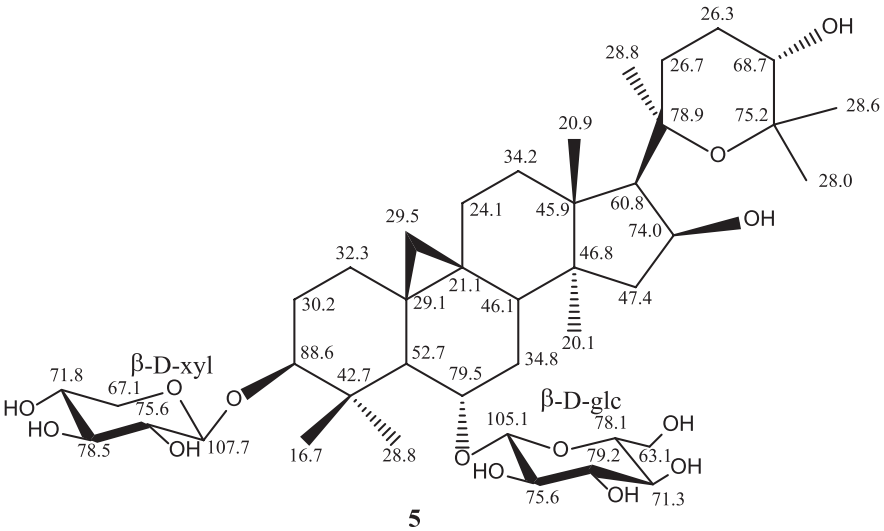
Compound **1**; $C_{43}H_{72}O_{14}$; $[\alpha]^{20}_D$ −5.0° (*c* 0.28, MeOH); ν^{KBr}_{max} cm^{-1}: 3400 (OH), 1735 (ester carbonyl); δ_C (CD_3OD); FABMS *m/z* $[M + Na]^+$ 835. Compound **2**; $C_{41}H_{70}O_{13}$; $[\alpha]^{20}_D$ +2.8° (*c* 0.58, MeOH); ν^{KBr}_{max} cm^{-1}: 3400 (OH); δ_C (CD_3OD); FABMS *m/z* $[M + Na]^+$ 793. Compound **3**; $C_{42}H_{72}O_{14}$; $[\alpha]^{20}_D$ +15° (*c* 0.32, MeOH); ν^{KBr}_{max} cm^{-1}: 3400 (OH); δ_C (CD_3OD); FABMS *m/z* $[M + Na]^+$ 823. Compound **4**; $C_{46}H_{78}O_{17}$; $[\alpha]^{20}_D$ −1.0° (*c* 0.32, MeOH); ν^{KBr}_{max} cm^{-1}: 3400 (OH); δ_C (CD_3OD); FABMS *m/z* $[M + Na]^+$ 925. The structures and ^{13}C NMR data of the aglycone moiety of **1** are provided below together with sugar residues identified for compounds **1**–**4**.



Calis et al. isolated eight known saponins: astrasieversianins II (Gan et al. 1986) and X (Gan et al. 1986); astragalosides I (Kitagawa et al. 1983a), II (Kitagawa et al. 1983a), IV (Kitagawa et al. 1983a), and VI (Kitagawa et al. 1983b); and cyclocanthosides E (Isaev et al. 1992) and G (Isaev et al. 1992) from the roots of *Astragalus melanophrurius* (Sect. Christiana). The metabolites were examined for their bioactivities; referring to the results, they were found to have modest antibiotic activity toward *Escherichia coli*, *Bacillus subtilis*, and *Micrococcus luteus.* On the other hand, all compounds exhibited immunomodulatory activity via stimulation of human lymphocyte proliferation in the concentration range of 0.01–10 μg/mL (Çalış et al. 1997).

A novel cycloartane-type glycoside, cyclocephaloside I (**5**), was reported from the roots of *Astragalus microcephalus* in addition to known glycosides: cyclocanthoside E (Isaev et al. 1992) and astragaloside IV (Kitagawa et al. 1983a). The structure of **5** was elucidated on the basis of spectral (IR, ^{1}H and ^{13}C NMR, and FABMS) and chemical (acetylation) methods and established as 20,25-epoxy-3*β*-(*β*-D-xylopyranosyl)oxy-6*α*-(*β*-D-glucopyranosyl)oxy-cycloartane-16*β*,24*α*-diol (Bedir et al. 1998a).

Compound **5**; $C_{41}H_{68}O_{14}$; $[\alpha]^{20}_{D}$ +6.1° (*c* 0.42, MeOH); ν^{KBr}_{max} cm^{-1}: (KBr) 3400 (OH); δ_C (C_5D_5N); FABMS *m/z* $[M + Na]^+$ 807. The structure and ^{13}C chemical shifts are given below for compound **5**.

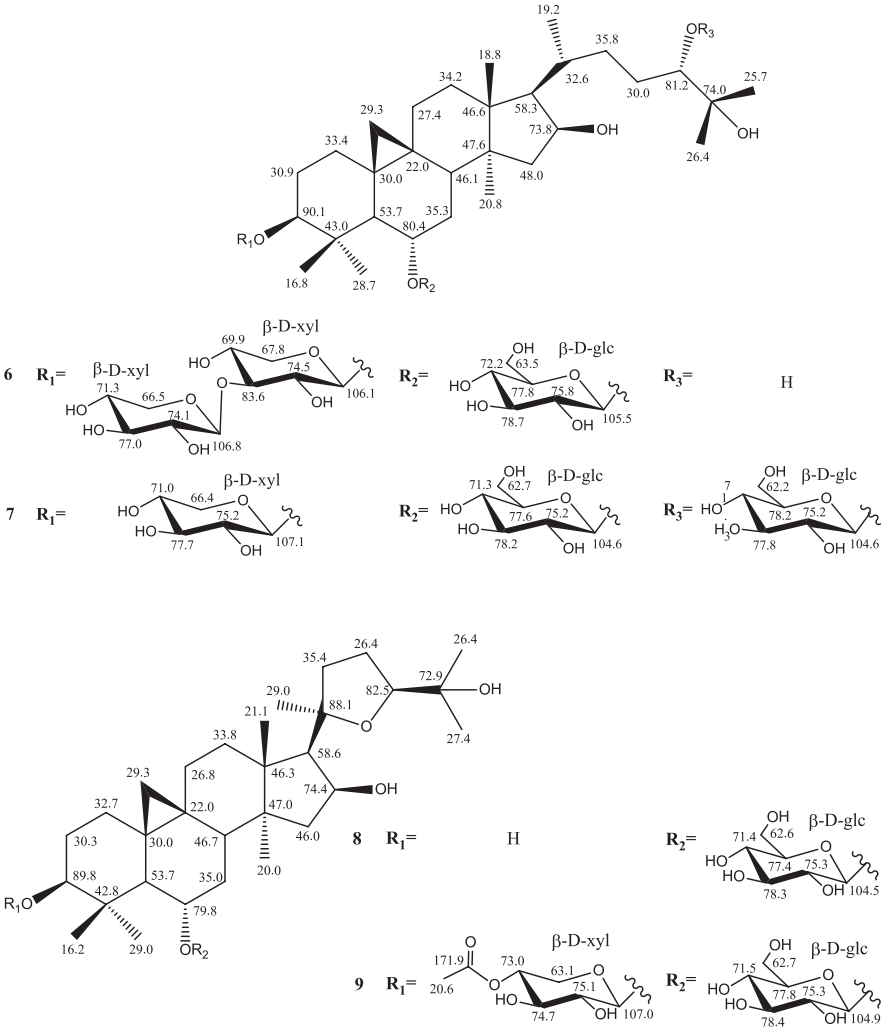


5

In 1998, a report described three new cycloartane-type triterpene glycosides, brachyosides A (**6**), B (**8**), and C (**7**), from the roots of *Astragalus brachypterus* (Sect. Pterophorus) and one new glycoside, cyclocephaloside II (**9**), from the roots of *Astragalus microcephalus* (Sect. Rhacophorus) together with five known saponins, astragalosides I (Kitagawa et al. 1983a), II (Kitagawa et al. 1983a), and IV (Kitagawa et al. 1983a), cyclocanthoside E (Isaev et al. 1992), and cycloastragenol (Kitagawa et al. 1983a). The structures of the new compounds were established by detailed spectral analysis, as 3-*O*-[*β*-D-xylopyranosyl(1→3)-*β*-D-xylopyranosyl-6-*O*-*β*-D-glucopyranosyl-3*β*,6*α*,16*β*,24(*S*),25-pentahydroxycycloartane (**6**), 3-*O*-*β*-D-xylopyranosyl-6-*O*-*β*-D-glucopyranosyl-24-*O*-*β*-D-glucopyranosyl-3*β*,6*α*,16*β*,24(*S*),25-pentahydroxycycloartane (**7**), 20(*R*),24(*S*)-epoxy-6-*O*-*β*-D-glucopyranosyl-3*β*,6*α*,16*β*,25-tetrahydroxycycloartane (**8**), and 20(*R*),24(*S*)-epoxy-3-*O*-(4′-*O*-acetyl)-*β*-D-xylopyranosyl-6-*O*-*β*-D-glucopyranosyl-3*β*,6*α*,16*β*,25-tetrahydroxycycloartane (**9**) (Bedir et al. 1998b).

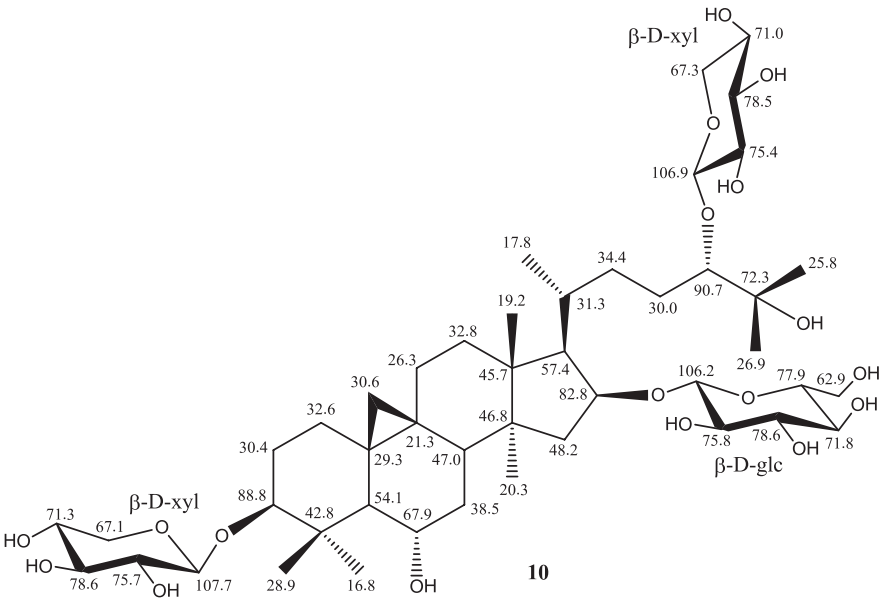
Compound **6**; $C_{46}H_{78}O_{18}$; $[\alpha]^{25}_{D}$ +15.5° (*c* 0.1, MeOH); δ_C (CD_3OD); FABMS *m/z* $[M–H]^-$ 917. Compound **7**; $C_{47}H_{80}O_{19}$; $[\alpha]^{25}_{D}$ +12.5° (*c* 0.1, MeOH); δ_C (CD_3OD); FABMS *m/z* $[M–H]^-$ 947. Compound **8**; $C_{36}H_{60}O_{10}$; $[\alpha]^{25}_{D}$ +40.1° (*c* 0.1, MeOH); δ_C (CD_3OD); FABMS *m/z* $[M–H]^-$ 651. Compound **9**; $C_{43}H_{70}O_{15}$; $[\alpha]^{25}_{D}$ +19.6° (*c* 0.1, MeOH); δ_C (CD_3OD); FABMS *m/z* $[M–H]^-$ 825.

The structures and ^{13}C NMR data of the aglycone moieties of **6** and **9** are provided below together with sugar residues determined for compounds **6**–**9**.



In 1999, a new tridesmosidic cycloartane-type glycoside cephalotoside A (**10**) was isolated from the roots of *Astragalus cephalotes* var. *brevicalyx* (Sect. Rhacophorus) in addition to the known glycosides cyclocanthosides A (Fadeev et al. 1988), D (Isaev et al. 1992), and E (Isaev et al. 1992). The structure of the new compound was established on the basis of spectral data and chemical (acetylation) methods as 3*β*-(*β*-D-xylopyranosyl)oxy-16*β*-(*β*-D-glucopyranosyl)oxy-24-(*β*-D-xylopyranosyl)oxy-cycloartane-6*α*,25-diol (Çalış et al. 1999).

Compound **10**; $C_{46}H_{78}O_{18}$; $[\alpha]^{20}_{D}$ +20.9° (*c* 0.53, MeOH); ν^{KBr}_{max} cm^{-1}: (KBr) 3400 (OH), 2927 (CH), 1170 and 1044 (C–O–C); δ_C (C_5D_5N); FABMS *m/z* $[M + Na]^+$ 941. The structure and ^{13}C chemical shifts of compound **10** are presented below.

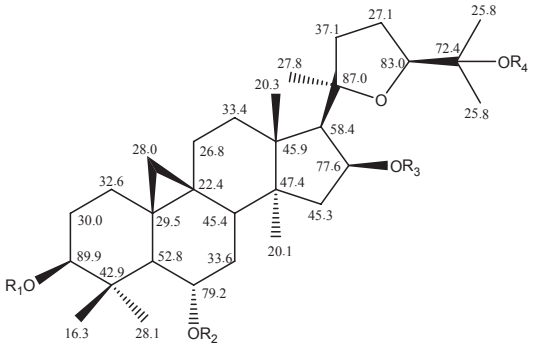


10

The study of the chemical constituents of *Astragalus trojanus* roots (Sect. Pterophorus) resulted in the isolation of six novel cycloartane-type glycosides (**11**–**16**). In addition, a new oleanane glycoside (**17**) and a new tryptophan derivative (**18**) were also isolated and characterized. MS, IR, ^{1}H NMR, and ^{13}C NMR experiments established the structures of compounds **11–17** as 3-*O*-*β*-D-xylopyranosyl-6-*O*-*β*-D-glucopyranosyl-16-*O*-acetoxy-(20*R*,24*S*)-epoxy-3*β*,6*α*,25-trihydroxycycloartane (trojanosideA),3-*O*-*β*-D-xylopyranosyl-6-*O*-*β*-D-xylopyranosyl-25-*O*-*β*-D-glucopyranosyl-(20*R*,24*S*)-epoxy-3*β*,6*α*,16*β*,25-tetrahydroxycycloartane (trojanoside B), 3-*O*-[*α*-L-rhamnopyranosyl-(1→2)-*β*-D-xylopyranosyl]-24-*O*-*β*-D-glucopyranosyl-3*β*,6*α*,16*β*,(24*S*),25-pentahydroxycycloartane (trojanoside C), 3-*O*-*β*-D-glucopyranosyl-6-*O*-*β*-D-glucopyranosyl-24-*O*-*β*-D-glucopyranosyl-3*β*,6α,16*β*,(24*S*), 25-pentahydroxycycloartane (trojanoside D), 3-*O*-[*α*-L-rhamnopyranosyl-(1→2)-*β*-D-xylopyranosyl]-6-*O*-*β*-D-glucopyranosyl-24-*O*-*β*-D-glucopyranosyl-3*β*,6*α*,16*β*,(24*S*),25-pentahydroxycycloartane (trojanoside E), 3-*O*-[*α*-L-arabinopyranosyl-(1→2)-*β*-D-xylopyranosyl]-6-*O*-*β*-D-glucopyranosyl-24-*O*-*β*-D-glucopyranosyl-3*β*,6*α*,16*β*,(24*S*),25-pentahydroxycycloartane (trojanoside F), and 3-*O*-[*α*-L-rhamnopyranosyl-(1→2)-*β*-D-xylopyranosyl-(1→2)-*β*-D-glucuronopyranosyl]-29-*O*-*β*-D-glucopyranosyl-3*β*,22*β*,24,29-tetrahydroxyolean-12-en (astrojanoside A), respectively. The structure of compound **18**, named as achillamide trivially, was determined as *N*-[3-hydroxy-3-methyl-glutaroyl]-tryptophan (Bedir et al. 1999a). Besides, the known compounds astrasieversianin I (Gan et al. 1986), astrasieversianin II (Gan et al. 1986), astragaloside I (Kitagawa et al. 1983a), astragaloside IV (Kitagawa et al. 1983a), astragaloside VII (Kitagawa et al. 1983c), and brachyoside C (Bedir et al. 1998b) were also purified and identified from the roots of *A. trojanus*. According to the authors, tetraglycosidic-type cycloartanes such

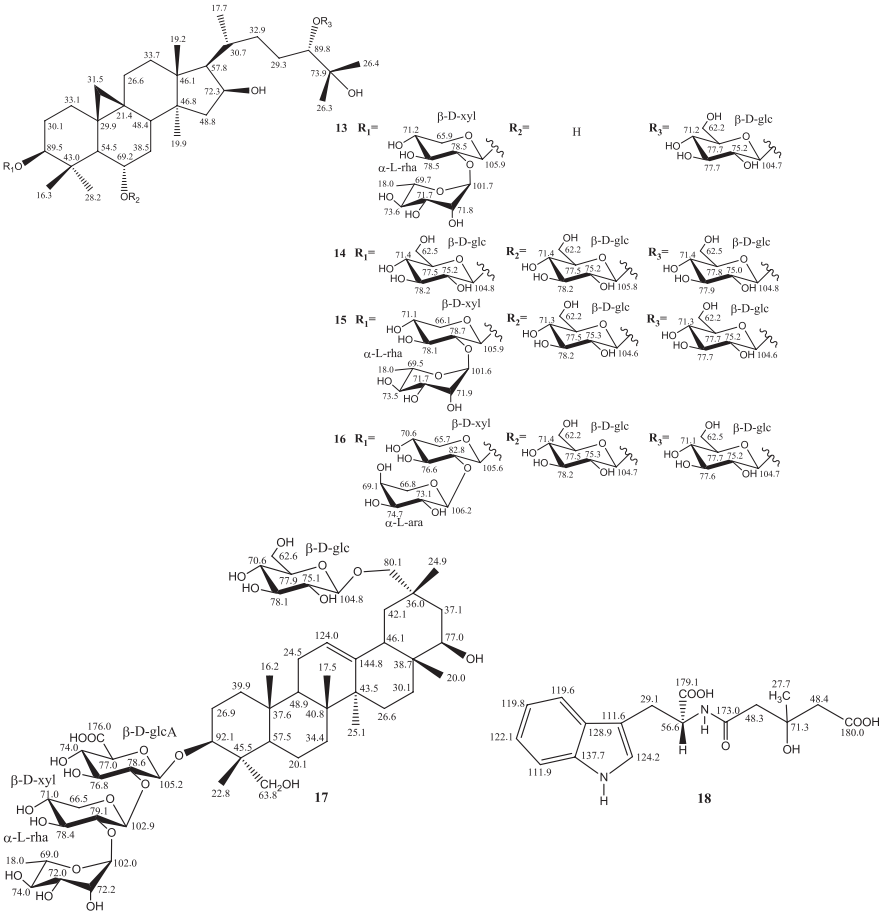
as trojanosides E and F were isolated from *Astragalus* genus for the first time (Bedir et al. 1999a).

Compound **11**; $C_{43}H_{70}O_{15}$; $[\alpha]^{25}_{D}$ +20.1° (*c* 0.1, MeOH); ν^{KBr}_{max} cm^{-1}: 3420 (OH), 1735 (ester carbonyl), 1260 and 1049 (C–O–C); δ_C (CD_3OD); FABMS *m/z* $[M–H]^-$ 825. Compound **12**; $C_{46}H_{76}O_{18}$; $[\alpha]^{25}_{D}$ +13.2° (*c* 0.1, MeOH); ν^{KBr}_{max} cm^{-1}: 3420 (OH), 1270 and 1040 (C–O–C); δ_C (CD_3OD); FABMS *m/z* $[M–H]^-$ 915. Compound **13**; $C_{47}H_{80}O_{18}$; $[\alpha]^{25}_{D}$ −5.0° (*c* 0.1, MeOH); ν^{KBr}_{max} cm^{-1}: 3420 (OH), 2933 (CH), 1250 and 1024 (C–O–C); δ_C (CD_3OD); FABMS *m/z* $[M–H]^-$ 931. Compound **14**; $C_{48}H_{82}O_{20}$; $[\alpha]^{25}_{D}$ +22.5° (*c* 0.1, MeOH); ν^{KBr}_{max} cm^{-1}: 3392 (OH), 2935 (CH), 1257 and 1044 (C–O–C); δ_C (CD_3OD); FABMS *m/z* $[M–H]^-$ 977. Compound **15**; $C_{53}H_{90}O_{23}$; $[\alpha]^{25}_{D}$ +2.6° (*c* 0.1, MeOH); ν^{KBr}_{max} cm^{-1}: 3392 (OH), 2933 (CH), 1257 and 1044 (C–O–C) cm^{-1}; δ_C (CD_3OD); FABMS *m/z* $[M–H]^-$ 1093. Compound **16**; $C_{52}H_{88}O_{23}$; $[\alpha]^{25}_{D}$ +5.2° (*c* 0.1, MeOH); ν^{KBr}_{max} cm^{-1}: 3420 (OH), 2924 (CH), 1270 and 1040 (C–O–C); δ_C (CD_3OD); FABMS *m/z* $[M–H]^-$ 1079. Compound **17**; $C_{53}H_{86}O_{23}$; $[\alpha]^{25}_{D}$ +16.7° (*c* 0.1, MeOH); ν^{KBr}_{max} cm^{-1}: 3393 (OH), 2926 (CH), 1749 (C=O), 1636 (C=C), 1271 and 1045 (C–O–C); δ_C (CD_3OD); FABMS *m/z* $[M–H]^-$ 1089. Compound **18**; $C_{17}H_{20}O_6N_2$; $[\alpha]^{25}_{D}$ −29.0° (*c* 0.1, MeOH); ν^{KBr}_{max} cm^{-1}: 3500 (NH), 3392 (OH), 1748, 1733, 1683 (CO-NH, C=O); δ_C (CD_3OD); FABMS *m/z* $[M–H]^-$ 347. The structures and ^{13}C NMR data of compounds **11**–**18** are provided below.



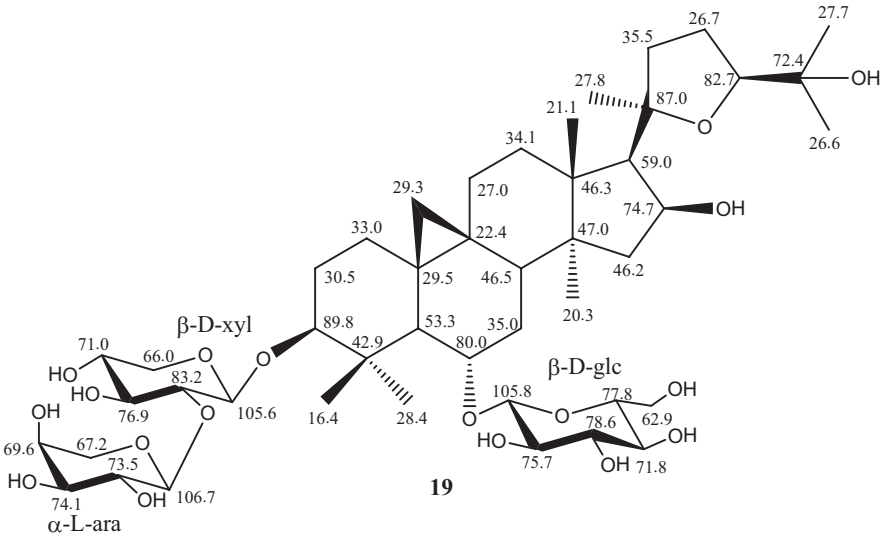
11 **R$_1$**= β-D-xyl (70.9, 66.5, 75.1, 77.8, 107.1) **R$_2$**= β-D-glc (62.7, 70.4, 77.6, 75.1, 78.1, 104.7) **R$_3$**= (20.6, 171.9) **R$_4$**= H

12 **R$_1$**= β-D-xyl (70.8, 66.1, 75.0, 77.6, 105.2) **R$_2$**= β-D-xyl (70.8, 66.1, 75.0, 77.6, 105.2) **R$_3$**= H **R$_4$**= β-D-glc (62.5, 71.1, 77.5, 75.0, 77.9, 98.4)



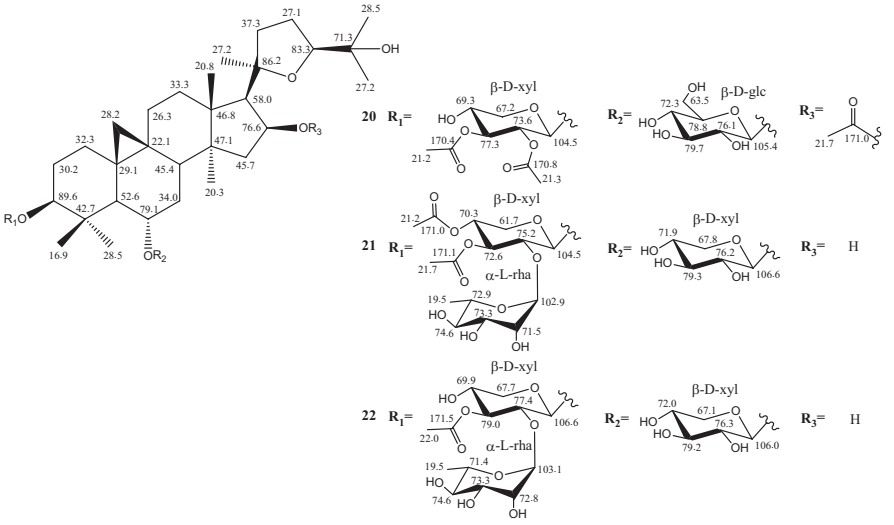
Isolation of trojanoside H (**19**) was also reported from the aerial parts of *Astragalus trojanus* along with six known glycosides astragaloside II (Kitagawa et al. 1983a), astragaloside IV (Kitagawa et al. 1983a), astragaloside VII (Bedir et al. 1999a), brachyoside B (Bedir et al. 1998b), brachyoside C (Bedir et al. 1998b), and the pterocarpan derivative maackiain (Chaudhuri et al. 1995). The structure of trojanoside H was confirmed by spectral methods (1-D and 2-D NMR and FABMS) and established as 3-*O*-*β*-[*α*-L-arabinopyranosyl-(1→2)-*β*-D-xylopyranosyl]-6-*O*-*β*-D-glucopyranosyl-20(*R*),24(*S*)-epoxy-3*β*,6*α*,16*β*,25-tetrahydroxycycloartane (Bedir et al. 1999b).

Compound **19**; $C_{46}H_{76}O_{18}$; $[\alpha]^{25}_{D}$ +14.2°; δ_C (CD_3OD); FABMS *m/z* $[M–H]^-$ 915. The ^{13}C chemical shifts are given below for compound **19**.



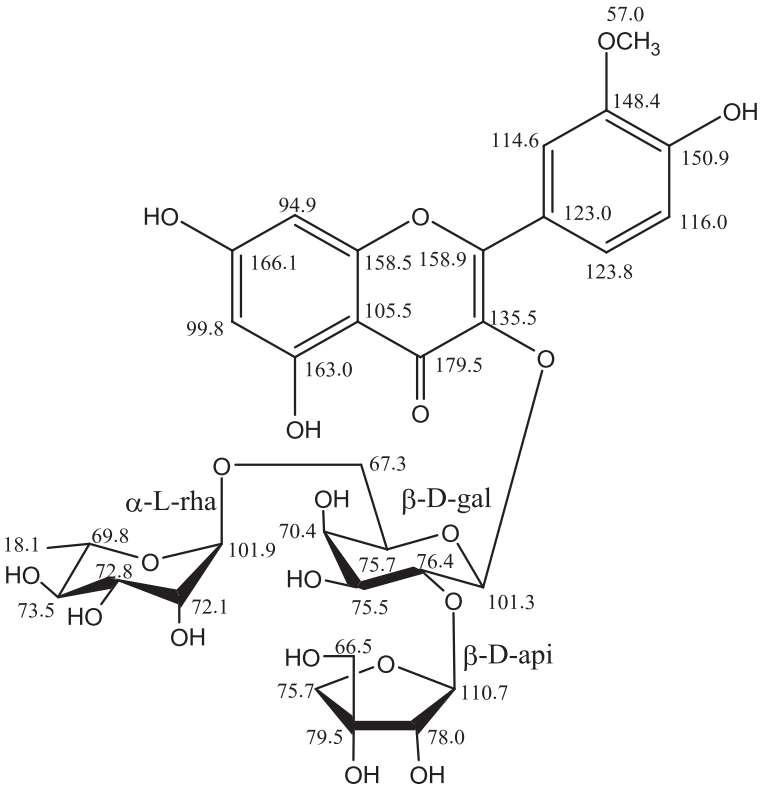
Another series of cycloartane saponins, trojanosides I–K (**20, 21,** and **22**), were also isolated from the aerial parts of *Astragalus trojanus*. The structures of three new saponins were established as 3-*O*-*β*-(2′,3′-di-*O*-acetyl)-D-xylopyranosyl-6-*O*-*β*-D-glucopyranosyl-16-*O*-acetoxy-20(*R*),24(*S*)-epoxycycloartane-3*β*,6*α*,16*β*,25-tetrol (**20**), 3-*O*-[*α*-L-rhamnopyranosyl-(1→2)-*β*-(3′,4′-di-*O*-acetyl)-D-xylopyranosyl]-6-*O*-*β*-D-xylopyranosyl-20(*R*),24(*S*)-epoxycycloartane-3*β*,6*α*,16*β*,25-tetrol (**21**), and 3-*O*-*β*-D-xylopyranosyl-6,16-di-*O*-*β*-D-glucopyranosyl-20(*R*),24(*S*)-epoxycycloartane-3*β*,6*α*,16*β*,25-tetrol (**22**) (Bedir et al. 2001a). Astrasieversianin I (Gan et al. 1986), astrasieversianin II (Gan et al. 1986), astrasieversianin XV (Gan et al. 1986), astragaloside I (Kitagawa et al. 1983a), astragaloside II (Kitagawa et al. 1983a), astragaloside IV (Kitagawa et al. 1983a), astragaloside VII (Kitagawa et al. 1983c), and trojanoside H (Bedir et al. 1999b) were also isolated and identified on the basis of their HR-ESI-MS and NMR (^{1}H– and ^{13}C–) data, in comparison with literature values (Bedir et al. 2001a).

Compound **20**; $C_{47}H_{74}O_{17}$; ν^{KBr}_{max} cm^{-1}: 3419, 2936, 1726, 1461, 1379, 1262, 1078 and 1041; δ_C (C_5D_5N); HR-ESI-MS at *m/z* [M + Na]$^+$ 933.3207. Compound **21**; $C_{50}H_{80}O_{19}$; ν^{KBr}_{max} cm^{-1}: 3408, 2928, 1745, 1371, 1244 and 1042; δ_C (C_5D_5N); HR-ESI-MS at *m/z* [M + Na]$^+$ 1007.3524. Compound **22**; $C_{48}H_{78}O_{18}$; ν^{KBr}_{max} cm^{-1}: 3395, 2934, 1726, 1461, 1373, 1262 and 1057; δ_C (C_5D_5N); HR-ESI-MS at *m/z* [M + Na]$^+$ 965.5188. The structures and ^{13}C NMR data of the compounds (**20**–**22**) are shown below.



Bedir et al. reported on the isolation and characterization of a new flavonol glycoside, isorhamnetin 3-*O*-*β*-D-apiofuranosyl-(1→2)-[*α*-L-rhamnopyranosyl-(1→6)]-*β*-D-galactopyranoside (**23**), and a known glycoside, isorhamnetin 3-*O*-*α*-L-rhamnopyranosyl-(1→6)-*β*-D-galactopyranoside (Burasheva et al. 1975), from the aerial parts of *Astragalus vulneraria* (Sect. Vulneraria) (Bedir et al. 2000b).

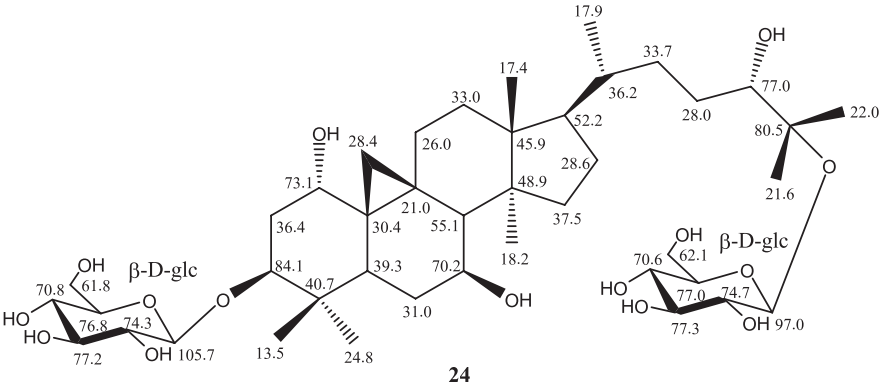
Compound **23**; $[\alpha]^{25}_{D}$ +38.4° (c 0.1, MeOH); δ_C (CD_3OD); FABMS *m/z* $[M–H]^-$ 755. The structure and ^{13}C chemical shifts are given below for compound **23**.



23

The same year, macrophyllosaponin E (**24**), a novel cycloartane-type triterpene, has been isolated from the roots of *Astragalus oleifolius* (Bedir et al. 2000c).The structure and ^{13}C chemical shifts are provided below for compound **24**.

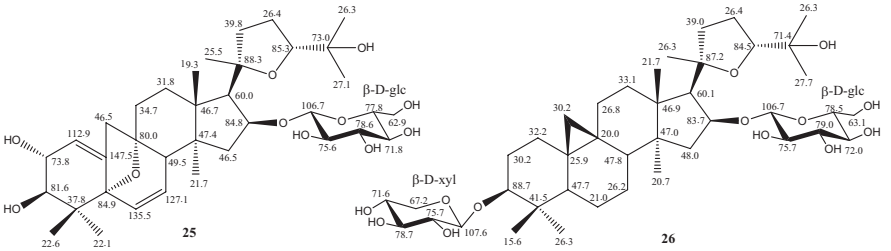
Compound **24**; $C_{42}H_{72}O_{15}$; δ_C (CD_3OD); HR-ESI-FT-MS at *m/z* $[M + Na]^+$ 839.4721.



24

In 2001, Bedir et al. reported on the isolation and structural elucidation of two novel cycloartane-type glycosides, 16-*O*-*β*-D-glucopyranosyl-20(*S*),24(*R*)-5*α*-9-diepoxy-2*α*,3*β*,16*β*,25-tetrahydroxy-9,10-seco-cycloarta-1(10),6(7)-diene (**25**) and 3-*O*-*β*-D-xylopyranosyl-16-*O*-*β*-D-glucopyranosyl-20(*S*),24(*R*)-epoxy-3*β*,16*β*,25-trithydroxycycloartane (**26**) from the roots of *Astragalus prusianus*. In the paper, a unique 5-α-9-epoxy structural feature in **25** was reported that was encountered for the first time for triterpene chemistry in nature (Bedir et al. 2001b).

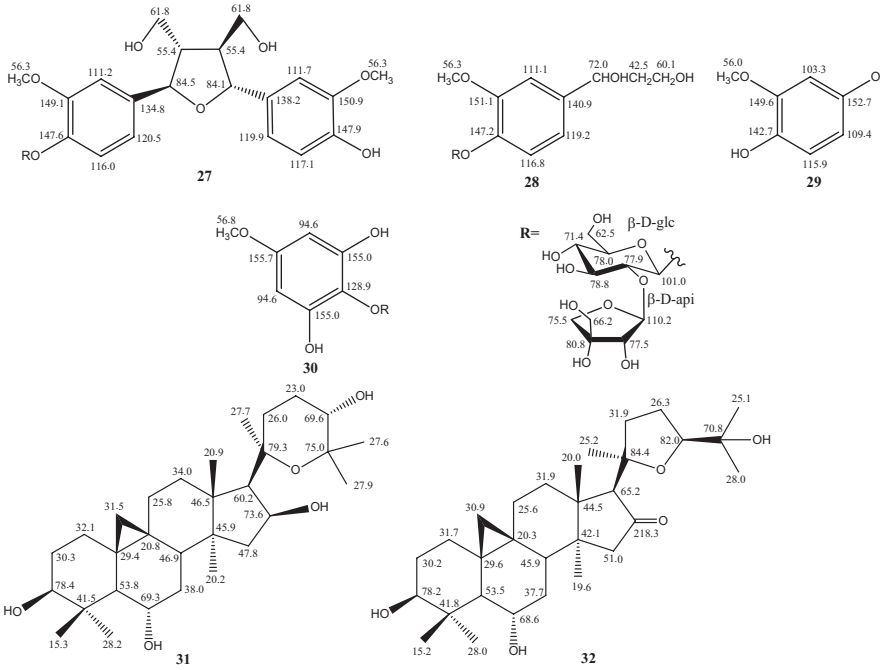
Compound **25**; $C_{36}H_{56}O_{11}$; $[\alpha]^{25}_D$ −112.5° (*c* 0.004, MeOH); ν^{KBr}_{max} cm^{-1}: 3396, 2927, 2395, 2358, 2339, 1738, 1593, 1382, 1256, 1165, 1073 and 1032; δ_C (CD_3OD); HR-ESI-FT-MS *m/z* [M + Na]$^+$ 687.2429. Compound **26**; $C_{41}H_{68}O_{13}$; $[\alpha]^{25}_D$ +20.0° (*c* 0.004, MeOH); ν^{KBr}_{max} cm^{-1}: 3376, 2933, 2870, 2363, 1726, 1459, 1381, 1166, 1071 and 1045; δ_C (C_5D_5N); HR-ESI-FT-MS *m/z* [M + Na]$^+$ 791.4842. The structures and ^{13}C chemical shifts are presented below for compounds **25** and **26**.



In another study in 2001, four new phenolic glycosides, *β*-apiofuranosyl-(1→2)-*β*-glucopyranosides (**27**–**30**), along with the new cycloartane triterpenes 20(*R*),25-epoxy-3*α*,6*β*,16*α*,24*β*-tetrahydroxycycloartane (**31**) and 20(*R*),24(*S*)-epoxy-3*β*,6*α*,25-trihydroxycycloartan-16-one (**32**) were isolated and purified from the roots of *Astragalus zahlbruckneri* (Sect. Rhacophorus). Structures of the new compounds were established as (+)-neo-olivil 4-*O*-*β*-apiofuranosyl-(1→2)-*β*-glucopyranoside (**27**), 7,8-dihydro-7-hydroxyconiferyl alcohol 4-*O*-*β*-apiofuranosyl-(1→2)-*β*-glucopyranoside (**28**), 2-methoxyphenol-4-*O*-*β*-apiofuranosyl-(1→2)-*β*-glucopyranoside (**29**), 3-hydroxy-5-methoxyphenol-2-*O*-*β*-apiofuranosyl-(1→2)-*β*-glucopyranoside (**30**), 20(*R*), 25-epoxy-3*β*,6*α*,16*β*,24*α*-tetrahydroxycycloartane (**31**), and 20(*R*),24(*S*)-epoxy-3*β*,6*α*,25-trihydroxycycloartan-16-one (**32**). Additionally, a known cycloartane sapogenin, namely, cycloastragenol (Kitagawa et al. 1983a), was isolated from the apolar fraction of *A. zahlbruckneri*. Compound **32** was reported before as a cycloartane derivative obtained by chemical oxidation of cycloastragenol (Kitagawa et al. 1983a, b, d). The structures were elucidated by a combination of spectroscopic data (^1H NMR, ^{13}C NMR, HMBC, HMQC, MS, and IR) (Çalış et al. 2001).

Compound **27**; $C_{31}H_{42}O_{16}$; $[\alpha]^{25}_D$ −69.9° (c 0.5, MeOH); δ_C (CD_3OD); FABMS *m/z* [M–H]$^-$ 669. Compound **28**; $[\alpha]^{25}_D$ −56.0 (c 0.5, MeOH); δ_C (CD_3OD); FABMS *m/z* [M–H]$^-$ 491. Compound **29**; $[\alpha]^{25}_D$ −59.0° (c 0.5, MeOH); δ_C (CD_3OD); FABMS *m/z* [M–H]$^-$ 433. Compound **30**; $[\alpha]^{25}_D$ −40.5° (c 0.5, MeOH); δ_C (CD_3OD);

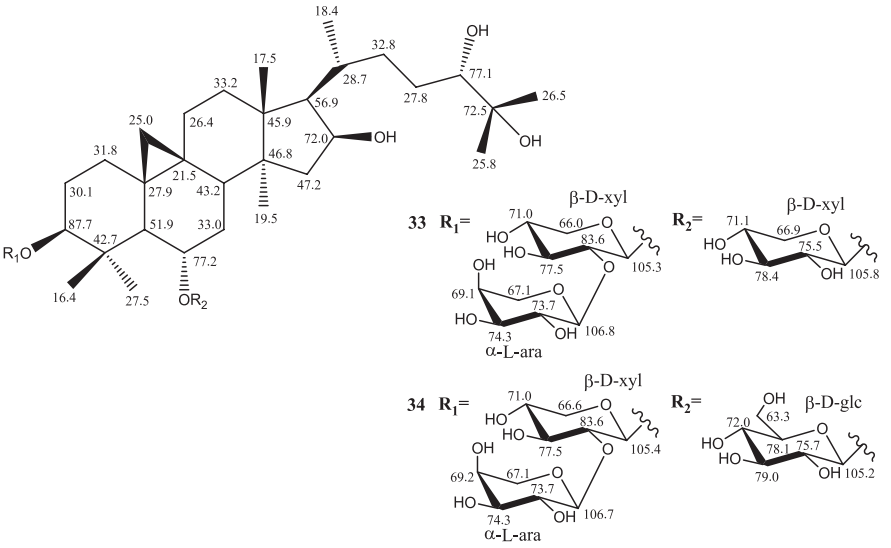
FABMS *m/z* $[M–H]^-$ 449. Compound **31**; $C_{30}H_{50}O_5$; $[\alpha]^{25}_D$ +23.3° (c 0.5, $CHCl_3$); δ_C ($CDCl_3$); FABMS *m/z* $[M–H]^-$ 489. Compound **32**; $C_{30}H_{48}O_5$; $[\alpha]^{25}_D$ +10.0° (c 0.5, $CHCl_3$); δ_C ($CDCl_3$); FABMS *m/z* $[M–H]^-$ 487. The structures and ^{13}C chemical shifts of compounds **27–32** are shown below.



Another phytochemical investigation was published in 2005, and two new cycloartane-type glycosides oleifoliosides A (**33**) and B (**34**) were isolated from the lower stem parts of *Astragalus oleifolius*. Structures of the new compounds were established as 3-*O*-[β-xylopyranosyl-(1→2)-α-arabinopyranosyl]-6-*O*–β-xylopyranosyl-3β,6α,16β,24(*S*),25-pentahydroxycycloartane and 3-*O*-[β-xylopyranosyl-(1→2)-α-arabinopyranosyl]-6-*O*–β-glucopyranosyl-3β,6α,16β,24(*S*),25-pentahydroxycycloartane, by using 1D- and 2D-NMR techniques and mass spectrometry. Additionally, three known cycloartane glycosides cyclocanthoside E (Isaev et al. 1992), astragaloside II (Kitagawa et al. 1983a), and astragaloside IV (Kitagawa et al. 1983a) were also isolated and characterized. These compounds were tested for their cytotoxicities on primary mammalian (L6) cells along with in vitro antiplasmodial, leishmanicidal, and trypanocidal activities. All the compounds showed good growth inhibitory activity opposed to *Leishmania donovani* with IC_{50} values ranging from 13.2 to 21.3 μg/mL. While all compounds were inactivite against *Trypanosoma cruzi* and *Plasmodium falciparum*, only two known compounds, namely, astragaloside II (IC_{50} 66.6 μg/mL) and cyclocanthoside E (IC_{50} 85.2 μg/mL), showed weak activity against *Trypanosoma brucei rhodesiense*. None of the compounds had toxicity to mammalian cells (IC_{50}'s > 90 μg/mL). In this study, leishmanicidal and trypanocidal

activities of cycloartane-type triterpene glycosides were reported for the first time (Özipek et al. 2005).

Compound **33**; $C_{45}H_{76}O_{17}$; $[\alpha]^{27}_D$ +18.9° (*c* 0.1, MeOH); ν^{KBr}_{max} cm^{-1}: 3427 (OH), 2922 (CH), 1048; δ_C (C_5D_5N); ESI-MS *m/z* $[M + Na]^+$ 911. Compound **34**; $C_{46}H_{78}O_{18}$; $[\alpha]^{27}_D$ +21.9° (*c* 0.1, MeOH); ν^{KBr}_{max} cm^{-1}: 3423 (OH), 2923 (CH), 1167, 1078; δ_C (C_5D_5N); ESI-MS *m/z* $[M + Na]^+$ 941. The structures and ^{13}C NMR data of the compounds (**33**–**34**) are presented below.

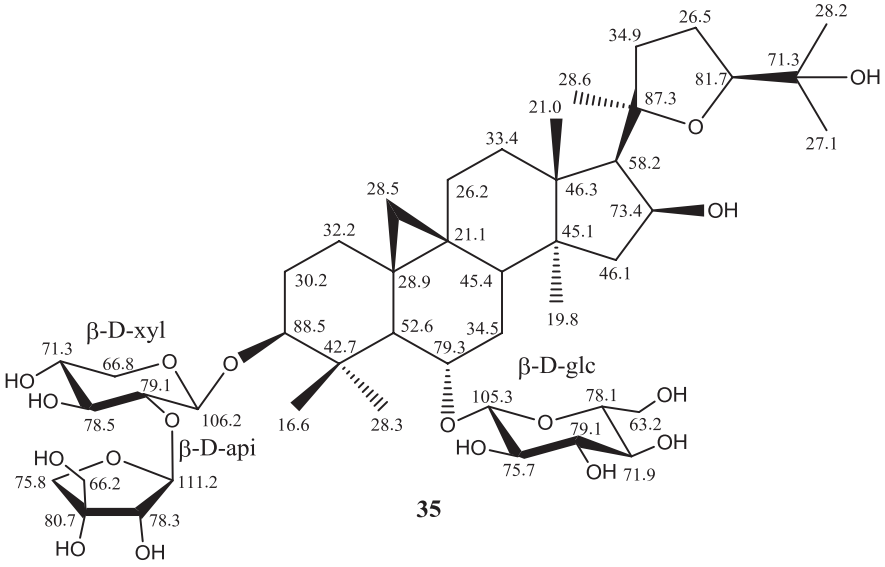


In 2005, a study was performed on another Turkish species; *Astragalus gilvus* (Sect. Christiana) and known cycloartane-type saponins, astrasieversianins I (Gan et al. 1986), II (Gan et al. 1986), VI (Gan et al. 1986), VIII (Gan et al. 1986), and X (Gan et al. 1986) and astragaloside IV (Kitagawa et al. 1983a), were isolated. As in *Astragalus gilvus*, the other studied species of Christiana section, viz., *Astragalus melanophrurius*, was also rich in acetylated sugar residues attached to cycloartane nucleus. Thus the authors made a comment about the probable chemotaxonomic significance of the acetylated compounds for Christiana section, implying overexpression of acetyl transferase genes in the plants of this section (Tabanca et al. 2005).

A new cycloartane-type triterpene glycoside, namely, (20*R*,24*S*)-3-*O*-[*β*-D-apiofuranosyl-(1→2)-*β*-D-xylopyranosyl]-6-*O*-*β*-D-glucopyranosyl-*3β*,6*α*,16*β*,25-tetrahydroxy-20,24-epoxycycloartane, named baibutoside (**35**), was isolated from the roots of *Astragalus baibutensis* (Sect. Pterophorus) along with four known glycosides, acetylastragaloside I (Kitagawa et al. 1983a) and astragalosides I (Kitagawa et al. 1983a), II (Kitagawa et al. 1983a), and IV (Kitagawa et al. 1983a). The authors commented that the apiose unit in cycloartane glycosides as in baibutoside was a very unusual finding. The evaluation of antiprotozoal activities of all the compounds on a panel of parasites including *Trypanosoma brucei rhodesiense*, *Trypanosoma cruzi*, *Leishmania donovani*, and *Plasmodium falciparum* was also studied. The

selective cytotoxicity tests versus primary L6 mammalian cells (rat skeletal myoblasts) revealed toxicity only for acetylastragaloside I with narrow selectivity index values of 2.5 and 4.8. Moreover, the compounds had no activity against *L. donovani* and *P. falciparum*. Almost all purified metabolites, except baibutoside, showed some growth inhibitory effect on *T. brucei rhodesiense*; among all, acetylastragaloside I was reported as the most active compound with IC_{50} value of 9.5 μg/mL. On the other hand, acetylastragaloside I also showed significant activity against *T. cruzi* (IC_{50} value of 5.0 μg/mL). Antiprotozoal activity of the cycloartane-type glycosides was reported for the first time in this study (Çalış et al. 2006).

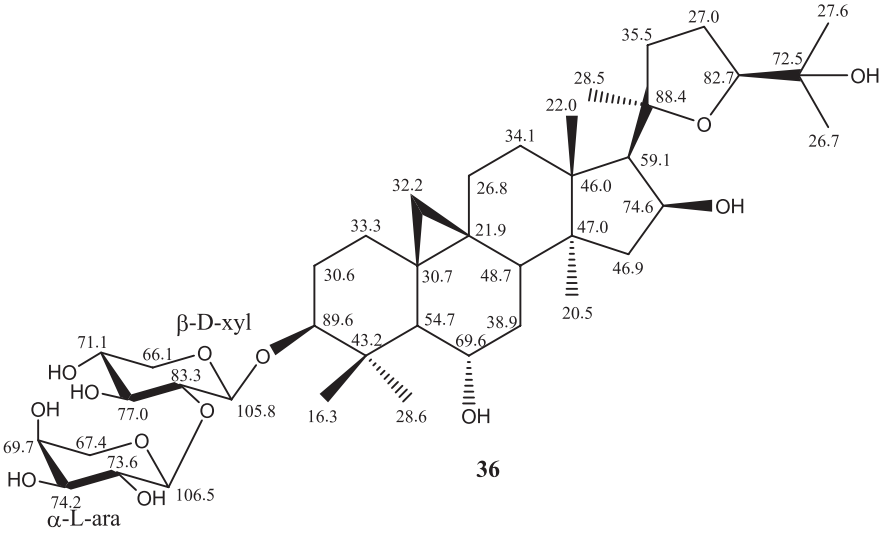
Compound **35**; $C_{46}H_{76}O_{18}$; $[\alpha]^{20}_{D}$ −7.0° (*c* 0.1, MeOH); ν^{KBr}_{max} cm^{-1}: 3400 (OH), 2933 (CH), 1166, 1077, 1042; δ_C (C_5D_5N); ESI-MS *m/z* $[M + Na]^+$ 939.6. The structure and ^{13}C chemical shifts are provided below for compound **35**.



35

A new monodesmosidic cycloartane-type glycoside together with two known cycloartane-type glycosides was obtained from *Astragalus elongatus* (Sect. Proselius) and was identified as elongatoside (3-*O*-[*α*-arabinopyranosyl-(1→2)-*β*-xylopyranosyl]-cycloastragenol) (**36**), askendosides D (3-*O*-[*α*-arabinopyranosyl-(1→2)-*β*-xylopyranosyl]-6-*O*-*β*-xylopyranosyl-cycloastragenol) (Isaev et al. 1983a), and G (3-*O*-[*α*-arabinopyranosyl-(1→2)-*β*-xylopyranosyl]-16-*O*-*β*-glucopyranosyl-3*β*,6*α*,16*β*,24(*R*),25-pentahydroxycycloartane) (Isaev 1996), on the basis of NMR experiments and mass spectrometry. For all pure compounds, human microvascular endothelial cell line (HMEC-1) was used to measure the inhibition of proliferation and ICAM-1 expression in vitro. Compound **36** was reported to possess weak activity in the ICAM-1 assay (Çalış et al. 2008a).

Compound **36**; $C_{40}H_{66}O_{13}$; $[\alpha]^{31}_D$ +31.0° (*c* 0.1, MeOH); δ_C (CD_3OD); HR-FAB-MS *m/z* $[M + H]^+$ 755.4564 (calcd. for $C_{40}H_{67}O_{13}$ 755.4582 $[M + H]^+$). The structure and ^{13}C chemical shifts are shown below for compound **36**.

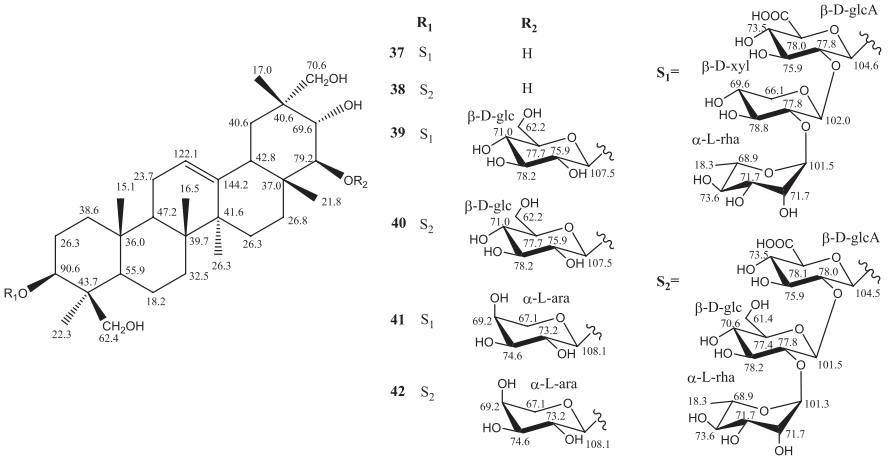


36

Avunduk et al. (2008) reported on the isolation and structural elucidation of six new triterpene saponins, 3-*O*-*α*-L-rhamnopyranosyl-(1→2)-*β*-D-xylopyranosyl-(1→2)-*β*-D-glucuronopyranosyl-21-epi-kudzusapogenol A (**37**), 3-*O*-*α*-L-rhamnopyranosyl-(1→2)-*β*-D-glucopyranosyl-(1→2)-*β*-D-glucuronopyranosyl-21-epi-kudzusapogenol A (**38**), 3-*O*-*α*-L-rhamnopyranosyl-(1→2)-*β*-D-xylopyranosyl-(1→2)-*β*-D-glucuronopyranosyl-22-*O*-*β*-D-glucopyranosyl-21-epi-kudzusapogenol A (**39**), 3-*O*-*α*-L-rhamnopyranosyl-(1→2)-*β*-D-glucopyranosyl-(1→2)-*β*-D-glucuronopyranosyl-22-*O*-*β*-D-glucopyranosyl-21-epi-kudzusapogenol A (**40**), 3-*O*-*α*-L-rhamnopyranosyl-(1→2)-*β*-D-xylopyranosyl-(1→2)-*β*-D-glucuronopyranosyl-22-*O*-*α*-L-arabinopyranosyl-21-epi-kudzusapogenol A (**41**), and 3-*O*-*α*-L-rhamnopyranosyl-(1→2)-*β*-D-glucopyranosyl-(1→2)-*β*-D-glucuronopyranosyl-22-*O*-*α*-L-arabinopyranosyl-21-epi-kudzusapogenol A (**42**), from the roots of *Astragalus flavescens* (Sect. Eustales) (Avunduk et al. 2008) together with five known compounds named trojanoside B (Bedir et al. 1999a), azukisaponin V (Kitagawa et al. 1983e), and astragalosides IV (Kitagawa et al. 1983a), VII (Kitagawa et al. 1983c), and VIII (Kitagawa et al. 1983c).

Compound **37**; $C_{47}H_{76}O_{19}$; $[\alpha]^{25}_D$ −8.0° (*c* 0.05, MeOH); δ_C (C_5D_5N); HR-ESI-MS *m/z* $[M + Na]^+$ 967.4884 (calcd. 967.4879). Compound **38**; $C_{48}H_{78}O_{20}$; $[\alpha]^{25}_D$ −7.5° (*c* 0.05, MeOH); δ_C (C_5D_5N); HR-ESI-MS *m/z* $[M + Na]^+$ 997.4980 (calcd. 997.4984). Compound **39**; $C_{53}H_{86}O_{24}$; $[\alpha]^{25}_D$ −6.2° (*c* 0.05, MeOH); δ_C (C_5D_5N); HR-ESI-MS *m/z* $[M + Na]^+$ 1129.5402 (calcd. 1129.5407). Compound **40**; $C_{54}H_{88}O_{25}$; $[\alpha]^{25}_D$ −4.5° (*c* 0.05, MeOH); δ_C (C_5D_5N); HR-ESI-MS *m/z* $[M + Na]^+$ 1159.5508 (calcd. 1159.5512). Compound **41**; $C_{52}H_{84}O_{23}$; $[\alpha]^{25}_D$ +3.1° (*c* 0.05,

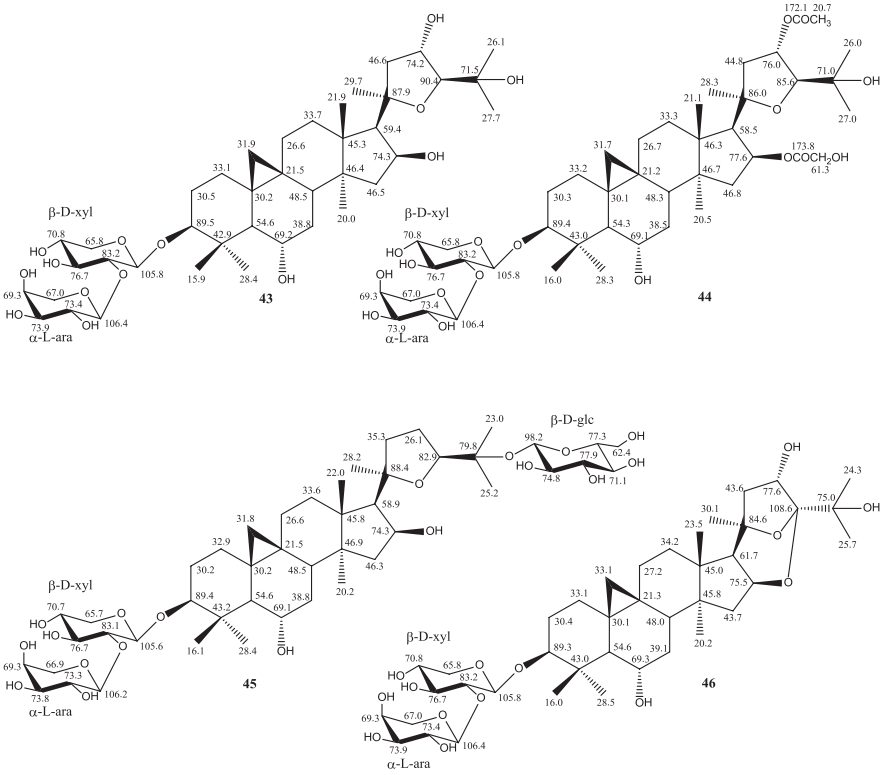
MeOH); δ_C (C_5D_5N); HR-ESI-MS *m/z* [M + Na]$^+$ 1099.5295 (calcd. 1099.5301). Compound **42**; $C_{53}H_{86}O_{24}$; $[\alpha]^{25}_D$ +8.5° (*c* 0.05, MeOH); δ_C (C_5D_5N); HR-ESI-MS *m/z* [M + Na]$^+$ 1129.5409 (calcd. 1129.5407). The structures and carbon NMR data of the compounds are provided below (**37–42**).



Another phytochemical work was performed in 2008. In this research, four new cycloartane glycosides, including 3-*O*-[*α*-L-arabinopyranosyl-(1→2)-*β*-D-xylopyranosyl]-3*β*,6*α*,16*β*,23*α*,25-pentahydroxy-20(*R*),24(*S*)-epoxycycloartane (**43**), 3-*O*-[*α*-L-arabinopyranosyl-(1→2)-*β*-D-xylopyranosyl]-16-*O*-hydroxyacetoxy-23-*O*-acetoxy-3*β*,6*α*,25-trihydroxy-20(*R*),24(*S*)-epoxycycloartane (**44**), 3-*O*-[*α*-L-arabinopyranosyl-(1→2)-*β*-D-xylopyranosyl]-25-*O*-*β*-D-glucopyranosyl-3*β*,6*α*,16*β*,25-tetrahydroxy-20(*R*),24(S)-epoxycycloartane (**45**), and 3-*O*-[*α*-L-arabinopyranosyl-(1→2)-*β*-D-xylopyranosyl]-3*β*,6*α*,23*α*,25-tetrahydroxy-20(*R*),24(*R*)-16*β*,24;20,24-diepoxycycloartane (**46**), along with three known cycloartane glycosides, 3-*O*-[*α*-L-arabinopyranosyl-(1→2)-*β*-D-xylopyranosyl]-3*β*,6*α*,16*β*,25-tetrahydroxy-20(*R*),24(*S*)-epoxycycloartane (Isaev et al. 1983a), askendoside C (Isaev et al. 1983b), and askendoside G (Isaev 1996), were isolated from the MeOH extract of the roots of *Astragalus campylosema* ssp. *campylosema* (Sect. Hololeuce). Their structures were established by the extensive use of 1D- and 2D-NMR experiments along with ESIMS and HRMS analysis. The authors commented that the presence of the hydroxyl function at position 23 (**43**–**44**) and the ketalic function at C-24 (**46**) were very rare in the cycloartane-type triterpene class (Çalış et al. 2008b).

Compound **43**; $C_{40}H_{66}O_{14}$; $[\alpha]^{35}_D$ +40.0° (*c* 0.1, MeOH); ν^{KBr}_{max} cm^{-1}: 3472 (>OH), 3035 (cyclopropane ring), 2932 (>CH), 1273 and 1040 (C–O–C); δ_C (CD_3OD); HR-MALDITOF-MS *m/z* [M + Na]$^+$ 793.4355 (calcd. for $C_{40}H_{66}O_{14}Na$, 793.4350). Compound **44**; $C_{44}H_{70}O_{17}$; $[\alpha]^{35}_D$ +67.0° (*c* 0.1, MeOH); ν^{KBr}_{max} cm^{-1}: 3480 (>OH), 3044 (cyclopropane ring), 2928 (>CH), 1734 (C=O), 1277-1035 (C–O–C);δ_C (CD_3OD); HR-MALDITOF-MS *m/z* [M + Na]$^+$ 893.4519 (calcd. for

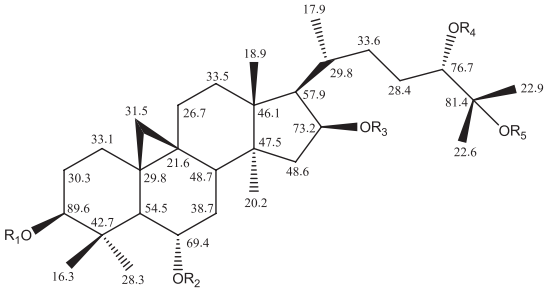
$C_{44}H_{70}O_{17}Na$, 893.4511). Compound **45**; $C_{46}H_{76}O_{18}$; $[\alpha]^{35}_{D}$ +24.0° (*c* 0.1, MeOH); ν^{KBr}_{max} cm^{-1}: 3475 (>OH), 3047 (cyclopropane ring), 2925 (>CH), 1270-1043 (C–O–C); HR-MALDITOF-MS *m/z* $[M + Na]^+$ 939.4934 (calcd. for $C_{46}H_{76}O_{18}Na$, 939.4929). Compound **46**; $C_{40}H_{64}O_{14}$; $[\alpha]^{35}_{D}$ +20.0° (*c* 0.1, MeOH); ν^{KBr}_{max} cm^{-1}: 3470 (>OH), 3040 (cyclopropane ring), 2930 (>CH), 1280-1045 (C–O–C); HR-MALDITOF-MS *m/z* $[M + Na]^+$ 791.4199 (calcd. for $C_{40}H_{64}O_{14}Na$, 791.4194). The structures and ^{13}C chemical shifts are presented below for compounds **43–46**.

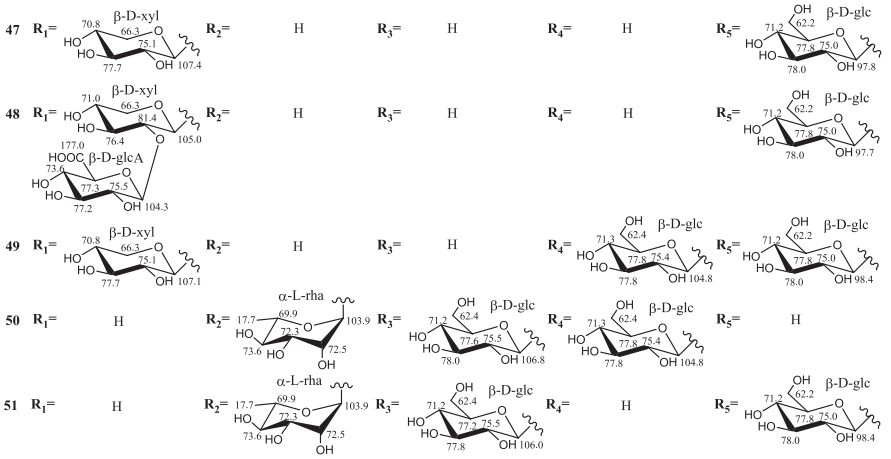


In 2009, on the basis of extensive spectroscopic analysis (IR, HR-MALDITOF-MS, ^1H NMR, ^{13}C NMR, HSQC, and HMBC), Polat et al. reported on the isolation and structural elucidation of five new cycloartane-type triterpene glycosides from the methanolic extract of the roots of *Astragalus amblolepis* Fischer (Sect. Rhacophorus) along with one known saponin, 3-*O*-*β*-D-xylopyranosyl-16-*O*-*β*-D-glucopyranosyl-3*β*,6*α*,16*β*,24(*S*),25-pentahydroxycycloartane (Karimov et al. 1998). Structures of the new compounds were established as 3-*O*-*β*-D-xylopyranosyl-25-*O*-*β*-D-glucopyranosyl-3*β*,6*α*,16*β*,24(*S*),25-pentahydroxycycloartane (**47**), 3-*O*-[*β*-D-glucuronopyranosyl-(1→2)-*β*-D-xylopyranosyl]-25-*O*-*β*-D-glucopyranosyl-3*β*,6*α*,16*β*,24(*S*),25-pentahydroxycycloartane (**48**), 3-*O*-*β*-D-xylopyranosyl-24,25-di-*O*-*β*-D-glucopyranosyl-3*β*,6*α*,16*β*,24(*S*),25-pentahydroxycycloartane (**49**),6-*O*-*α*-L-rhamnopyranosyl-16,24-di-*O*-*β*-D-glucopyranosyl-3*β*,6*α*,16*β*,24(*S*),25-pentahydroxycycloartane (**50**), and 6-*O*-*α*-L-rhamnopyranosyl-16,25-di-*O*-*β*-D-glucopyranosyl-3*β*,6*α*,16*β*,24(*S*),25-pentahydroxycycloartane (**51**). The authors

commented that the absence of sugar residue at C-3 position of cycloartane glycosides such as compounds **50** and **51** was rather unusual in nature. Moreover, a rhamnosyl moiety at C-6 position was reported for the first time in cyclocanthogenol skeleton, one of the most common aglycones in *Astragalus* genus together with cycloastragenol. In this study, the presence of glucuronic acid moiety was reported for the first time in cycloartane chemistry (Polat et al. 2009).

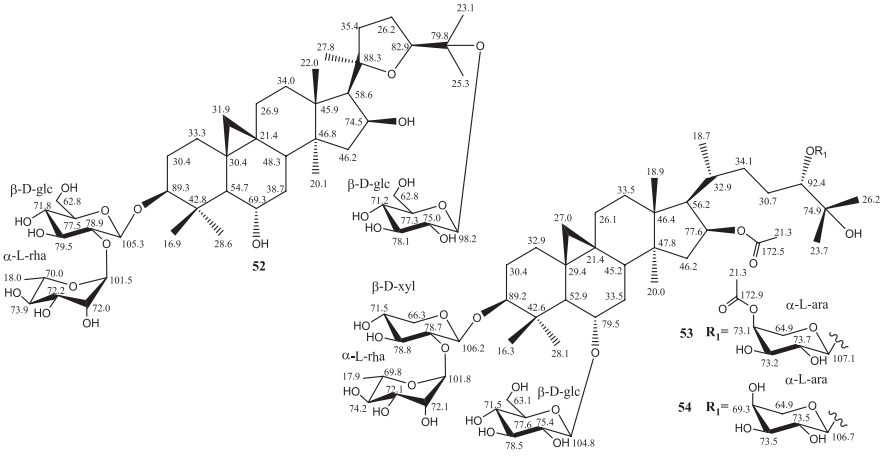
Compound **47**; $C_{41}H_{70}O_{14}$; $[\alpha]^{25}_{D}$ +34.0° (*c* 0.1, MeOH); ν^{KBr}_{max} cm^{-1}: 3480 (>OH), 3025 (cyclopropane ring), 2870 (>CH), 1290-1030 (C–O–C); δ_C (CD_3OD); HR-MALDITOF-MS *m/z* $[M + Na]^+$ 809.4669 (calcd. for $C_{41}H_{70}O_{14}Na$, 809.4663). Compound **48**; $C_{47}H_{78}O_{20}$; $[\alpha]^{25}_{D}$ +15.8° (*c* 0.1, MeOH); ν^{KBr}_{max} cm^{-1}: 3477 (>OH), 3048 (cyclopropane ring), 2895 (>CH), 1285-1043 (C–O–C); δ_C (CD_3OD); HR-MALDITOF-MS *m/z* $[M + Na]^+$ 985.4979 (calcd. for $C_{47}H_{78}O_{20}Na$, 985.4984). Compound **49**; $C_{47}H_{80}O_{19}$; $[\alpha]^{25}_{D}$ +22.7° (*c* 0.1, MeOH); ν^{KBr}_{max} cm^{-1}: 3483 (>OH), 3027 (cyclopropane ring), 2877 (>CH), 1279-1038 (C–O–C); δ_C (CD_3OD); HR-MALDITOF-MS *m/z* $[M + Na]^+$ 971.5199 (calcd. for $C_{47}H_{80}O_{19}Na$, 971.5192). Compound **50**; $C_{48}H_{82}O_{19}$; $[\alpha]^{25}_{D}$ +17.9° (*c* 0.1, MeOH); ν^{KBr}_{max} cm^{-1}: 3472 (>OH), 3040 (cyclopropane ring), 2883 (>CH), 1283-1029 (C–O–C); δ_C (CD_3OD); HR-MALDITOF-MS *m/z* $[M + Na]^+$ 985.5350 (calcd. for $C_{48}H_{82}O_{19}Na$, 985.5348). Compound **51**; $C_{48}H_{82}O_{19}$; $[\alpha]^{25}_{D}$ +24.4° (*c* 0.1, MeOH); ν^{KBr}_{max} cm^{-1}: 3486 (>OH), 3035 (cyclopropane ring), 2874 (>CH), 1292-1040 (C–O–C); δ_C (CD_3OD); HR-MALDITOF-MS *m/z* $[M + Na]^+$ 985.5341 (calcd. for $C_{48}H_{82}O_{19}Na$, 985.5348). The structures and carbon NMR data of compounds **47**–**51** were provided below.





Three new cycloartane-type triterpene glycosides were reported from *Astragalus wiedemannianus* (Sect. Pterophorus) in addition to known secondary metabolites, namely, cycloastragenol (Kitagawa et al. 1983a), cycloascauloside B (Alaniya et al. 2008), astragaloside IV (Kitagawa et al. 1983a), astragaloside VIII (Kitagawa et al. 1983c), brachyoside B (Bedir et al. 1998b), astragaloside II (Kitagawa et al. 1983c), astrachrysoside A (Wang et al. 1990), and astrasieversianin X (Gan et al. 1986). The structures of the new compounds were elucidated on the basis of 1D- and 2D-NMR techniques and established as 3-*O*-[*α*-L-rhamnopyranosyl-(1→2)-*β*-D-glucopyranosyl]-25-*O*-*β*-D-glucopyranosyl-20(*R*),24(*S*)-epoxy-3*β*,6*α*,16*β*,25-tetrahydroxycycloartane (**52**), 3-*O*-[*α*-L-rhamnopyranosyl-(1→2)-*β*-D-xylopyranosyl]-6-*O*-*β*-D-glucopyranosyl-24-*O*-*α*-(4′-*O*-acetoxy)-L-arabinopyranosyl-16-*O*-acetoxy-3*β*,6*α*,16*β*,24(*S*),25-pentahydroxycycloartane(**53**),and3-*O*-[*α*-L-rhamnopyranosyl-(1→2)-*β*-D-xylopyranosyl]-6-*O*-*β*-D-glucopyranosyl-24-*O*-*α*-L-arabinopyranosyl-16-*O*-acetoxy-3*β*,6*α*,16*β*,24(*S*),25-pentahydroxycycloartane (**54**). In this study, the presence of an arabinose moiety on the acyclic side chain of cycloartanes was encountered for the first time (Polat et al. 2010).

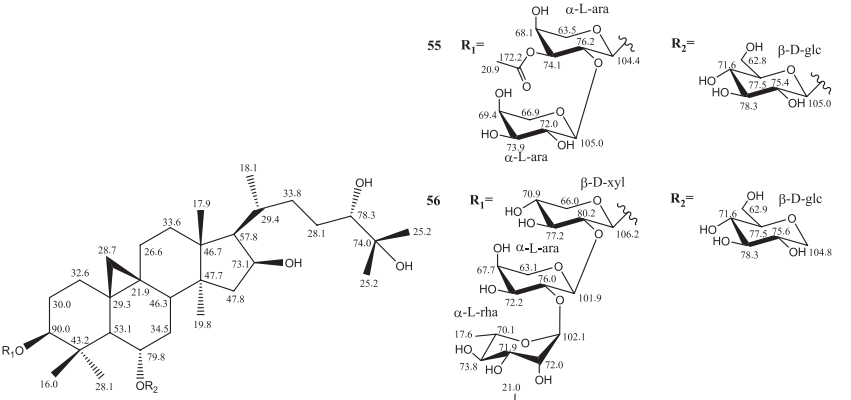
Compound **52**; $C_{48}H_{80}O_{19}$; $[\alpha]^{25}_{D}$ +25.2° (*c* 0.1, MeOH); ν^{KBr}_{max} cm^{-1}: 3474 (>OH), 3042 (cyclopropane ring), 2934 (>CH), 1271-1024 (C–O–C); δ_C (CD_3OD); HR-MALDITOF-MS *m/z* [M + Na]$^+$ 983.5196 (calcd. for $C_{48}H_{80}O_{19}Na$, 983.5192). Compound **53**; $C_{56}H_{92}O_{24}$; $[\alpha]^{25}_{D}$ +35.8° (*c* 0.1, MeOH); ν^{KBr}_{max} cm^{-1}: 3481 (>OH), 3035 (cyclopropane ring), 2941 (>CH), 1737 (C=O), 1264-1038 (C–O–C); δ_C (CD_3OD); HR-MALDITOF-MS *m/z* [M + Na]$^+$ 1171.5880 (calcd. for $C_{56}H_{92}O_{24}Na$, 1171.5876). Compound **54**; $C_{54}H_{90}O_{23}$; $[\alpha]^{25}_{D}$ +38.7° (*c* 0.1, MeOH); ν^{KBr}_{max} cm^{-1}: 3486 (>OH), 3047 (cyclopropane ring), 2930 (>CH), 1728 (C=O), 1260-1031 (C–O–C); δ_C (CD_3OD); HR-MALDITOF-MS *m/z* [M + Na]$^+$ 1129.5778 (calcd. for $C_{54}H_{90}O_{23}Na$, 1129.5771). The structures and ^{13}C NMR data of **52**–**54** are shown below.

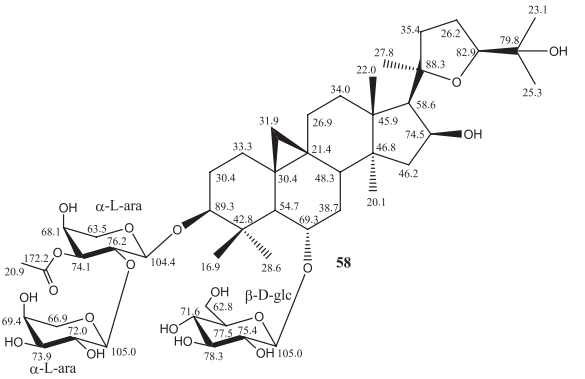


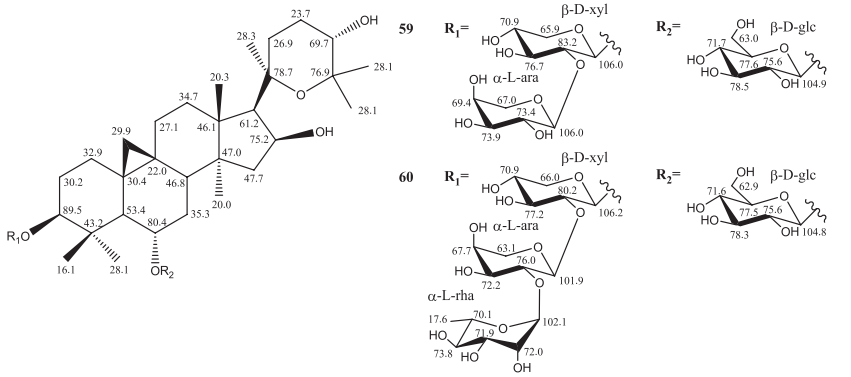
In 2010, a report described six new cycloartane-type triterpene glycosides from *Astragalus icmadophilus* (Sect. Acanthophace) along with two known cycloartane-type glycosides, five known oleanane-type triterpene glycosides, and one known

flavonol glycoside. The structures of the new compounds were established by detailed spectral analysis as 3-*O*-[*α*-L-arabinopyranosyl-(1→2)-*O*-3-acetoxy-*α*-L-arabinopyranosyl]-6-*O*-*β*-D-glucopyranosyl-3*β*,6*α*,16*β*,24(*S*),25-pentahydroxycycloartane (**55**), 3-*O*-[*α*-L-rhamnopyranosyl-(1→2)-*O*-α-L-arabinopyranosyl-(1→2)-*O*-*β*-D-xylopyranosyl]-6-*O*-*β*-D-glucopyranosyl-3*β*,6*α*,16*β*,24(*S*),25-pentahydroxycycloartane (**56**), 3-*O*-[*α*-L-arabinopyranosyl-(1→2)-*O*-3,4-diacetoxy-*α*-L-arabinopyranosyl]-6-*O*-*β*-D-glucopyranosyl-3*β*,6*α*,16*β*,24(*S*),25-pentahydroxycycloartane(**57**),3-*O*-[*α*-L-arabinopyranosyl-(1→2)-*O*-3-acetoxy-*α*-L-arabinopyranosyl]-6-*O*-*β*-D-glucopyranosyl-3*β*,6*α*,16*β*,25-tetrahydroxy-20(*R*),24(*S*)-epoxycycloartane (**58**), 3-*O*-[*α*-L-arabinopyranosyl-(1→2)-*O*-*β*-D-xylopyranosyl]-6-*O*-*β*-D-glucopyranosyl-3*β*,6*α*,16*β*,24α-tetrahydroxy-20(*R*),25-epoxycycloartane (**59**), and 3-*O*-[*α*-L-rhamnopyranosyl-(1→2)-*O*-*α*-L-arabinopyranosyl-(1→2)-*O*-*β*-D-xylopyranosyl]-6-*O*-*β*-D-glucopyranosyl-3*β*,6*α*,16*β*,24*α*-tetrahydroxy-20(*R*),25-epoxycycloartane (**60**). The authors stated that compounds **59–60** were based on cyclocephalogenin as aglycone, more unusual in the plant kingdom, so far reported only from *Astragalus* spp. (Bedir et al. 1998a; Agzamova and Isaev 1999; Sukhina et al. 2007). In addition, two known cycloartane-type glycosides, oleifolioside B (Özipek et al. 2005) and astragaloside I (Kitagawa et al. 1983a); five known oleanane-type triterpene glycosides, azukisaponin V (Kitagawa et al. 1983e), azukisaponin V methyl ester (Mohamed et al. 1995), astragaloside VIII (Kitagawa et al. 1983c), astragaloside VIII methyl ester (Cui et al. 1992a), and 22-*O*-[*β*-D-glucopyranosyl-(1→2)-*O*-*α*-L-arabinopyranosyl]-3*β*,22*β*,24-trihydroxy-olean-12-ene (Yoshikawa et al. 1985); and the flavonol glycoside narcissin (Senatore et al. 2000) were characterized (Horo et al. 2010).

Compound **55**; $C_{48}H_{80}O_{19}$; $[\alpha]^{25}_{D}$ +28.4° (*c* 0.1, MeOH); ν^{KBr}_{max} cm^{-1}: 3477 (>OH), 3041 (cyclopropane ring), 2940 (>CH), 1739 (C=O), 1264 and 1059 (C–O–C); δ_C (CD_3OD); HR-MALDITOF-MS *m/z* $[M + Na]^+$ 983.5198 (calcd. for $C_{48}H_{80}O_{19}Na$, 983.5192). Compound **56**; $C_{52}H_{88}O_{22}$; $[\alpha]^{25}_{D}$ +37.2° (*c* 0.1, MeOH); ν^{KBr}_{max} cm^{-1}: 3484 (>OH), 3037 (cyclopropane ring), 2949 (>CH), 1260 and 1064 (C–O–C); δ_C (CD_3OD); HR-MALDITOF-MS *m/z* $[M + Na]^+$ 1087.5669 (calcd. for $C_{52}H_{88}O_{22}Na$, 1087.5665). Compound **57**; $C_{50}H_{82}O_{20}$; $[\alpha]^{25}_{D}$ +31.8° (*c* 0.1, MeOH); ν^{KBr}_{max} cm^{-1}: 3471 (>OH), 3032 (cyclopropane ring), 2931 (>CH), 1741 (C=O), 1258-1050 (C–O–C); δ_C (CD_3OD); HR-MALDITOF-MS *m/z* $[M + Na]^+$ 1025.5294 (calcd. for $C_{50}H_{82}O_{20}Na$, 1025.5297). Compound **58**; $C_{48}H_{78}O_{19}$; $[\alpha]^{25}_{D}$ +22.1° (*c* 0.1, MeOH); ν^{KBr}_{max} cm^{-1}: 3488 (>OH), 3035 (cyclopropane ring), 2927 (>CH), 1732 (C=O), 1275 and 1030 (C–O–C); δ_C (CD_3OD); HR-MALDITOF-MS *m/z* $[M + Na]^+$ 981.5041 (calcd. for $C_{48}H_{78}O_{19}Na$, 981.5035). Compound **59**; $C_{46}H_{76}O_{18}$; $[\alpha]^{25}_{D}$ +8.5° (*c* 0.1, MeOH); ν^{KBr}_{max} cm^{-1}: 3481 (>OH), 3045 (cyclopropane ring), 2934 (>CH), 1280-1040 (C–O–C); δ_C (CD_3OD); HR-MALDITOF-MS *m/z* $[M + Na]^+$ 939.4932 (calcd. for $C_{46}H_{76}O_{18}Na$, 939.4929). Compound **60**; $C_{52}H_{86}O_{22}$; $[\alpha]^{25}_{D}$ +11.7° (*c* 0.1, MeOH); ν^{KBr}_{max} cm^{-1}: 3474 (>OH), 3049 (cyclopropane ring), 2937 (>CH), 1266-1052 (C–O–C); δ_C (CD_3OD); HR-MALDITOF-MS *m/z* $[M + Na]^+$ 1085.5511 (calcd. for $C_{52}H_{86}O_{22}Na$, 1085.5508). The structures and ^{13}C NMR data of the compounds (**55–60**) are presented below.


55
56
57
α-L-ara
β-D-xyl
β-D-glc
α-L-rha
R1=
R2=

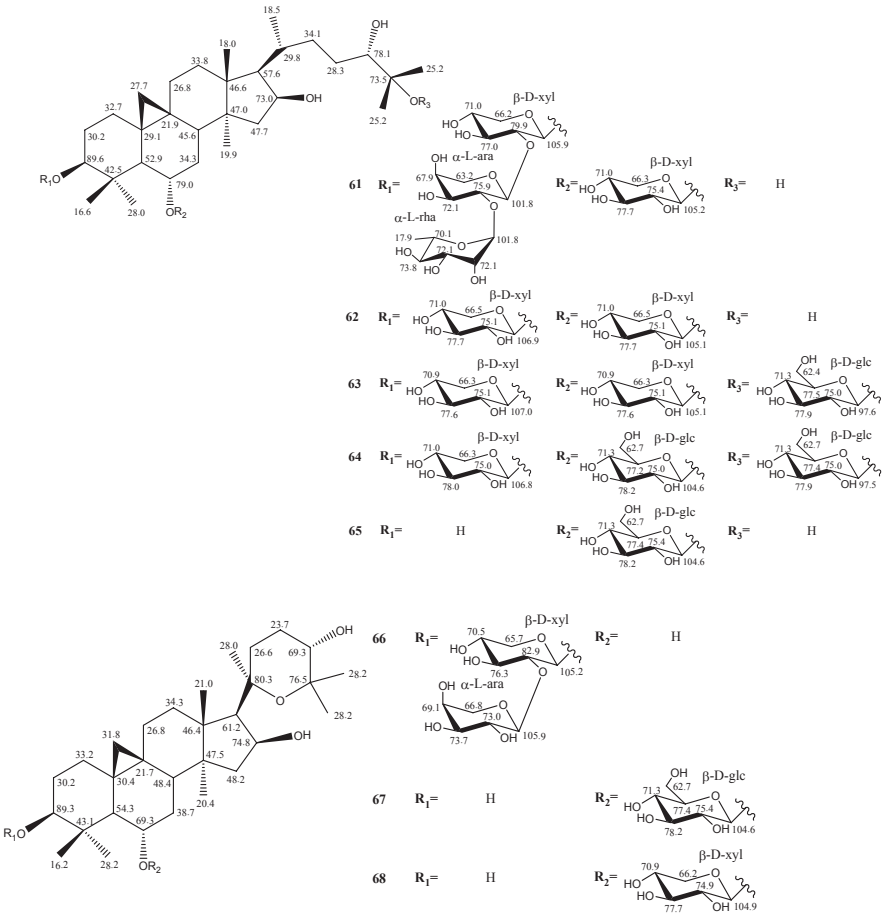

58
α-L-ara
β-D-glc


59
60
β-D-xyl
α-L-ara
α-L-rha
β-D-glc
R1=
R2=

In 2011, from the roots of *Astragalus ptilodes* Boiss. var. *cariensis* Boiss. (Sect. Pterophorus), five known compounds, astragaloside VII [(3*β*,6*α*,16*β*,20*R*,24*S*)-20,24-epoxy-16-hydroxy-3-(*β*-D-xylopyranosyloxy)-9,19-cyclolanostane-6,25-diyl bis[*β*-D-glucopyranoside] (Bedir et al. 1999a), cyclosiversioside E (3*β*,6*α*,16*β*,20*R*,24*S*)-20,24-epoxy-16,25-dihydroxy-9,19-cyclolanostane-3,6-diyl bis[*β*-D-xylopyranoside]) (Svechnikova et al. 1982a), cyclosiversioside F (3*β*,6*α*,16*β*,20*R*,24*S*)-20,24-epoxy-16,25-dihydroxy-3-(*β*-D-xylopyranosyloxy)-9,19-cyclolanostan-6-yl *β*-D-glucopyranoside) (Svechnikova et al. 1982a), astragaloside I (3*β*,6*α*,16*β*,20*R*,24*S*)-3-[(2,3-di-*O*-acetyl-*β*-D-xylopyranosyl)oxy]-20,24-epoxy-16,25-dihydroxy-9,19-cyclolanostan-6-yl *β*-D-glucopyranoside) (Kitagawa et al. 1983a), and cyclosiversioside A (3*β*,6*α*,16*β*,20*R*,24*R*)-3-[(2,3-di-*O*-acetyl-*β*-D-xylopyranosyloxy]-20,24-epoxy-16,25-dihydroxy-9,19-cyclolanostan-6-yl *β*-D-xylopyranoside) (Svechnikova et al. 1982b), were isolated (Linnek et al. 2011).

In another study in 2011, eight new cycloartane-type triterpene glycosides, 3-*O*-[α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl-(1→2)-β-D-xylopyranosyl]-6-*O*-β-D-xylopyranosyl-3β,6α,16β,24(*S*),25-pentahydroxycycloartane (**61**), 3,6-di-*O*-β-D-xylopyranosyl-3β,6α,16β,24(*S*),25-pentahydroxycycloartane (**62**), 3,6-di-*O*-β-D-xylopyranosyl-25-*O*-β-D-glucopyranosyl-3β,6α,16β,24(*S*),25-pentahydroxycycloartane (**63**), 3-*O*-β-D-xylopyranosyl-6,25-di-*O*-β-D-glucopyranosyl-3β,6α,16β,24(*S*),25-pentahydroxycycloartane (**64**), 6-*O*-β-D-glucopyranosyl-3β,6α,16β,24(*S*),25-pentahydroxycycloartane (**65**), 3-*O*-[α-L-arabinopyranosyl-(1→2)-β-D-xylopyranosyl]-3β,6α,16β,24α-tetrahydroxy-20(*R*),25-epoxycycloartane (**66**), 6-*O*-β-D-glucopyranosyl-3β,6α,16β,24α-tetrahydroxy-20(*R*),25-epoxy cycloartane (**67**), and 6-*O*-β-D-xylopyranosyl-3β,6α,16β,24α-tetrahydroxy-20(*R*),25-epoxycycloartane (**68**), were isolated from *Astragalus aureus* Willd (Sect. Adiaspastus), along with ten known cycloartane-type glycosides, namely, 3-*O*-[α-L-rhamnopyranosyl-(1→2)-*O*-α-L-arabinopyranosyl-(1→2)-*O*-β-D-xylopyranosyl]-6-*O*-β-D-glucopyranosyl-3β,6α,16β,24(*S*),25-pentahydroxycycloartane (Horo et al. 2010), oleifolioside B (Özipek et al. 2005), cyclocanthoside G (Isaev et al. 1992), cyclocanthoside E (Isaev et al. 1992), 3-*O*-[α-L-rhamnopyranosyl-(1→2)-*O*-α-L-arabinopyranosyl-(1→2)-*O*-β-D-xylopyranosyl]-6-*O*-β-D-glucopyranosyl-3β,6α,16β,24α-tetrahydroxy-20(*R*),25-epoxycycloartane (Horo et al. 2010), 3-*O*-[α-L-arabinopyranosyl-(1→2)-*O*-β-D-xylopyranosyl]-6-*O*-β-D-glucopyranosyl-3β,6α,16β,24α-tetrahydroxy-20(*R*),25-epoxycycloartane (Horo et al. 2010), cyclocanthoside F (Agzamova and Isaev 1999), cyclocephaloside I (Bedir et al. 1998a), cyclotrisectoside (Sukhina et al. 2007), and macrophyllosaponin B (Çalış et al. 1996). The authors stated that aminoglycosides of cyclocanthogenin (**65**) and cyclocephalogenin (**67**, **68**) were encountered for the first time. In this study, a number of cancer cell lines were used for measuring cytotoxic activities of all compounds, among which only compound **68** showed moderate cytotoxic activity against human breast cancer (MCF7) at 45 μM concentration (Gülcemal et al. 2011).

Compound **61**; $C_{51}H_{86}O_{21}$; $[\alpha]^{25}_{D}$ +37.2° (*c* 0.1, MeOH); ν^{KBr}_{max} cm^{-1}: 3474 (>OH), 3035 (cyclopropane ring), 2953 (>CH), 1255 and 1068 (C–O–C); δ_C (CD_3OD); HR-MALDITOF-MS *m/z* $[M + Na]^+$ 1057.5563 (calcd. for $C_{51}H_{86}O_{21}Na$, 1057.5559). Compound **62**; $C_{40}H_{68}O_{13}$; $[\alpha]^{25}_{D}$ +29.2° (*c* 0.1, MeOH); ν^{KBr}_{max} cm^{-1}: 3487 (>OH), 3043 (cyclopropane ring), 2950 (>CH), 1269 and 1059 (C–O–C); δ_C (CD_3OD); HR-MALDITOF-MS *m/z* $[M + Na]^+$ 779.4561 (calcd. for $C_{40}H_{68}O_{13}Na$, 779.4558). Compound **63**; $C_{46}H_{78}O_{18}$; $[\alpha]^{25}_{D}$ +35.6° (*c* 0.1, MeOH); ν^{KBr}_{max} cm^{-1}: 3482 (>OH), 3040 (cyclopropane ring), 2945 (>CH), 1259 and 1051 (C–O–C); δ_C (CD_3OD); HR-MALDITOF-MS *m/z* $[M + Na]^+$ 941.5090 (calcd. for $C_{46}H_{78}O_{18}Na$, 941.5086). Compound **64**; $C_{47}H_{80}O_{19}$; $[\alpha]^{25}_{D}$ +32.5° (*c* 0.1, MeOH); ν^{KBr}_{max} cm^{-1}: 3477 (>OH), 3038 (cyclopropane ring), 2934 (>CH), 1263 and 1054 (C–O–C); δ_C (CD_3OD); HR-MALDITOF-MS *m/z* $[M + Na]^+$ 971.5197 (calcd. for $C_{47}H_{80}O_{19}Na$, 971.5192). Compound **65**; $C_{36}H_{62}O_{10}$; $[\alpha]^{25}_{D}$ +27.8° (*c* 0.1, MeOH); ν^{KBr}_{max} cm^{-1}: 3485 (>OH), 3047 (cyclopropane ring), 2930 (>CH), 1254 and 1061 (C–O–C); δ_C (CD_3OD); HR-MALDITOF-MS *m/z* $[M + Na]^+$ 677.4246 (calcd. for $C_{36}H_{62}O_{10}Na$, 677.4241). Compound **66**; $C_{40}H_{66}O_{13}$; $[\alpha]^{25}_{D}$ +10.5° (*c* 0.1, MeOH); ν^{KBr}_{max} cm^{-1}: 3488 (>OH), 3053 (cyclopropane ring), 2939 (>CH), 1274 and 1050 (C–O–C); δ_C (CD_3OD); HR-MALDITOF-MS *m/z* $[M + Na]^+$ 777.4405 (calcd. for $C_{40}H_{66}O_{13}Na$, 777.4401). Compound **67**; $C_{36}H_{60}O_{10}$; $[\alpha]^{25}_{D}$ +15.2° (*c* 0.1, MeOH); ν^{KBr}_{max} cm^{-1}: 3479 (>OH), 3048 (cyclopropane ring), 2947 (>CH), 1257 and 1065 (C–O–C); δ_C (CD_3OD); HR-MALDITOF-MS *m/z* $[M + Na]^+$ 675.4089 (calcd. for $C_{36}H_{60}O_{10}Na$, 675.4084). Compound **68**; $C_{35}H_{58}O_9$; $[\alpha]^{25}_{D}$ +8.2° (*c* 0.1, MeOH); ν^{KBr}_{max} cm^{-1}: 3471 (>OH), 3050 (cyclopropane ring), 2943 (>CH), 1268 and 1057 (C–O–C); δ_C (CD_3OD); HR-MALDITOF-MS *m/z* $[M + Na]^+$ 645.3983 (calcd. for $C_{35}H_{58}O_9Na$, 645.3979). The structures and ^{13}C NMR data of compounds **61**–**68** are given below.



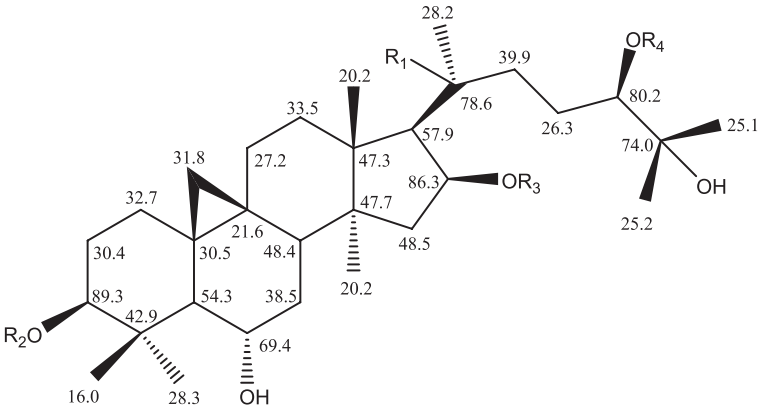
The study of the chemical constituents of *Astragalus pycnocephalus* var. *pycnocephalus* (Sect. Rhacophorus) has resulted in the isolation of four known cycloartane-type glycosides. Their structures were established as trojanoside H (Bedir et al. 1999b), astragaloside IV (Kitagawa et al. 1983a), astragaloside VIII (Kitagawa et al. 1983c), and astrasieversianin X (Gan et al. 1986) by the extensive use of 1D- and 2D-NMR experiments along with HRMS analyses and by comparison with literature values. In this report, the inhibitory activities of all compounds were tested against the metalloenzyme carbonic anhydrase (CA, EC 4.2.1.1). All compounds showed strong inhibition against α-glucosidase, and they exhibited mild activity against *β*-glucosidase (Koz et al. 2011).

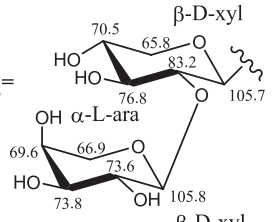
Studies on *Astragalus stereocalyx* Bornm (Sect. Stereocalyx) resulted the isolation of six new cycloartane-type triterpene glycosides. Structures of the newcompounds were determined as 3-*O*-[α-L-arabinopyranosyl-(1→2)-β-D-xylopyranosyl]-16-*O*-β-D-glucopyranosyl-3β,6α,16β,20(*S*),24(*R*), 25-hexahydroxycycloartane (**69**), 3-*O*-[α-L-

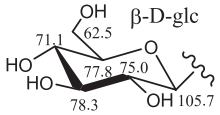
arabinopyranosyl-(1→2)-β-D-xylopyranosyl]-3β,6α,16β,20(*S*),24(*R*),25-hexahydroxycycloartane (**70**), 3-*O*-[α-L-arabinopyranosyl-(1→2)-β-D-glucopyranosyl]-3β,6α,16β,20(*S*),24(*R*),25-hexahydroxycycloartane(**71**),3-*O*-[α-L-arabinopyranosyl-(1→2)-β-D-glucopyranosyl]-24-*O*-β-D-glucopyranosyl-3β,6α,16β,24(*R*),25-pentahydroxycycloartane (**72**), 3-*O*-[α-L-arabinopyranosyl-(1→2)-β-D-glucopyranosyl]-16-*O*-β-D-glucopyranosyl-3β,6α,16β,24(*R*),25-pentahydroxycycloartane(**73**),and3-*O*-{α-L-rhamnopyranosyl-(1→4)-[α-L-arabinopyranosyl-(1→2)]-β-D-glucopyranosyl}-3β,6α,16β,24(*R*),25-pentahydroxycycloartane (**74**). Additionally, six known cycloartane-type glycosides, askendoside C (Isaev et al. 1983b), askendoside F (Isaev 1995), askendoside G (Isaev 1996), 3-*O*-β-D-glucopyranosyl-16-*O*-β-D-glucopyranosyl-3β,6α,16β,24(R),25-pentahydroxycycloartane (Verotta et al. 2002), elongatoside (Çalış et al. 2008a), and trojanoside H (Bedir et al. 1999b), were isolated. The authors stressed that compounds **69**–**71** were based on an aglycone possessing an unusual hydroxyl group at position 20. In this research, some cell lines including Hela (human cervical cancer), HT-29 (human colon cancer), U937 (human leukemia), and H446 (human lung cancer) were utilized for testing antiproliferative activity of the obtained compounds. A concentration range between 1 and 50 μM was chosen for testing, where only a few compounds showed weak activities. Hela was the only susceptible cell line to the compounds. While the compound 3-*O*-β-D-glucopyranosyl-16-*O*-β-D-glucopyranosyl-3β,6α,16β,24(R),25-pentahydroxycycloartane displayed an IC_{50} value of 10 μM against Hela cells, compounds **74**, askendoside C, and askendoside G exhibited IC_{50} values of 29.9, 31.5, and 24.4 μM, respectively (Yalçın et al. 2012).

Compound **69**; $C_{46}H_{78}O_{19}$; $[\alpha]^{25}_{D}$ +27.2° (*c* 0.1, MeOH); ν^{KBr}_{max} cm^{-1}: 3480 (>OH), 3039 (cyclopropane ring), 2961 (>CH), 1250 and 1072 (C–O–C); δ_C (CD_3OD); HR-MALDITOF-MS *m/z* $[M + Na]^+$ 957.5038 (calcd. for $C_{46}H_{78}O_{19}Na$, 957.5035). Compound **70**; $C_{40}H_{68}O_{14}$; $[\alpha]^{25}_{D}$ +25.2° (*c* 0.1, MeOH); ν^{KBr}_{max} cm^{-1}: 3475 (>OH), 3048 (cyclopropane ring), 2955 (>CH), 1260 and 1057 (C–O–C); δ_C (CD_3OD); HR-MALDITOF-MS *m/z* $[M + Na]^+$ 795.4509 (calcd. for $C_{40}H_{68}O_{14}Na$,

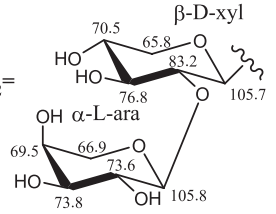
795.4507). Compound **71**; $C_{41}H_{70}O_{15}$; $[\alpha]^{25}_{D}$ +28.6° (*c* 0.1, MeOH); ν^{KBr}_{max} cm^{-1}: 3477 (>OH), 3036 (cyclopropane ring), 2948 (>CH), 1252 and 1067 (C–O–C); δ_C (CD_3OD); HR-MALDITOF-MS *m/z* $[M + Na]^+$ 825.4615 (calcd. for $C_{41}H_{70}O_{15}Na$, 825.4612). Compound **72**; $C_{47}H_{80}O_{19}$; $[\alpha]^{25}_{D}$ +12.7° (*c* 0.1, MeOH); ν^{KBr}_{max} cm^{-1}: 3482 (>OH), 3043 (cyclopropane ring), 2952 (>CH), 1263 and 1060 (C–O–C); δ_C (CD_3OD); HR-MALDITOF-MS *m/z* $[M + Na]^+$ 971.5196 (calcd. for $C_{47}H_{80}O_{19}Na$, 971.5192). Compound **73**; $C_{47}H_{80}O_{19}$; $[\alpha]^{25}_{D}$ +10.8° (*c* 0.1, MeOH); ν^{KBr}_{max} cm^{-1}: 3488 (>OH), 3045 (cyclopropane ring), 2940 (>CH), 1258 and 1064 (C–O–C); δ_C (CD_3OD); HR-MALDITOF-MS *m/z* $[M + Na]^+$ 971.5194 (calcd. for $C_{47}H_{80}O_{19}Na$, 971.5192). Compound **74**; $C_{47}H_{80}O_{18}$; $[\alpha]^{25}_{D}$ +14.5° (*c* 0.1, MeOH); ν^{KBr}_{max} cm^{-1}: 3470 (>OH), 3051 (cyclopropane ring), 2943 (>CH), 1268 and 1054 (C–O–C); δ_C (CD_3OD); HR-MALDITOF-MS *m/z* $[M + Na]^+$ 955.5246 (calcd. for $C_{47}H_{80}O_{18}Na$, 955.5242). The structures and ^{13}C NMR data of **69–74** are shown below.



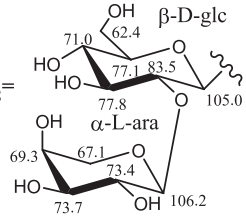
69 R_1= OH R_2= 

R_3= 

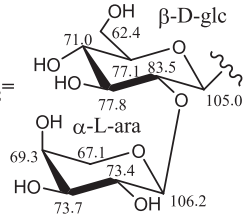
R_4= H

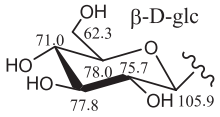
70 R_1= OH R_2= 

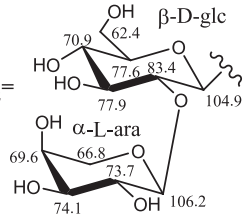
R_3=R_4=H

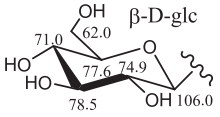
71 R_1= OH R_2= 

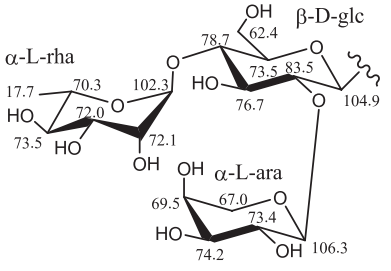
R_3=R_4=H

72 R_1=R_3=H R_2= 

R_4= 

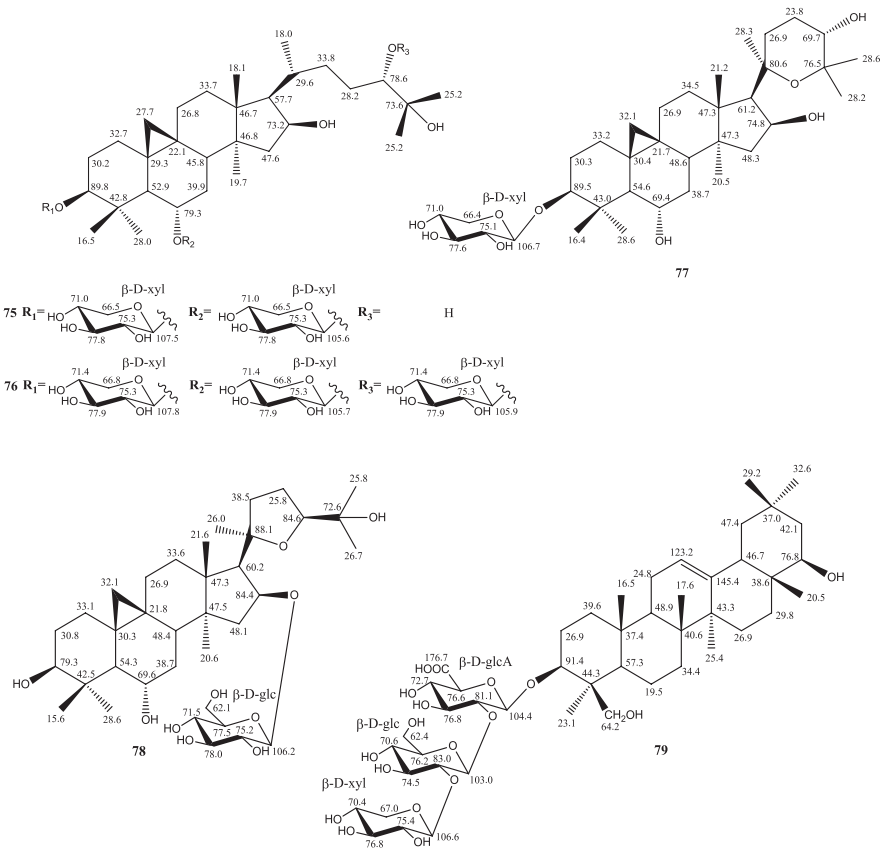
73 R_1=R_4=H R_2= 

R_3= 

74 R_1=R_3=R_4=H R_2= 

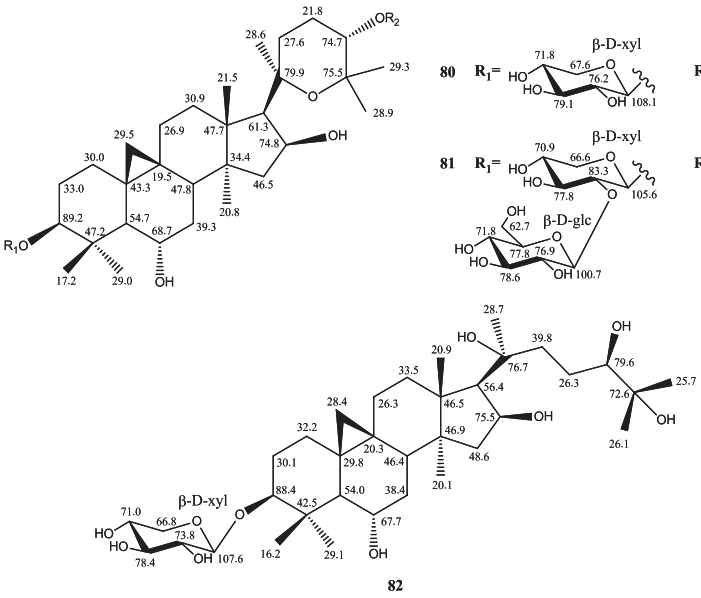
In 2012, another phytochemical study was performed on *Astragalus hareftae* (Sect. Acanthophace), which was resulted in isolation of four new cycloartanes (hareftosides A–D) and a new oleanane-type triterpenoid (hareftoside E) along with 11 known compounds. Structures of the new compounds were established as 3,6-di-*O*-*β*-D-xylopyranosyl-3*β*,6*α*,16*β*,24(*S*),25-pentahydroxycycloartane (**75**), 3,6,24-tri-*O*-*β*-D-xylopyranosyl-3*β*,6*α*,16*β*,24(*S*),25-pentahydroxycycloartane (**76**), 3-*O*-*β*-D-xylopyranosyl-3*β*,6*α*,16*β*,25-tetra-hydroxy-20(*R*),25(*S*)-epoxycycloartane (**77**), 16-*O*-*β*-D-glucopyranosyl-3*β*,6*α*,16*β*,25-tetrahydroxy-20(*R*),24(*S*)-epoxycycloartane (**78**), and 3-*O*-[*β*-D-xylopyranosyl-(1→2)-*O*-*β*-D-glucopyranosyl-(1→2)-*O*-*β*-D-glucuronopyranosyl] soyasapogenol B (**79**) by the extensive use of 1D- and 2D-NMR experiments along with ESI-MS and HR-MS analyses. Known compounds were identified as cyclocanthoside E (Isaev et al. 1992), macrophyllosaponin B (Çalış et al. 1996), 3-*O*-*β*-D-xylopyranosyl-6,25-di-*O*-*β*-D-glucopyranosyl-3*β*,6*α*,16*β*,24(*S*),25-pentahydroxycycloartane (Gülcemal et al. 2011), oleifolioside B (Özipek et al. 2005), cyclocephaloside I (Bedir et al. 1998a), astrasieversianin X (Gan et al. 1986), trojanoside B (Bedir et al. 1999a), cycloastragenol (Kitagawa et al. 1983a), astragaloside IV (Kitagawa et al. 1983a), brachyoside B (Bedir et al. 1998b), and cyclodissectoside (Sukhina et al. 2007) and four known oleanane-type triterpene glycosides, azukisaponin V (Kitagawa et al. 1983e), dehydroazukisaponin V (Mohamed et al. 1995), wistariasaponin D (Konoshima et al. 1991), and astragaloside VIII (Kitagawa et al. 1983c), respectively (Horo et al. 2012).

Compound **75**; $C_{40}H_{68}O_{13}$; $[\alpha]^{25}_{D}$ +27.3° (*c* 0.1, MeOH); ν^{KBr}_{max} cm^{-1}: 3460 (>OH), 3035 (cyclopropane ring), 2945 (>CH), 1260 and 1058 (C–O–C); δ_C (CD_3OD); HR-MALDITOF-MS *m/z* $[M + Na]^+$ 779.4564 (calcd. for $C_{40}H_{68}O_{13}Na$, 779.4558). Compound **76**; $C_{45}H_{76}O_{17}$; $[\alpha]^{25}_{D}$ +24.3° (*c* 0.1, MeOH); ν^{KBr}_{max} cm^{-1}: 3470 (>OH), 3030 (cyclopropane ring), 2950 (>CH), 1264 and 1060 (C–O–C); δ_C (CD_3OD); HR-MALDITOF-MS *m/z* $[M + Na]^+$ 911.4984 (calcd. for $C_{45}H_{76}O_{17}Na$, 911.4980). Compound **77**; $C_{35}H_{58}O_9$; $[\alpha]^{25}_{D}$ +19.8° (*c* 0.1, MeOH); ν^{KBr}_{max} cm^{-1}: 3480 (>OH), 3030 (cyclopropane ring), 2945 (>CH), 1260 and 1055 (C–O–C); δ_C (CD_3OD); HR-MALDITOF-MS *m/z* $[M + Na]^+$ 645.3982 (calcd. for $C_{35}H_{58}O_9Na$, 645.3979). Compound **78**; $C_{36}H_{60}O_{10}$; $[\alpha]^{25}_{D}$ +20.8° (*c* 0.1, MeOH); ν^{KBr}_{max} cm^{-1}: 3460 (>OH), 3040 (cyclopropane ring), 2935 (>CH), 1250 and 1050 (C–O–C); δ_C (CD_3OD); HR-MALDITOF-MS *m/z* $[M + Na]^+$ 675.4086 (calcd. for $C_{36}H_{60}O_{10}Na$, 675.4084). Compound **79**; $C_{47}H_{76}O_{18}$; $[\alpha]^{25}_{D}$ +12.1° (*c* 0.1, MeOH); ν^{KBr}_{max} cm^{-1}: 3448 (>OH), 2934 (>CH), 1658 (C=C); δ_C (CD_3OD); HR-MALDITOF-MS *m/z* $[M + Na]^+$ 951.4932 (calcd. for $C_{47}H_{76}O_{18}Na$, 951.4929). The structures and ^{13}C NMR data of compounds **75–79** are provided below.



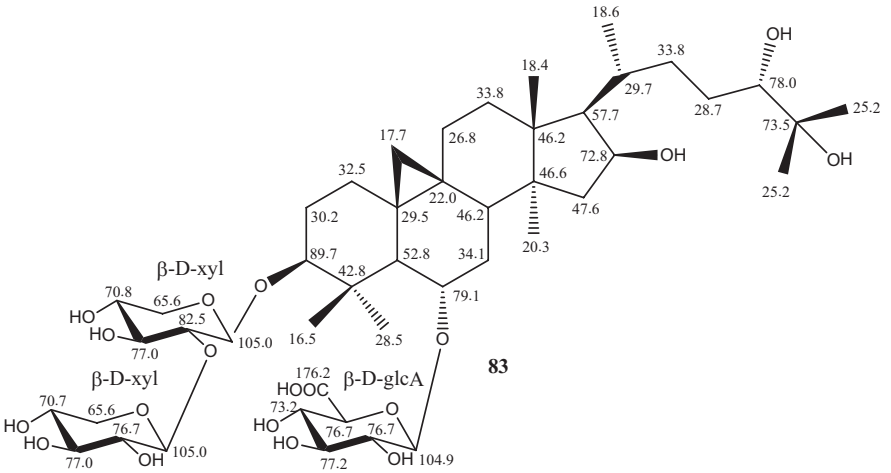
Another study conducted in Karabey et al. (2012) reported on the isolation and structural elucidation of three new cycloartane-type triterpene glycosides from the roots of *Astragalus schottianus* Boiss. (Sect. Rhacophorus). By means of spectroscopic methods (IR, 1D- and 2D-NMR, HR-ESI-MS), their structures were established as 20(*R*),25-epoxy-3-*O*-*β*-D-xylopyranosyl-24-*O*-*β*-D-glucopyranosyl-3*β*,6*α*,16*β*,24*α*-tetrahydroxycycloartane (**80**), 20(*R*),25-epoxy-3-*O*-[*β*-D-glucopyranosyl(1→2)]-*β*-D-xylopyranosyl-24-*O*-*β*-D-glucopyranosyl-3*β*,6*α*,16*β*,24*α*-tetrahydroxycycloartane (**81**), and 3-*O*-*β*-D-xylopyranosyl-3*β*,6*α*,16*β*,20(*S*),24(*S*),25-hexahydroxycycloartane (**82**) (Karabey et al. 2012). According to the authors, compound **82** represents the second entry of the series of cycloartane-type compound possessing a 20-OH functional group in *Astragalus* genus. Moreover, in nature, only five compounds, obtained from *Oxytropis bicolor* and *Astragalus stereocalyx*, were reported to have such a substitution at C-20 and 3*β*,16*β*,20(*S*),24(*S*),25-pentahydroxycycloartane framework (Rong-Qi et al. 1991; Sun and Chen 1997; Yalçın et al. 2012).

Compound **80**; $C_{41}H_{68}O_{14}$; δ_C (C_5D_5N); HR-ESI-MS *m/z* $[M + Na]^+$ 807.46042 (calcd. for $C_{41}H_{68}O_{14}Na$, 807.45068). Compound **81**; $C_{47}H_{78}O_{20}$; δ_C (C_5D_5N); HR-ESI-MS *m/z* $[M–Cl]^-$ 981.48275 (calcd. for $C_{47}H_{78}O_{20}Cl$). Compound **82**; $C_{35}H_{60}O_{10}$; δ_C (C_5D_5N); HR-ESI-MS *m/z* $[M–Cl]^-$ 675.38677 (calcd. for $C_{35}H_{60}O_{10}Cl$, 675.38750). The structures and carbon chemical shifts of compounds **80**, **81**, and **82** are presented below.



Phytochemical investigation of *Astragalus erinaceus* (Sect. Rhacophorus) was resulted in isolation of a new cycloartane-type saponin, 3-*O*-[*β*-D-xylopyranosyl-(1→2)-*β*-D-xylopyranosyl]-6-*O*-*β*-D-glucuronopyranosyl-3*β*,6*α*,16*β*,24(*S*),25-pentahydroxycycloartane (**83**). Additionally, five known saponins, cyclodissectoside (Sukhina et al. 2007), cycloastragenol (Kitagawa et al. 1983a), 6-*O*-*β*-D-glucopyranosyl-3*β*,6*α*,16*β*,24(*S*),25-pentahydroxycycloartane (Gülcemal et al. 2011), oleifolioside B (Özipek et al. 2005), and 3,6-di-*O*-*β*-D-xylopyranosyl-3*β*,6*α*,16*β*,24(*S*),25-pentahydroxycycloartane (Gülcemal et al. 2011), were also identified. The authors stated that the glucuronic acid moiety was an unusual finding, and compound **83** was representing second example of such framework in cycloartane chemistry (Savran et al. 2012).

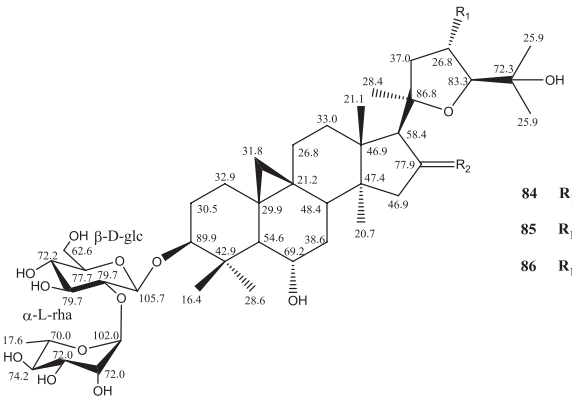
Compound **83**; $C_{46}H_{77}O_{19}$; $[\alpha]^{25}_D$ +30.8° (*c* 0.1, MeOH); δ_C (CD_3OD); HR-ESI-MS *m/z* $[M + Na]^+$ 955.4882 (calcd. for $C_{46}H_{77}O_{19}Na$, 955.4879). The structure and ^{13}C chemical shifts are provided below for **83**.



Another phytochemical study was performed on *Astragalus angustifolius* (Sect. Melanocercis) which was resulted in isolation of six cycloartane- (**84**–**89**) and four oleanane-type triterpenoids (**90**–**93**) together with five known triterpene glycosides. Structures of the compounds were established as 3-*O*-[*α*-L-rhamnopyranosyl-(1→2)-*β*-D-glucopyranosyl]-16-*O*-hydroxyacetoxy-3*β*,6*α*,16*β*,25 tetrahydroxy-20(*R*),24(*S*)-epoxycycloartane (**84**), 3-*O*-[*α*-L-rhamnopyranosyl-(1→2)-*β*-D-glucopyranosyl]-16-*O*-hydroxyacetoxy-3*β*,6*α*,16*β*,23*α*,25-pentahydroxy-20(*R*),24(*S*)-epoxycycloartane (**85**), 3-*O*-[α-L-rhamnopyranosyl-(1→2)-*β*-D-glucopyranosyl]-3*β*,6*α*,25-trihydroxy-20(*R*),24(*S*)-epoxycycloartane-16-one (**86**), 3-*O*-[*α*-L-rhamnopyranosyl-(1→2)-*β*-D-glucopyranosyl]-3*β*,6*α*,16*β*,25-tetrahydroxy-20(*R*),24(*R*)-epoxycycloartane (**87**), 3-*O*-*β*-D-xylopyranosyl-6-*O*-*α*-L-rhamnopyranosyl-3*β*,6*α*,16*β*,25-tetrahydroxy-20(*R*),24(*R*)-epoxycycloartane (**88**), 6-*O*-*α*-L-rhamnopyranosyl-3*β*,6*α*,16*β*,25-tetrahydroxy-20(*R*),24(*R*)-epoxy cycloartane (**89**), 3-*O*-[*α*-L-rhamnopyranosyl-(1→2)-*β*-D-xylopyranosyl-(1→2)-*β*-D-glucuronopyranosyl]-3*β*,21*β*,22*α*,24,29-pentahydroxyolean-12-ene (**90**), 3-*O*-[*α*-L-rhamnopyranosyl-(1→2)-*β*-D-xylopyranosyl-(1→2)-*β*-D-glucuronopyranosyl]-3*β*,22*β*,24-trihydroxyolean-12-en-29-oic acid (**91**), 3-*O*-[*α*-L-rhamnopyranosyl-(1→2)-*β*-D-xylopyranosyl-(1→2)-*β*-D-glucuronopyranosyl]-22-*O*-*α*-L-arabinopyranosyl-3*β*,22*β*,24-trihydroxyolean-12-ene (**92**), and 29-*O*-*β*-D-glucopyranosyl-3*β*,22*β*,24,29-tetrahydroxyolean-12-ene (**93**) by the extensive use of 1D- and 2D-NMR experiments along with ESIMS and HRMS analysis. Known

compounds were identified as 25-*O*-glucopyranosylcycloastragenol (Bedir et al. 1999a), cycloaraloside D (Isaev 1991), 3-*O*-[α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyl]-25-*O*-β-D-glucopyranosyl-20(*R*),24(*S*)-epoxy-3β,6α,16β,25-tetrahydroxycycloartane (Polat et al. 2010), astrojanoside A (Bedir et al. 1999a), and astragaloside VIII (Kitagawa et al. 1983c). The authors reported that compounds **84–86** were glycosides of cycloastragenol, while compounds **87–89** had the C-24 epimer of cycloastragenol as aglycone, reported for the first time in nature. In this study, antiproliferative activity in Hela, H-446, HT-29, and U937 cell lines was tested for all compounds. Compound **91** was the only compound that displayed a weak activity against both Hela and HT-29 cell lines with IC_{50} values of 36 and 50 μM, respectively (Gülcemal et al. 2012).

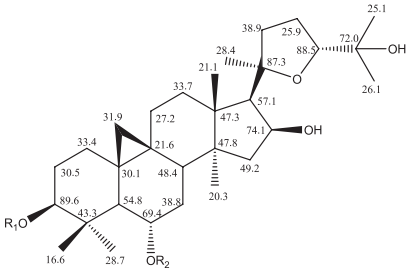
Compound **84**; $C_{44}H_{72}O_{16}$; $[\alpha]^{25}_D$ +19.01° (*c* 0.1, MeOH); ν^{KBr}_{max} cm^{-1}: 3470, 3040, 2950, 1260 and 1058; δ_C (CD_3OD); HR-MALDITOF-MS *m/z* $[M + Na]^+$ 879.4722 (calcd. for $C_{44}H_{72}O_{16}Na$, 879.4718). Compound **85**; $C_{44}H_{72}O_{17}$; $[\alpha]^{25}_D$ +41.4° (*c* 0.1, MeOH); ν^{KBr}_{max} cm^{-1}: 3480, 3035, 2945, 1260 and 1055; δ_C (CD_3OD); HR-MALDITOF-MS *m/z* $[M + Na]^+$ 895.4669 (calcd. for $C_{44}H_{72}O_{17}Na$, 895.4667). Compound **86**; $C_{42}H_{68}O_{14}$; $[\alpha]^{25}_D$ −23.1° (*c* 0.1, MeOH); ν^{KBr}_{max} cm^{-1}: 3475, 3030, 2948, 1740, 1258 and 1050; δ_C (CD_3OD); HR-MALDITOF-MS *m/z* $[M + Na]^+$ 819.4512 (calcd. for $C_{42}H_{68}O_{14}Na$, 819.4507). Compound **87**; $C_{42}H_{70}O_{14}$; $[\alpha]^{25}_D$ −5.21° (*c* 0.1, MeOH); ν^{KBr}_{max} cm^{-1}: 3460, 3038, 2935, 1250 and 1050; δ_C (CD_3OD); HR-MALDITOF-MS *m/z* $[M + Na]^+$ 821.4666 (calcd. for $C_{42}H_{70}O_{14}Na$, 821.4663). Compound **88**; $C_{41}H_{68}O_{13}$; $[\alpha]^{25}_D$ +15.1° (*c* 0.1, MeOH); ν^{KBr}_{max} cm^{-1}: 3460, 3035, 2940, 1240 and 1050; δ_C (CD_3OD); HR-MALDITOF-MS *m/z* $[M + Na]^+$ 791.4561 (calcd. for $C_{41}H_{68}O_{13}Na$, 791.4558). Compound **89**; $C_{36}H_{60}O_9$; $[\alpha]^{25}_D$ −8.09° (*c* 0.1, MeOH); ν^{KBr}_{max} cm^{-1}: 3450, 3042, 2938, 1250 and 1052; δ_C (CD_3OD); HR-MALDITOF-MS *m/z* $[M + Na]^+$ 659.4139 (calcd. for $C_{36}H_{60}O_9Na$, 659.4135). Compound **90**; $C_{47}H_{76}O_{19}$; $[\alpha]^{25}_D$ −10.9° (*c* 0.1, MeOH); ν^{KBr}_{max} cm^{-1}: 3455, 2945, 1666, 1252 and 1052; δ_C (CD_3OD); HR-MALDITOF-MS *m/z* $[M + Na]^+$ 967.4881 (calcd. for $C_{47}H_{76}O_{19}Na$, 967.4879). Compound **91**; $C_{47}H_{74}O_{19}$; $[\alpha]^{25}_D$ +7.12° (*c* 0.1, MeOH); ν^{KBr}_{max} cm^{-1}: 3451, 2937, 1735, 1658, 1248 and 1048; δ_C (CD_3OD); HR-MALDITOF-MS *m/z* $[M + Na]^+$ 965.4726 (calcd. for $C_{47}H_{74}O_{19}Na$, 965.4722). Compound **92**; $C_{52}H_{84}O_{21}$; $[\alpha]^{25}_D$ −8.54° (*c* 0.1, MeOH); ν^{KBr}_{max} cm^{-1}: 3446, 2930, 1730, 1648, 1245 and 1052; δ_C (CD_3OD); HR-MALDITOF-MS *m/z* $[M + Na]^+$ 1067.5406 (calcd. for $C_{52}H_{84}O_{21}Na$, 1067.5403). Compound **93**; $C_{36}H_{60}O_9$; $[\alpha]^{25}_D$ +27.7° (*c* 0.1, MeOH); ν^{KBr}_{max} cm^{-1}: 3440, 2933, 1645, 1240 and 1044; δ_C (CD_3OD); HR-MALDITOF-MS *m/z* $[M + Na]^+$ 659.4139 (calcd. for $C_{36}H_{60}O_9Na$, 659.4135). The structures and carbon chemical shifts of compounds **84–93** are shown below.

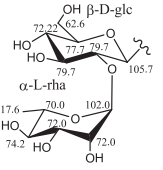


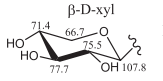
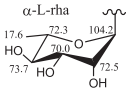
84 R_1= H R_2= β-$OCOCH_2OH$ α-H

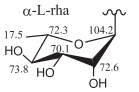
85 R_1= OH R_2= β-$OCOCH_2OH$ α-H

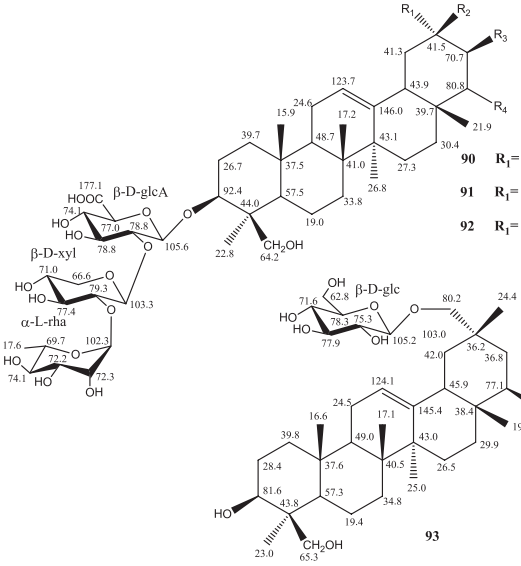
86 R_1= H R_2= O



87 R_1=  R_2= H

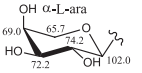
88 R_1=  R_2= 

89 R_1= H R_2= 



90 R_1= CH_2OH R_2= CH_3 R_3= OH R_4= α-OH

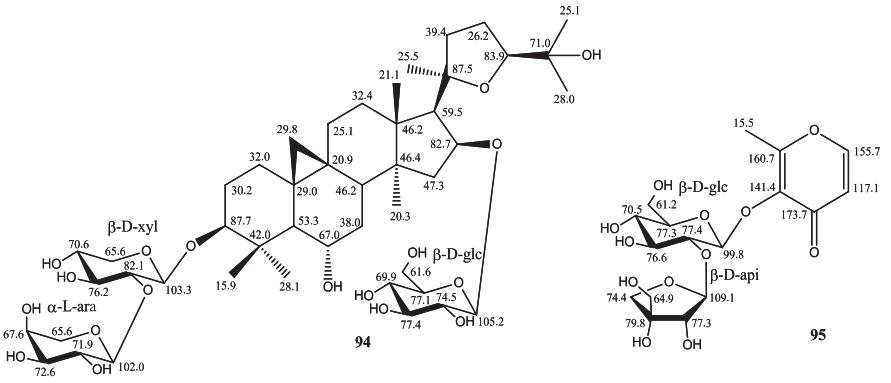
91 R_1= CH_3 R_2= COOH R_3= H R_4= β-OH

92 R_1= CH_3 R_2= CH_3 R_3= H R_4= β-O- 

93

Another phytochemical investigation was published in 2013, and a new cycloartane-type glycoside, (20*R*,24*S*)-3-*O*-[*α*-L-arabinopyranosyl-(1→2)-*β*-D-xylopyranosyl]-20,24-epoxy-16-*O*-*β*-D-glucopyranosyl-3*β*,6*α*,16*β*,25-tetrahydroxycycloartane (**94**), and a new glycoside, 3-*O*-[*β*-D-apiofuranosyl-(1→2)-*β*-D-glucopyranosyl]maltol (**95**), were isolated from *Astragalus halicacabus* (Sect. Halicacabus) together with seven known cycloartane-type glycosides, i.e., cyclocanthoside D (Isaev et al. 1992); askendosides D (Isaev et al. 1983a), F (Isaev 1995), and G (Isaev 1996); cyclosieversioside G (Svechnikova et al. 1983); cyclostipuloside A (Karimov et al. 1998); and elongatoside (Çalış et al. 2008a), and a known maltol glucoside, 3-*O*-β-D-glucopyranosylmaltol (Sala et al. 2001). The authors commented that maltol glycoside was reported for the first time in the *Astragalus* genus and even in the Fabaceae family (Djimtombaye et al. 2013).

Compound **94**; $C_{46}H_{76}O_{18}$; $[\alpha]^{25}_{D}$ +41.4° (*c* 0.1, MeOH); ν^{KBr}_{max} cm^{-1}: 3480 (>OH), 3035 (cyclopropane ring), 2945 (>CH), 1260 and 1055 (C–O–C); δ_C (CD_3OD); HR-ESI-MS *m/z* $[M + Na]^+$ 939.4991 (calcd. for $C_{46}H_{76}O_{18}Na$, 939.4990). Compound **95**; $C_{17}H_{24}O_{12}$; $[\alpha]^{25}_{D}$ −78.9° (*c* 0.1, MeOH); ν^{KBr}_{max} cm^{-1}: 3376, 1650; δ_C (CD_3OD); HR-ESI-MS *m/z* $[M + Na]^+$ 443.1199 (calcd. for $C_{17}H_{24}O_{12}Na$, 443.1182). The structures and ^{13}C chemical shifts are given below for **94** and **95**.

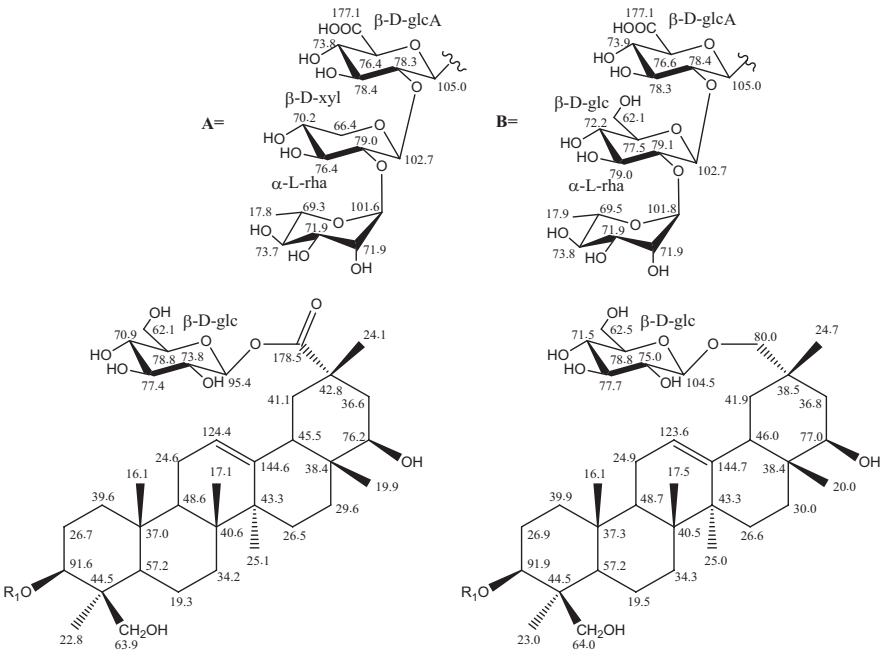


From *Astragalus tauricolus* (Sect. Malacothrix), on the basis of the results of the online screening by HPLC–ESIMS^n, ten new oleanane-type triterpene glycosides were isolated and established by spectral methods, 3-*O*-[*α*-L-rhamnopyranosyl-(1→2)-*β*-D-xylopyranosyl-(1→2)-*β*-D-glucuronopyranosyl]-29-*O*-*β*-D-glucopyranosyl-3*β*,22*β*,24-trihydroxyolean-12-en-29-oic acid (**96**), 3-*O*-[*α*-L-rhamnopyranosyl-(1→2)-*β*-D-glucopyranosyl-(1→2)-*β*-D-glucuronopyranosyl]-29-*O*-*β*-D-glucopyranosyl-3*β*,22*β*,24,29-tetrahydroxyolean-12-ene (**97**), 3-*O*-[*α*-L-rhamnopyranosyl-(1→2)-*β*-D-xylopyranosyl-(1→2)-*β*-D-glucuronopyranosyl]-21-*O*-*α*-L-rhamnopyranosyl-3*β*,21*β*,22*α*,24-tetrahydroxyolean-12-ene (**98**), 3-*O*-[*α*-L-rhamnopyranosyl-(1→2)-*β*-D-glucopyranosyl-(1→2)-*β*-D-glucuronopyranosyl]-21-*O*-*α*-L-rhamnopyranosyl-3*β*,21*β*,22*α*,24-tetrahydroxyolean-12-ene (**99**), 3-*O*-[*α*-L-rhamnopyranosyl-(1→2)-*β*-D-glucopyranosyl-(1→2)-*β*-D-glucuronopyranosyl]-29-

O-*β*-D-glucopyranosyl-3*β*,22*β*,24,-trihydroxyolean-12-en-29-oic acid (**100**), 3-*O*-[*α*-L-rhamnopyranosyl-(1→2)-*β*-D-xylopyranosyl-(1→2)-*β*-D-glucuronopyranosyl]-22-*O*-*α*-L-rhamnopyranosyl-3*β*,22*β*,24-trihydroxyolean-12-ene (**101**), 3-*O*-[*α*-L-rhamnopyranosyl-(1→2)-*β*-D-glucopyranosyl-(1→2)-*β*-D-glucuronopyranosyl]-3*β*,24-dihydroxyolean-12-ene-22-oxo-29-oic acid (**102**), 3-*O*-[*α*-L-rhamnopyranosyl-(1→2)-*β*-D-glucopyranosyl-(1→2)-*β*-D-glucuronopyranosyl]-3*β*,21*β*,22*α*,24,29-pentahydroxyolean-12-ene (**103**), 3-*O*-[*β*-D-glucopyranosyl-(1→2)-*β*-D-glucuronopyranosyl]-29-*O*-*β*-D-glucopyranosyl-3*β*,22*β*,24,-trihydroxyolean-12-en-29-oic acid (**104**), and 3-*O*-[*β*-D-xylopyranosyl-(1→2)-*β*-D-glucuronopyranosyl]-29-*O*-*β*-D-glucopyranosyl-3*β*,22*β*,24,-trihydroxyolean-12-en-29-oic acid (**105**), along with 12 known oleanane-type glycosides, namely, astrojanoside A (Bedir et al. 1999a), melilotus-saponin O2 (Hirakawa et al. 2000), 3-*O*-[*α*-L-rhamnopyranosyl-(1→2)-*β*-D-xylopyranosyl-(1→2)-*β*-D-glucuronopyranosyl]-3*β*,21*β*,22*α*,24,29-pentahydroxyolean-12-ene (Gülcemal et al. 2012), azukisaponin V (Kitagawa et al. 1983e), wistariasaponin B2 (Konoshima et al. 1989), astragaloside VIII (Kitagawa et al. 1983c), wistariasaponin B1 (Konoshima et al. 1989), cloversaponin IV (Sakamato et al. 1992), azukisaponin II (Kitagawa et al. 1983f), wistariasaponin D (Konoshima et al. 1991), dehydroazukisaponin V (Mohamed et al. 1995), and 3-*O*-*β*-D-glucuronopyranosyl-soyasapogenin B (Udayama et al. 1998). The authors commented that the phytochemical investigation of *A. tauricolus* showed only oleanane-type triterpene glycosides and no cycloartane-type glycosides, the main constituents of most *Astragalus* spp., sharing this peculiar feature with a limited group of *Astragalus* spp. [*A. hamosus* (Ionkova 1991), *A. complanatus* (Cui et al. 1992a), *A. sinicus* (Cui et al. 1992b), and *A. corniculatus* (Krasteva et al. 2006, 2007)]. Moreover, an HPLC–ESIMS^n approach was used to characterize astragalosides in Radix Astragali [Xu et al. 2007; Zu et al. 2009; Chu et al. 2010), but no HPLC–ESIMS^n study was addressed to oleanane-type triterpene saponins in *Astragalus* spp. In this study, antiproliferative activity of the compounds was also investigated against some cell lines including human breast cancer (MCF-7), human lung adenocarcinoma (A549), human prostate cancer (PC-3), and human leukemia (U937) cell lines. Compound **103** was the only compound exhibiting moderate activity with an IC_{50} of 22 μM against human leukemia cell line (U937) (Gülcemal et al. 2013).

Compound **96**; $C_{53}H_{84}O_{24}$; $[\alpha]^{25}_D$ +13.1° (*c* 0.1, MeOH); ν^{KBr}_{max} cm^{-1}: 3428 (>OH), 2934 (>CH), 1680 (C=O), 1658 (C=C); δ_C (CD_3OD); HR-MALDITOF-MS *m/z* $[M + Na]^+$ 1127.5262 (calcd. for $C_{53}H_{84}O_{24}Na$, 1127.5250). Compound **97**; $C_{54}H_{88}O_{24}$; $[\alpha]^{25}_D$ +9.3° (*c* 0.1, MeOH); ν^{KBr}_{max} cm^{-1}: 3442 (>OH), 2930 (>CH), 1670 (C=O), 1655 (C=C); δ_C (CD_3OD); HR-MALDITOF-MS *m/z* $[M + Na]^+$ 1143.5573 (calcd. for $C_{54}H_{88}O_{24}Na$, 1143.5563). Compound **98**; $C_{53}H_{86}O_{22}$; $[\alpha]^{25}_D$ +22.4° (*c* 0.1, MeOH); ν^{KBr}_{max} cm^{-1}: 3450 (>OH), 2938 (>CH), 1680 (C=O), 1660 (C=C); δ_C (CD_3OD); HR-MALDITOF-MS *m/z* $[M + Na]^+$ 1097.5515 (calcd. for $C_{53}H_{86}O_{22}Na$, 1097.5508). Compound **99**; $C_{54}H_{88}O_{23}$; $[\alpha]^{25}_D$ +21.1° (*c* 0.1, MeOH); ν^{KBr}_{max} cm^{-1}: 3430 (>OH), 2948 (>CH), 1670 (C=O), 1656 (C=C); δ_C (CD_3OD);

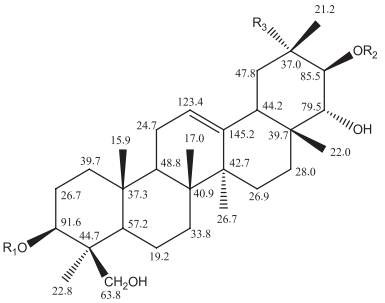
HR-MALDITOF-MS *m/z* $[M+Na]^+$ 1127.5625 (calcd. for $C_{54}H_{88}O_{23}Na$, 1127.5614). Compound **100**; $C_{54}H_{86}O_{25}$; $[\alpha]^{25}_D$ +15.6° (*c* 0.1, MeOH); ν^{KBr}_{max} cm^{-1}: 3435 (>OH), 2925 (>CH), 1660 (C=C); δ_C (CD_3OD); HR-MALDITOF-MS *m/z* $[M + Na]^+$ 1157.5363 (calcd. for $C_{54}H_{86}O_{25}Na$, 1157.5356). Compound **101**; $C_{53}H_{86}O_{21}$; $[\alpha]^{25}_D$ +19.6° (*c* 0.1, MeOH); ν^{KBr}_{max} cm^{-1}: 3443 (>OH), 2935 (>CH), 1668 (C=C); δ_C (CD_3OD); HR-MALDITOF-MS *m/z* $[M + Na]^+$ 1081.5567 (calcd. for $C_{53}H_{86}O_{21}Na$, 1081.5559). Compound **102**; $C_{48}H_{74}O_{20}$; $[\alpha]^{25}_D$ +9.8° (*c* 0.1, MeOH); ν^{KBr}_{max} cm^{-1}: 3430 (>OH), 2938 (>CH), 1650 (C=C); δ_C (CD_3OD); HR-MALDITOF-MS *m/z* $[M + Na]^+$ 993.4682 (calcd. for $C_{48}H_{74}O_{20}Na$, 993.4671). Compound **103**; $C_{48}H_{78}O_{20}$; $[\alpha]^{25}_D$ +16.8° (*c* 0.1, MeOH); ν^{KBr}_{max} cm^{-1}: 3438 (>OH), 2930 (>CH), 1655 (C=C); δ_C (CD_3OD); HR-MALDITOF-MS *m/z* $[M + Na]^+$ 997.4989 (calcd. for $C_{48}H_{78}O_{20}Na$, 997.4982). Compound **104**; $C_{48}H_{76}O_{21}$; $[\alpha]^{25}_D$ +16.8° (*c* 0.1, MeOH); ν^{KBr}_{max} cm^{-1}: 3445 (>OH), 2928 (>CH), 1655 (C=C); δ_C (CD_3OD); HR-MALDITOF-MS *m/z* $[M + Na]^+$ 1011.4785 (calcd. for $C_{48}H_{76}O_{21}Na$, 1011.4777). Compound **105**; $C_{47}H_{74}O_{20}$; $[\alpha]^{25}_D$ +11.5° (*c* 0.1, MeOH); ν^{KBr}_{max} cm^{-1}: 3440 (>OH), 2934 (>CH), 1650 (C=C); δ_C (CD_3OD); HR-MALDITOF-MS *m/z* $[M + Na]^+$ 981.4683 (calcd. for $C_{47}H_{74}O_{20}Na$, 981.4671). The structures and ^{13}C data of the compounds (**96–105**) are presented below.

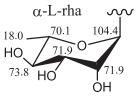


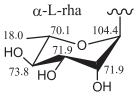
96 R_1= A

100 R_2= B

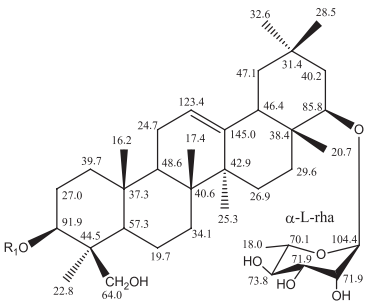
97 R_1= B



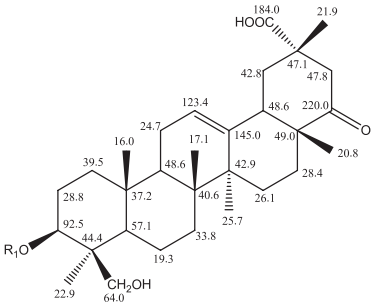
98 R_1= A R_2=  R_3= CH_3

99 R_2= B R_2=  R_3= CH_3

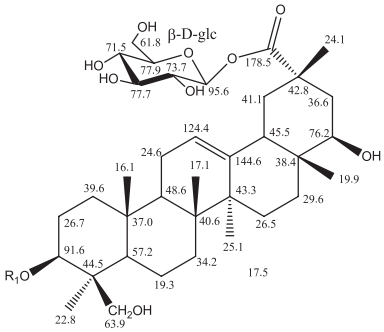
103 R_2= B R_2= H R_3= CH_2OH

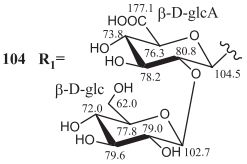


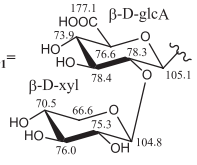
101 R_1= A



102 R_1= B



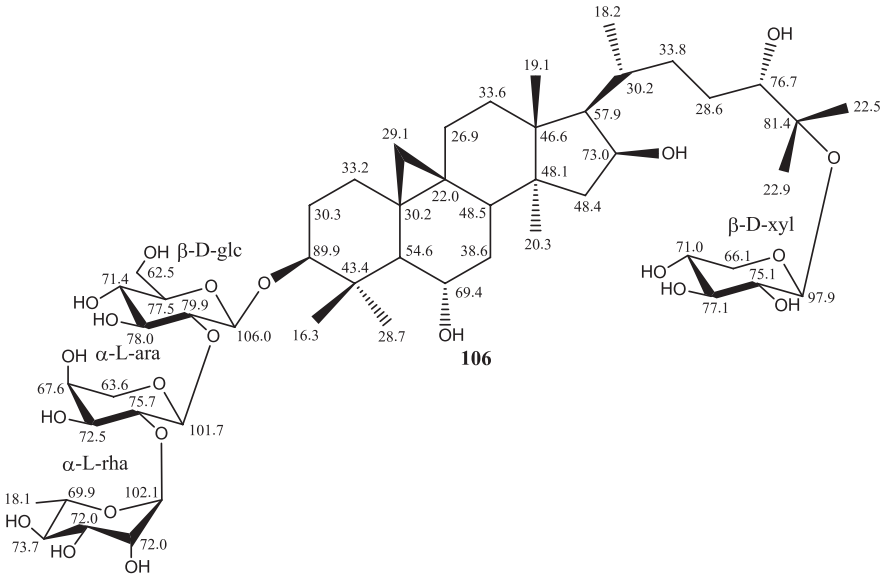
104 R_1= 

105 R_1= 

In 2014, a new cycloartane-type triterpene glycoside, 3-*O*-[*α*-L-rhamnopyranosyl-(1→2)-*α*-L-arabinopyranosyl-(1→2)-*β*-D-glucopyranosyl]-25-*O*-*β*-D-xylopyranosyl-3*β*,6*α*,16*β*,24(*S*),25-pentahydroxycycloartane (krugianoside A, **106**), was isolated from the roots of *Astragalus plumosus* var. *krugianus* Chamb.&Matthews (Sect. Rhacophorus), together with 15 known triterpene glycosides, namely, 6-*O*-*β*-D-glucopyranosyl-3*β*,6*α*,16*β*,24*α*-tetrahydroxy-20(*R*),25-epoxycycloartane (Gülcemal et al. 2011), cyclocephaloside I (Bedir et al. 1998a), 3-*O*-[*α*-L-arabinopyranosyl-(1→2)-*O*-*β*-D-xylopyranosyl]-6-*O*-*β*-D-glucopyranosyl-3*β*,6*α*,16*β*,24*α*-tetrahydroxy-20(*R*),25-epoxycycloartane (Horo

et al. 2010), 6-*O*-*β*-D-glucopyranosyl-3*β*,6*α*,16*β*,24(*S*),25-pentahydroxycycloartane (Gülcemal et al. 2011), 3-*O*-[*α*-L-arabinopyranosyl-(1→2)-*O*-*β*-D-xylopyranosyl]-3*β*,6*α*,16*β*,24*α*-tetrahydroxy-20(*R*),25-epoxycycloartane (Gülcemal et al. 2011), cyclocanthoside E (Isaev et al. 1992), oleifoliosides B (Özipek et al. 2005), 3-*O*-[*α*-L-rhamnopyranosyl-(1→2)-*α*-L-arabinopyranosyl-(1→2)-*O*-*β*-D-xylopyranosyl]-6-*O*-*β*-D-glucopyranosyl-3*β*,6*α*,16*β*,24(*S*),25-pentahydroxycycloartane (Horo et al. 2010), cycloastragenol (Kitagawa et al. 1983a), brachyoside B (Bedir et al. 1998b), cycloaraloside A (Isaev et al. 1989), cyclogaleginoside B (Alaniya et al. 1984), cycloaraloside D (Isaev 1991), elongatoside (Çalış et al. 2008a), and astragaloside IV (Kitagawa et al. 1983a). In this report, cytotoxic activity of all compounds was evaluated in human skin fibroblast WS1 cells, which revealed no cytotoxicity. The antioxidant potential was also examined for the compounds. Compounds **106** and oleifoliosides B prevented elevation of ROS induced by t-BOOH, emphasizing the potential activity of these compounds to protect fibroblasts from oxidative stress (Denizli et al. 2014).

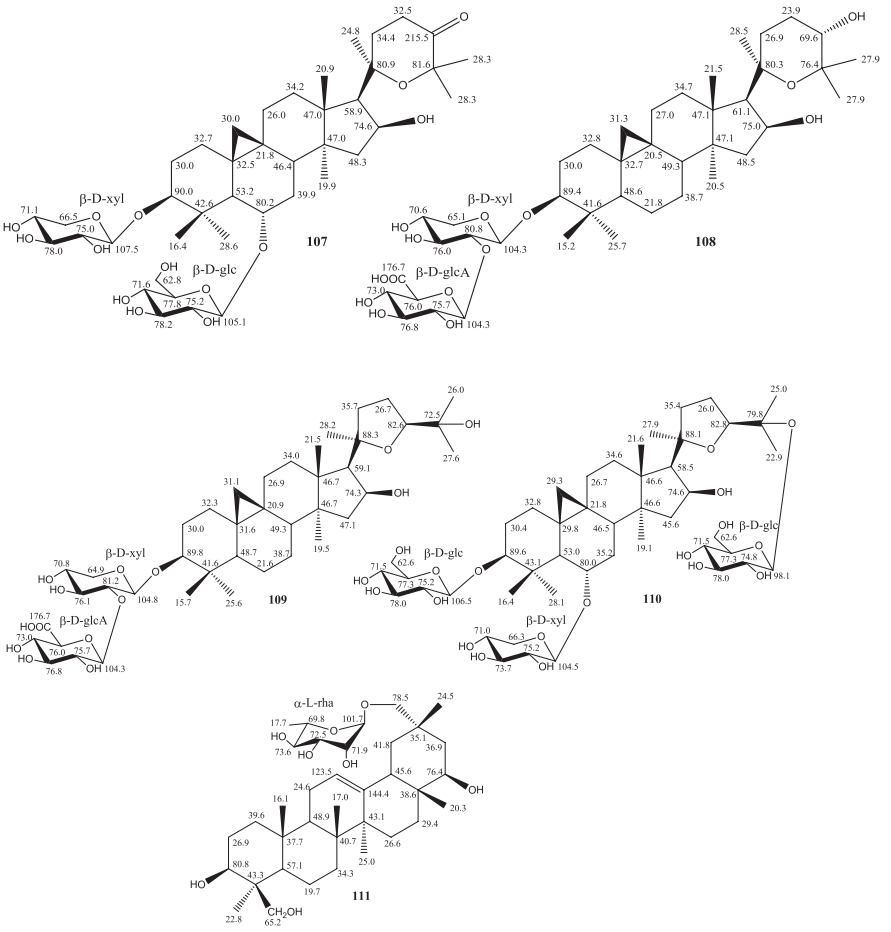
Compound **106**; $C_{52}H_{88}O_{22}$; $[\alpha]^{25}_{D}$ +25.6° (*c* 0.1, MeOH); ν^{KBr}_{max} cm^{-1}: 3470 (>OH), 3055 (cyclopropane ring), 2940 (>CH), 1265 and 1055 (C–O–C); δ_C (CD_3OD); HR-MALDITOF-MS *m/z* $[M + Na]^+$ 1087.5669 (calcd. for $C_{52}H_{88}O_{22}Na$, 1087.5665). The structure and ^{13}C chemical shifts are given below for compound **106**.



Another phytochemical study was performed on *Astragalus pennatulus* (Sect. Rhacophorus), which resulted in isolation of four new cycloartane- and a new oleanane-type triterpenoids together with five known cycloartane-type glycosides. Structures of the compounds were established as 3-*O*-*β*-D-xylopyranosyl-6-*O*-*β*-D-glucopyranosyl-3*β*,6*α*,16*β*-trihydroxy-24-oxo-20(*R*),25-epoxycycloartane (**107**),

3-*O*-[*β*-D-glucuronopyranosyl-(1→2)-*β*-D-xylopyranosyl]-3*β*,16*β*,24*α*-trihydroxy-20(*R*),25-epoxycycloartane (**108**), 3-*O*-[*β*-D-glucuronopyranosyl-(1→2)-*β*-D-xylopyranosyl]-3*β*,16*β*,25-trihydroxy-20(*R*),24(*S*)-epoxycycloartane (**109**), 3,25-di-*O*-*β*-D-glucopyranosyl-6-*O*-*β*-D-xylopyranosyl-3*β*,6*α*,16*β*,25-tetrahydrox-20(*R*),24(*S*)-epoxycycloartane (**110**), and 29-*O*-*α*-L-rhamnopyranosyl-abrisapogenol B (**111**) by the extensive use of 1D- and 2D-NMR experiments along with ESIMS and HRMS analysis. Known compounds were identified as cyclodissectoside (Sukhina et al. 2007), hareftoside C (Horo et al. 2012), 6-*O*-*β*-D-glucopyranosyl-3*β*,6*α*,16*β*,24*α*-tetrahydroxy-20(*R*),25-epoxycycloartane (Gülcemal et al. 2011), cyclocephaloside I (Bedir et al. 1998a), and astragaloside IV (Kitagawa et al. 1983a). The authors stated that the aglycone of compound **107**, 3*β*,6*α*,16*β*-trihydroxy-24-oxo-20(*R*),25-epoxycycloartane, was reported for the first time. In this report, three cancer cell lines including A549 (human lung adenocarcinoma), A375 (human melanoma), and DeFew (human B lymphoma) cells were used for testing cytotoxicity of the isolated compounds. There was no significant cytotoxicity in any of the tested compounds (Un et al. 2016).

Compound **107**; $C_{41}H_{66}O_{14}$; $[\alpha]^{25}_{D}$ −60.4° (*c* 0.03, MeOH); ν^{KBr}_{max} cm^{-1}: 3455 (>OH), 3030 (cyclopropane ring), 2940 (>CH), 1260 and 1055 (C–O–C); δ_C (CD_3OD); HR-MALDITOF-MS *m/z* $[M + Na]^+$ 805.4354 (calcd. for $C_{41}H_{66}O_{14}Na$, 805.4350). Compound **108**; $C_{41}H_{66}O_{14}$; $[\alpha]^{25}_{D}$ −50.7° (*c* 0.06, MeOH); ν^{KBr}_{max} cm^{-1}: 3460 (>OH), 3030 (cyclopropane ring), 2950 (>CH), 1264 and 1055 (C–O–C); δ_C (CD_3OD); HR-MALDITOF-MS *m/z* $[M + Na]^+$ 805.4353 (calcd. for $C_{41}H_{66}O_{14}Na$, 805.4350). Compound **109**; $C_{41}H_{66}O_{14}$; $[\alpha]^{25}_{D}$ −12.7° (*c* 0.07, MeOH); ν^{KBr}_{max} cm^{-1}: 3465 (>OH), 3030 (cyclopropane ring), 2955 (>CH), 1260 and 1060 (C–O–C); δ_C (CD_3OD); HR-MALDITOF-MS *m/z* $[M + Na]^+$ 805.4355 (calcd. for $C_{41}H_{66}O_{14}Na$, 805.4350). Compound **110**; $C_{47}H_{78}O_{19}$; $[\alpha]^{25}_{D}$ −117.3° (*c* 0.04, MeOH); ν^{KBr}_{max} cm^{-1}: 3455 (>OH), 3035 (cyclopropane ring), 2935 (>CH), 1250 and 1050 (C–O–C); δ_C (CD_3OD); HR-MALDITOF-MS *m/z* $[M + Na]^+$ 969.5037 (calcd. for $C_{47}H_{78}O_{19}Na$, 969.5035). Compound **111**; $C_{36}H_{60}O_8$; $[\alpha]^{25}_{D}$ −93.2° (*c* 0.04, MeOH); ν^{KBr}_{max} cm^{-1}: 3460 (>OH), 2934 (>CH), 1658 (C=C); δ_C (CD_3OD); HR-MALDITOF-MS *m/z* $[M + Na]^+$ 643.4190 (calcd. for $C_{36}H_{60}O_8Na$, 643.4186). The structures and ^{13}C chemical shifts are provided below for compounds **107–111**.



In 2016, a study was performed on another Turkish species; *Astragalus tmoleus* Boiss. var. *tmoleus* (Sect. Pterophorus) and known cycloartane-type saponins, astrasieversianins I (Gan et al. 1986) and II (Gan et al. 1986), astragaloside IV (Kitagawa et al. 1983a), cyclocephaloside II (Bedir et al. 1998b), and acetylastragaloside I (Kitagawa et al. 1983a), were isolated (Avunduk et al. 2016).

Phytochemical investigation of *Astragalus lycius* Boiss (Sect. Onobrychium) was resulted in isolation of eight known secondary metabolites. Their structures were established as 5,5′-dihydroxy-3′-methoxy-isoflavone-7-*O*-*β*-D-glucoside (Vitor et al. 2004), genistin (Vitor et al. 2004), sissotrin (Vitor et al. 2004),

5,4′-dimethoxy-isoflavone-7-*O*-*β*-D-glucopyranoside (Vitor et al. 2004), (7*S*,8*R*)-5-methoxydehydrodiconiferyl alcohol-4-*O*-*β*-D-glucopyranoside (Machida and Sakamoto 2009), 4-*O*-lariciresinol-glucoside (Kurkin et al. 1991), 2-phenylethyl-*β*-D-glucopyranoside (Wu et al. 2011), and *β*-sitosterol-3-*O*-*β*-D-glucopyranoside (Nyongha et al. 2010) by the extensive use of 1D- and 2D-NMR experiments along with HRMS analyses and by comparison with literature values. The authors reported that the first seven compounds were reported for the first time from *Astragalus* genus. Interestingly, no cycloartane- or oleanane-type triterpene glycoside, the main constituents of *Astragalus* spp., was isolated. This peculiar feature characterizes a very limited group of *Astragalus* spp. such as Sect. Hymenostegis [*A. lagurus* (Guzhva et al. 1984)] and Sect. Vulneraria [*A. vulneraria* (Bedir et al. 2000b), *A. onobrychis* Guzhva et al. 1992)]. In this study, all the isolated compounds were investigated for their cytotoxic activities against a number of cancer cell lines [PC3 (human prostate carcinoma), HT-29 (human colon carcinoma), MDA-MB-231 (human breast carcinoma)] and a transformed cell line [HEK 293 (human embryonic kidney 293)], in which only 4-*O*-lariciresinol-glucoside represented a strong and selective activity against human colon carcinoma (HT-29) at 2.69 μM concentration (Horo et al. 2016).

The immunostimulatory effect of 19 cycloartane-type triterpene glycosides was examined (Bedir et al.); furthermore the bioactivity of macrophyllosaponins B–D; cyclocanthoside D and E; astrasieversianin II and X; trojanoside A and H; cyclocephaloside I; astragaloside I, II, IV, VI, and VII; brachyoside B; askendoside G; and cephalatoside A; cycloastragenol which previously isolated from *A. oleifolius*, *A. prusianus*, *A. microcephalus*, *A. trojanus*, *A. cephalotes*, and *A. melanophrurius* was tested using a transcription-based bioassay for nuclear factor-kappa B (NF-kappa B) activation in a human macrophage/monocyte cell line, THP-1. All compounds were inactive at 100 μg/mL except astragaloside I, which increased NF-kappa B directed luciferase expression up to 65% compared with maximal stimulation by *E. coli* lipopolysaccharide (LPS) at 10 μg/mL. With addition of 50 ng/mL LPS to the compounds, none of them were active at low dosage levels (0.1 μg/mL). On the other hand, astragaloside I increased mRNA expression of the inflammatory cytokines interleukin-1β (IL-1β) and tumor necrosis factor-alpha (TNF-α), which was measured using reverse transcriptase-polymerase chain reaction (RT)-PCR (Bedir et al. 2000a).

In the year of 2005, the gastroprotective effect of astragaloside IV, a cycloartane-type triterpene glycoside isolated from *Astragalus zahlbruckneri*, was measured. In addition to this, rolls of prostaglandins, sulfhydryls, and nitric oxide were also investigated. For this, astragaloside IV (3–30 mg kg^{-1}) which was given orally to the tested mice depending on dose reduced the ethanol-induced gastric hemorrhagic lesions. The results suggested that the combination of N^G-nitro-largininemethyl ester (70 mg kg^{-1}, ip), a nitric oxide (NO)-synthase inhibitor, with astragaloside IV suspended in Tween 80 at 3, 10, and 30 mg kg^{-1} showed 15, 37, and 52% gastroprotection, respectively. Dose-dependent treatment confirmed that astragaloside IV with 30 mg kg^{-1} had the highest ulcer inhibition.

On the other hand, it was reported that the effect of astragaloside IV was not limited by inhibition of prostaglandin synthesis with indomethacin (10 mg kg^{-1}, s.c.) and the block of endogenous sulfhydryls with *N*-ethylmaleimide (NEM, 10 mg kg^{-1}, s.c.) (Navarrete et al. 2005).

In the year of 2005, another study was performed for the investigation of immunostimulating effect of 13 cycloartane- and 1 oleanane-type triterpene saponins isolated from Turkish species (*Astragalus brachypterus*, *A. cephalotes*, *A. microcephalus*, and *A. trojanus*), as well as methanol extracts from the roots of three *Astragalus* species (*A. cephalotes*, *A. oleifolius*, and *A. trojanus*). Cytokine concentrations of interleukins IL-1 (interleukin-1) and IL-8 (interleukin-8) and TNF-α after bacterial lipopolysaccharide (LPS) stimulation and IL-2 (interleukin-2) and IL-4 (interleukin-4) and INF-ɣ (interferon gamma) after phorbolacetate (PHA) stimulation were determined utilizing commercial enzyme-linked immunosorbent assay (ELISA) kits. All triterpene saponins, which were tested in the mentioned study (brachyoside A, brachyoside C, cycloastragenol, astragaloside I, brachyoside B, cyclocephaloside II, astragaloside II, astragaloside VII, trojanoside A, cyclocanthoside E, trojanoside H, cyclocephaloside I, astragaloside I, astrojanoside A: 3-*O*-(*α*-L-rhamnopyranosyl-(1→2)-*β*-D-xylopyranosyl-(1→2)-*β*-D-glucuronopyranosyl)-29-*O*-*β*-D-glucopyranosyl-3*β*,22*β*,24,29-tetrahydroxyolean-12-en), presented a substantial IL-2-inducing activity between 35.9% and 139.6%. IL-2 is a cytokine produced by activated T cells with powerful immunostimulatory and antineoplastic properties. Among the extracts, the highest activity was obtained from *A. oleifolius* (141.2%). Glycosides of 20,24-epoxy and 20,25-epoxy cycloartanes showed higher IL-2-inducing activity than those of acyclic-cycloartane derivatives; in addition to this, in aglycone of 20,24-epoxy, cycloartanes in cycloastragenol also showed higher IL-2-inducing activity. Especially the activity of astragaloside VII, a tridesmosidic glycoside of cycloastragenol, was the most remarkable. Additionally, the oleanane-type triterpene saponins represented a prominent IL-2-inducing activity (Yeşilada et al. 2005).

In the year of 2011, another study was carried out for determination of in vitro growth stimulatory and in vivo wound healing properties of four cycloartane-type saponins that are present in Turkish *Astragalus* species as major chemical entities (astragaloside IV, cycloastragenol, cyclocephaloside I, and cyclocanthoside E). The obtained results indicated that cycloartane-type saponins of *Astragalus* genus are able to promote wound healing based on proliferation and migration in scratch assay, proliferation in MTT assay, and in vivo wound model study. Although all the obtained compounds could increase both migration and fibroblast proliferation, the most prominent were for cycloastragenol (CA), astragaloside IV (AG), and cyclocanthoside E (CCE). CA at 1 ng/mL showed a remarkable effect upon migration, whereas AG and CCE had their highest activities at 10 ng/mL. Simultaneously, AG and CCE at 10 ng/mL and CA at 1 ng/mL showed the highest proliferation rates in MTT assay. The results also showed that the topical treatments of *Astragalus* cycloartanes improve healing of subsequently induced abrasion skin wounds in rats compared to the control. At the end of 14-day treatment period, it was reported that 5% CA preparation was found to be the most remarkable in the treated skin. Histological

researches also confirmed that the group treated by CA had a greater cell density, more newly formed blood vessels and more regularly organized dermis (linear alignment) compared to the other groups (Sevimli-Gür et al. 2011).

The evaluation of hemolytic activities referring to two immunomodulator *Astragalus* saponins macrophyllosaponin B (Mac B) from *Astragalus oleifolius* DC and astragaloside VII (Ast VII) from *Astragalus trojanus* Stev. and their adjuvant potentials on the cellular and humoral immune responses of Swiss albino mice against BSA (bovine serum albumin) were studied. The hemolytic activity of Mac B and Ast VII was measured using 0.5% rabbit red blood cell. According to the final results, no hemolytic activity was observed at concentrations of 2.5–500 μg/mL. Results referring to the effect of Ast VII and Mac B on mitogen- and BSA-stimulated splenocyte proliferation in BSA-immunized mice represented that LPS-stimulated splenocyte proliferation in the mice immunized with BSA/Ast VII and BSA/Mac B was significantly higher than that in the BSA control group. It was reported that the effect was dose dependent since BSA-induced splenocyte proliferation in the BSA-immunized mice was enhanced by Ast VII as well as Mac B in different doses. Mac B and Ast VII showed a slight hemolytic effect, with 0.42 and 0.54% values, respectively, while the results for the effects of Ast VII and Mac B on the BSA-specific serum antibody response represented that the serum IgG (immunoglobulin G), IgG1 (immunoglobulin G1), and IgG2b (immunoglobulin G2b), antibody levels immunized with BSA, were remarkably increased by Ast VII (120 μg), Mac B (90 μg), and Freund's comparing to the control group. For adjuvant activity, on days 1 and 15, Swiss albino mice were immunized with only BSA 100 μg or with BSA 100 μg dissolved in saline containing Ast VII (30, 60, 120, and 240 μg), Mac B (30, 60, 90, and 120 μg), or Freund's adjuvant; 2 weeks after the last immunization for concanavalin A (Con A)-, lipopolysaccharide (LPS)-, and BSA-stimulated splenocyte proliferation assay, sera and splenocytes were collected, and BSA-specific antibodies in serum were measured. Moreover, 2 weeks after the last immunization, the IFN-γ and IL-4 levels in the sera were detected using ELISA. Ast VII (120 μg) and Mac B (90 μg) were found to stimulate IFN-γ production such as Freund's 2 weeks after the last immunization as compared to the control. Results showed that Ast VII and Mac B generate essential specific antibody and cellular response against BSA in mice; it can be confirmed that the tested molecules have potentials as a new class saponin adjuvant (Nalbantsoy et al. 2011).

In the year of 2012, immunomodulatory properties and in vitro anti-inflammatory activities of cycloartane-type saponins from *Astragalus* species were studied in mice. For this, the ability of LPS + Ast VII and LPS + Mac B to induce IL-1β, TGF-1β, TNF-α, IL-2, IL-4, and IFN-γ production was tested. Groups of five male Swiss albino mice were immunized ip with LPS (12.5 μg)/AST VII (60 μg) and LPS (12.5 μg)/Mac B (60 μg) on day 1 and AST VII (60 μg) and Mac B (60 μg) alone on day 2. The cytokine levels in the sera were detected 4 h after the last immunization using ELISA; the results suggested that AST VII and Mac B increased the concentration of Th1 (T helper 1) cytokine release (IL-2 and IFN-γ) and suppressed the concentration of Th2 (T helper 2) cytokine production (IL-4) remarkably. The

results referring to the immunohistochemical studies exhibited that both IL-Rα (CD25) and CD69 surface receptors justifying the Th1 cytokine release were induced. According to the final results, compounds did not affect either NF-κB or NAG-1 (nonsteroidal anti-inflammatory drug-activated gene) activity; on the other hand inhibition of inducible nitric oxide synthase (iNOS) activity was inhibited by Mac B with half maximal inhibitory concentration (IC_{50}) of 156 μg/mL. Although Ast VII and Mac B had no significant effect on the inflammatory cellular targets in vitro, they resulted in strong immunoregulatory effects without the stimulation of inflammatory cytokines in mice (Nalbantsoy et al. 2012).

Chemotaxonomy

Until now, as mentioned before, 31 out of 447 Turkish *Astragalus* species, which were chosen from 14 different sections, have been investigated for their secondary metabolite contents. Most of the studied sections, namely, Sect. Adiaspastus [*A. aureus* (Gülcemal et al. 2011)], Sect. Christiana [*A. gilvus* Boiss (Tabanca et al. 2005), *A. melanophrurius* (Çalış et al. 1997)], Sect. Halicacabus [*A. halicacabus* (Djimtombaye et al. 2013)], Sect. Macrophyllium [*A. oleifolius* (Çalış et al. 1996; Bedir et al. 2000c; Özipek et al. 2005)], Sect. Proselius [*A. campylosema* Boiss. ssp. *campylosema* (Çalış et al. 2008b), *A. elongatus* (Çalış et al. 2008a)], Sect. Pterophorus [*A. brachypterus* (Bedir et al. 1998b), *A. baibutensis* (Çalış et al. 2006), *A. ptilodes* Boiss. var. *cariensis* Boiss (Linnek et al. 2011), *A. tmoleus* var. *tmoleus* (Avunduk et al. 2016)], Sect. Rhacophorus [*A. amblolepis* (Polat et al. 2009), *A. cephalotes* var. *brevicalyx* (Çalış et al. 1999), *A. erinaceus* (Savran et al. 2012), *A. microcephalus* (Bedir et al. 1998a, b), *A. plumosus* var. *krugianus* (Denizli et al. 2014), *A. prusianus* (Bedir et al. 2001b), *A. schottianus* (Karabey et al. 2012), *A. zahlbruckneri* (Çalış et al. 2001)], and Sect. Stereocalyx [A. *stereocalyx* Bornm. (Yalçın et al. 2012)], provided cycloartane glycosides; however, cycloartane- and oleanane-type saponins were encountered together in a few sections: Sect. Acanthophace [*A. hareftae* (Horo et al. 2012), *A. icmadophilus* (Horo et al. 2010)], Sect. Eustales [*A. flavescens* (Konoshima et al. 1989)], Sect. Melanocercis [*A. angustifolius* (Gülcemal et al. 2012)], Sect. Pterophorus [*A. wiedemannianus* Fischer (Polat et al. 2010), *A. trojanus* (Bedir et al. 1999a, b, 2001a)], and Sect. Rhacophorus [*A. pennatulus* (Un et al. 2016), *Astragalus pycnocephalus* var. *pycnocephalus* (Koz et al. 2011)]. The Sect. Malacothrix [*A. tauricolus* (Gülcemal et al. 2013)] provided oleanane-type saponins exclusively and no cycloartane-type glycosides, the main components of *Astragalus* spp., sharing this uncharacteristic feature with a limited group of *Astragalus* spp. [*A. hamosus* (Ionkova 1991), *A. complanatus* (Cui et al. 1992a), *A.* sinicus (Cui et al. 1992b), and *A. corniculatus* (Krasteva et al. 2006, 2007)]. *A. lycius* (Horo et al. 2016), belonging to Section Onobrychium, comprises only phenolic- and flavonoid-type glycosides. Moreover, *A. vulneraria* (Bedir et al. 2000b), belonging to Sect. Vulneraria, includes only flavonol

glycosides. Amusingly, no cycloartane- or oleanane-type triterpene glycoside, the main constituents of *Astragalus* spp., was isolated from *A. lycius* and *A. vulneraria*. This peculiar feature characterizes a very limited group of *Astragalus* spp. (Guzhva et al. 1984, 1992).

Cycloastragenol with 20(R),24(S)-epoxy side chain is the principal aglycone in the *Astragalus* species along with cyclocanthogenol. However, cyclocephalogenol is more uncommon in the genus so far reported only from four sections, viz., Acanthophace [*A. hareftae* (Horo et al. 2012), *A. icmadophilus* (Horo et al. 2010)], Adiaspastus [*A. aureus* (Gülcemal et al. 2011)], Pterophorus [*A. trojanus* (Bedir et al. 1999a, b, 2001a), *A. wiedemannianus* Fischer (Polat et al. 2010)], and Rhacophorus [*A. microcephalus* (Bedir et al. 1998a, b), *A. plumosus* var. *krugianus* (Denizli et al. 2014), *A. zahlbruckneri* (Çalış et al. 2001)]. *A. angustifolius* (Gülcemal et al. 2012), single studied species of Melanocercis section, provided the C-24 epimer of cycloastragenol as aglycone, which was reported for the first time in nature. *A. halicacabus* (Djimtombaye et al. 2013), single tested species of Halicacabus section, provided maltol glycosides, reported for the first time in the *Astragalus* genus and even in the Fabaceae family. *A. pennatulus* (Un et al. 2016), belonging to Sect. Rhacophorus, provided a cyclocephalogenin-alike glycoside characterized by the deficiency of the hydroxyl function at C-6 and the presence of a keto function at C-24, so far never reported in literature.

Structural Summary of Cycloartanes

Astragalus cycloartanes are classified based on the side chains extending from C-17 of the tetracyclic framework. In the case of Turkish *Astragalus* cycloartanes, three main aglycone structures including cycloastragenol, cyclocanthagenol, and cyclocephalogenol have been reported, deriving from 20,24-epoxy-, acyclic-, and 20,25-epoxy side chains, respectively (Isaev et al. 1983a, b, 1989; Isaev 1991, 1995, 1996; Mamedova and Isaev 2004).

20,24-Epoxy Side Chain Compounds

As shown in Fig. 1, 20,24-epoxy side chain cycloartanes undergo both oxidation and glycosylation reactions at different positions during biosynthesis. Carbons 3, 6, 16, 23, and 25 are susceptible to oxidation reactions, mainly involving regio- and stereo-specific hydroxylations catalyzed by P450 monooxygenase enzymes. In the case of glycosylation reactions, except C-23 position, all of the hydroxylated carbons (3, 6, 16, and 25) were found to be sugar attached. Particularly, bisdesmosidic saponins are common in *Astragalus* cycloartanes, where C-3(O) and C-6(O) are the major attachment positions. Moreover, in nature, tridesmosidic saponins have only been reported from *Astragalus* species as cycloartane saponins (i.e., astragaloside

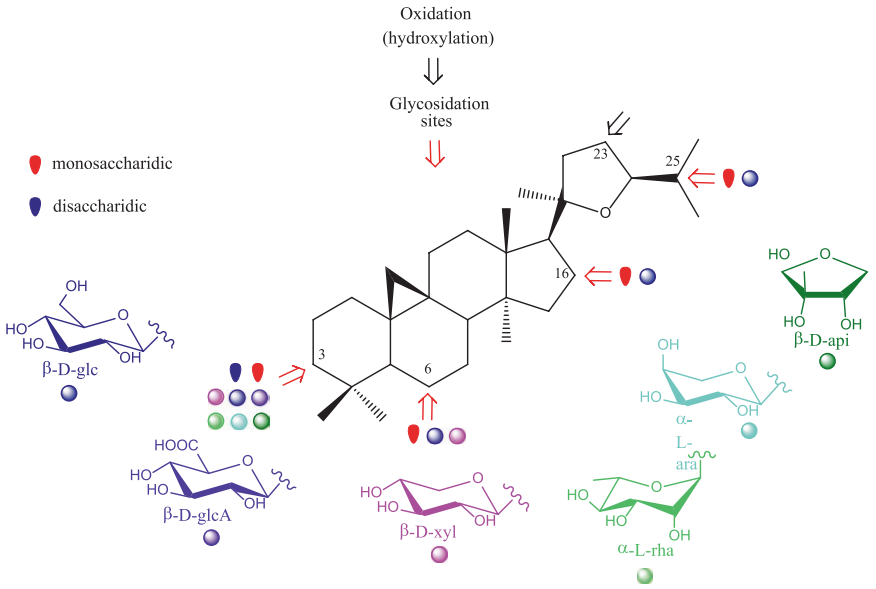


Fig. 1 General structure of 20,24-epoxy side chain compounds

VII, brachyoside C, cephalotoside A, trojanoside B, D, E, and F), whereas monodesmosidic and monosaccharidic compounds are encountered rarely compared to their counterparts bisdesmosidic/disaccharidic and trisaccharidic glycosides. Sugar diversity of the cycloartane class is much less in comparison to other saponin groups, such as oleananes, ursanes, lupanes, dammaranes, etc. Up to now, only six different sugar moieties have been found, viz., β-D-glucose, β-D-glucuronic acid, β-D-xylose, α-L-rhamnose, α-L-arabinose, and β-D-apiose. Although all the sugars mentioned above are found to be at C-3(O) position of 20,24-epoxy side chain cycloartanes, β-D-glucose is the only extending saccharide from C-16(O) and C-25(O) positions. Both β-D-glucuronic acid and β-D-xylose units have only been reported at C-6(O) position. While C-3(O) is found to include mono- or disaccharidic units, C-6, C-16, and C-25 positions are only glycosylated with a single sugar.

Acyclic Side Chain Compounds

Acyclic side chain derivatives have also been reported with similar oxidation and glycosylation patterns shown in Fig. 2. Whereas oxidations (mainly hydroxylations) are observed at C-1, C-3, C-6, C-7, C-16, C-20, C-24, and C-25, the glycosylation occurs on oxygenated carbons 3, 6, 16, 24, and 25. All glycosylated carbons are found to have at least one sugar unit. However, C-3(O) goes under further

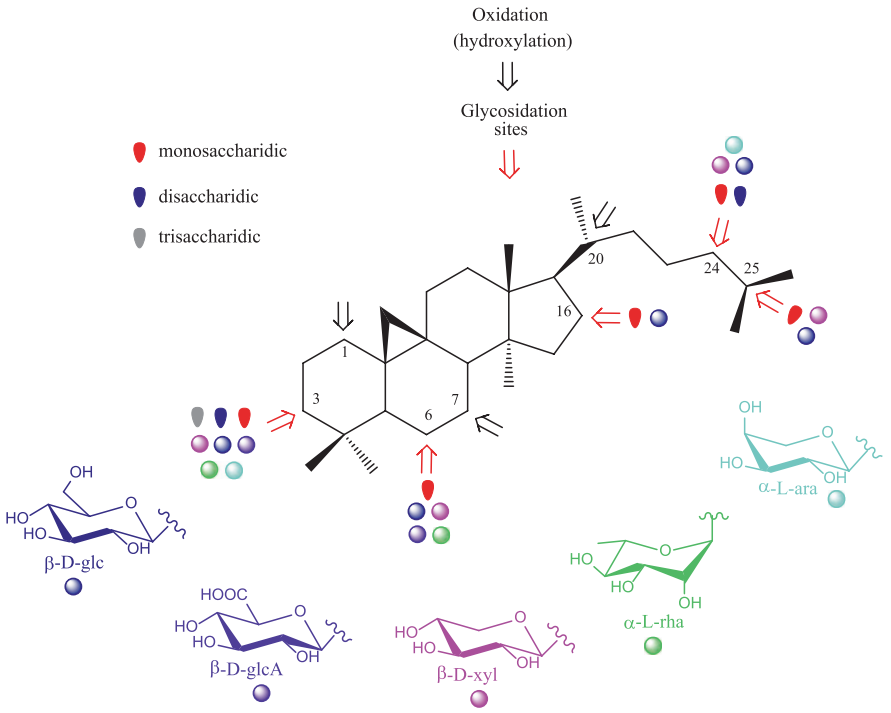


Fig. 2 General structure of acyclic side chain compounds

glycosylation reactions yielding di- and trisaccharidic sugar chains, whereas disaccharidic nature was also encountered for C-24(O). The sugars reported to be attached on acyclic side chain cyloartanes are β-D-glucose, β-D-glucuronic acid, β-D-xylose, α-L-rhamnose, and α-L-arabinose. As in 20,24-epoxy side chain structures, C-3 was found to contain all abovementioned sugar moieties attached, whereas, except arabinose unit, all the sugars were reported from the major glycosylation position C-6. C-16 had only β-D-glucose, while β-D-glucose and β-D-xylose units were branching from C-24 and C-25 positions. β-D-Glucose was recorded in all sugar sites, whereas the sugar α-L-rhamnose extended only from C-3 and C-24.

20,25-Epoxy Side Chain Compounds

20,25-Epoxy side chain cycloartanes are less common compared to the other side chains. This group of compounds is reported to have similar oxidation and glycosylation arrangement shown in Fig. 3. While hydroxylations are observed at C-3, C-6, C-16, and C-24, the glycosylation takes place on oxymethine carbons 3, 6, and 24. Up to now, five different sugars, viz., β-D-glucose, β-D-glucuronic acid, β-D-xylose,

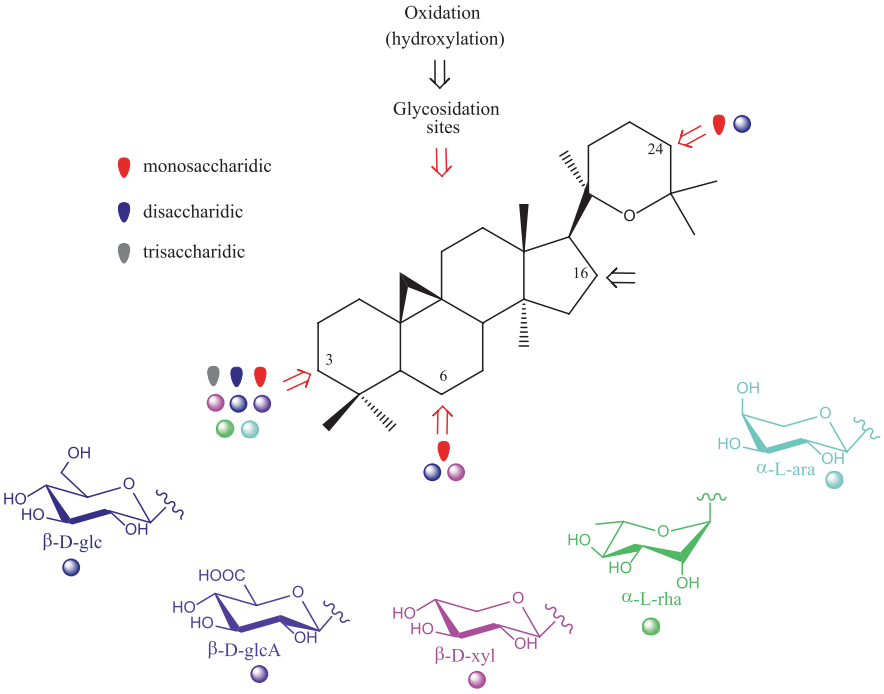


Fig. 3 General structure of 20,25-epoxy side chain compounds

α-L-rhamnose, and α-L-arabinose, have been reported for 20,25-epoxy side chain cycloartanes. C-6(O) and C-24(O) are found to have monosaccharidic units, while C-3(O) locates mono-, di-, and tetrasaccharidic sugar chains. Only β-D-glucose or β-D-xylose are attached from C-6(O), whereas β-D-glucose is reported as the only sugar branching from C-24(O).

Stereochemistry of *Astragalus* Cycloartanes

A total of 14 structurally distinct genins have been found in Turkish *Astragalus* species. Some of these have not been characterized as pure compounds but as the corresponding glycosides. β-Configuration of C-3 (OH) and C-16 (OH) and α-conuration of C-6 (OH) are consistent with all naturally occurring cycloartanes present in the genus *Astragalus* (Fig. 4).

20,24-Epoxycycloartanes, representing the largest group in Turkish *Astragalus* plants, are found in nature with three different stereoisomers, viz., 20*R*,24*S*, 20*S*,24*R* and 20*R*,24*R*. In the case of 20*S*,24*R* configuration, C-20 and C-24 resonate about 86.5–87.5 and 84.5–85.5, respectively, while, for the 20*R*,24*S* configuration, these carbons have chemical shifts of *ca.* 87.0–88.0 and 81.5–82.5. For 20*R*,24*R*

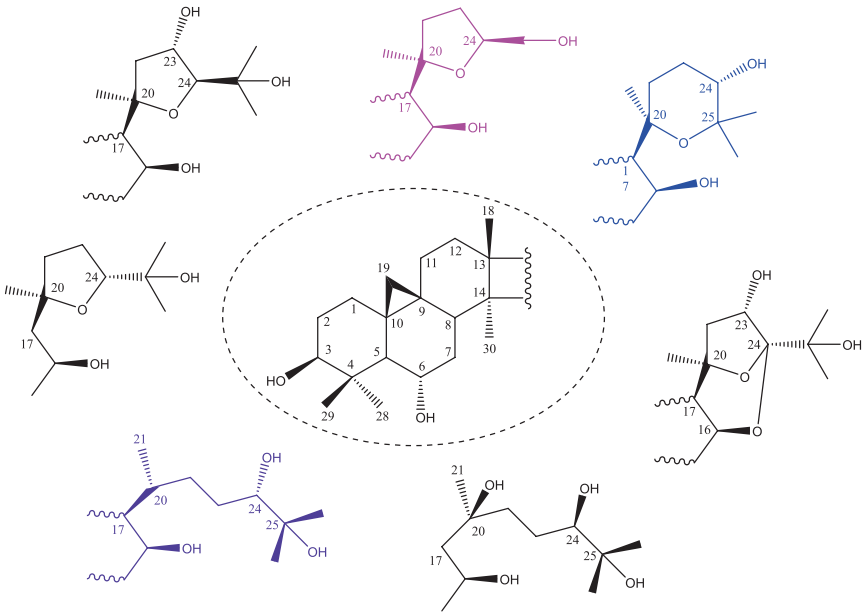


Fig. 4 Stereochemistry of *Astragalus* cycloartanes

configuration, C-20 and C-24 resonate around 87.3–88.8 and 88.4–85.5, respectively (Kitagawa et al. 1983a; Bedir et al. 2001b; Gülcemal et al. 2012). 16β,24;20,24-Diepoxycycloartane-type derivatives are very unusual in the plant kingdom. An examination of molecular models indicates that the heterocycles can be fused as the 20*R*, 24*R* and 20*S*, 24*S* configurations. Therefore, establishing the stereochemistry of one of these asymmetric centers defines the configuration of the other chiral atom (Çalış et al. 2008b).

For the acyclic side chain substituted cycloartane-type saponins, obtained from the *Astragalus* genus, the 24-position locates a hydroxyl group, meaning two possible stereoisomer (24*S* or 24*R*). The ^{13}C NMR chemical shift of C-24 could be used as a characteristic parameter in the determination of the configurations at C-24. In the case of the 24*R* configuration, the chemical shift of C-24 was found about 79.9–80.6 ppm (Isaev et al. 1983b), while for the 24S configuration of cyclocanthogenin, the same position's chemical shift ranged between 77.0 and 78.2 ppm (Isaev et al. 1992).

20,25-Epoxycycloartane genins are represented only with a single stereoisomer of cyclocephalogenin (20*R*,25-epoxycycloartane). The α orientation of the -OH group at C-24 can be determined by the nOe correlation between Me-27 and H-24β signals (Bedir et al. 1998a).

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Vetiveria zizanioides (L.) Nash: A Magic Bullet to Attenuate the Prevailing Health Hazards

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Introduction

The world has encountered the importance of plants since it came into existence. The use of medicinal plants by humankind is although ancient but seems to increase tremendously as modern medicinal system integrates the use of herbal medicines. Therefore, the cultivation of medicinal plants especially imperative medicinal plants is creating new extent in the field of agriculture. India remains one of the ancient witnesses in realizing the importance of plants as it upholds the major diversity in both flora and fauna. India ranks tenth among 12 mega biodiversity centers of the world with 2 major hot spots of endemic species including 49,000 plant species reported in 16 different agroclimatic zones. About 4900 species of flowering plants are endemic to the country. There are about 15,000–20,000 plant species reported to have unlimited treasure of medicinal value, with an estimated endemism of 30% (Trivedi 2007; Anonymous 2012). Among these, 7000–8000 are reported to be used by traditional communities and 1200–2000 in regulated systems of medicine in the department of Ayurveda, Yoga and Naturopathy, Unani, Siddha, Sowa Rigpa, and Homeopathy (AYUSH) (Joy et al. 1998; Qazi 2003; Anonymous 2012).

Aromatic plants contain essential oils, a product of secondary metabolism, containing volatile aroma compounds and isolated by distillation. Because the distilled oily extracts represent the "essence" of plants, they are termed as essential oils (EOs). EOs exhibit therapeutic potential due to which aromatic plants can also be categorized as medicinal plants, although their applications are much broader. Out of 18,664 plants of medicinal value reported in India, over 3000 plants have been identified as essential oil-bearing medicinal plants of the large number of plants

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belonging to 87 angiospermic families (Kochhar 2009; Farooqi and Sreeramu 2010). The EO is obtained from the vegetative organs: flowers, leaves, barks, woods, roots, rhizomes, fruits, seeds, etc. (Burt 2004; Celiktas et al. 2007; Skocibusic and Bezic 2006). EOs showcase multiple uses in the "beauty industry" as perfumes, cosmetics, or soaps, in the food industry as flavors for foods and drinks, as well as in the medical industry (Gupta 2007). They have antiseptic, antimicrobial, and antiviral activities (Reichling et al. 2009; Astani et al. 2010; Astani et al. 2011). As mosquito repellents, they bear the potential for malaria prevention (Pohlit et al. 2011). Moreover, they reveal considerable cytotoxicity toward cancer cells (Efferth et al. 2011; Sertel et al. 2011a, b).

India being the third largest producer of natural essential oils, next to the USA and Brazil, holds an important position in the world market. As per the reports, total production of essential oils at global level is estimated to be about 1,00,000–1,10,000 tons out of which India contributes with a share of 16–17%. Since India stands as the major mega biodiversity centers of the world representing prolific panoply of MAPs, the void between Indian trade and the world trade can be filled by coherently channelizing systematic research to improve and utilize the full potential of medicinal herbs (Duke 2002; Charles 2013). Apparently, the evolutionary intention for the production of these secondary metabolites was not to produce gentle medicines for human beings, but in contrast to use it as defense against herbivores and microorganisms (Efferth and Greten 2012). Therefore another aspect of pharmaceutical and pharmacological research should be aimed at distinguishing the fine line between toxic activities and pharmacologically valuable activities, which can be employed for therapeutic purposes.

In the following article, an array of the miraculous therapeutic properties of essential oil-bearing plant, vetiver (*Vetiveria zizanoides* L. Nash), are addressed outlining the morphology and distribution of the plant, essential oil biosynthesis, composition and its economics, and potential utilities of the plant.

Description of Plant

In the quest of exploring the potential utilities of different medicinal plants, *Vetiveria zizanoides* emerged as a "miracle grass" illustrating its omni-useful potentials. The commercial and social utility of this plant was first realized on account of its aromatic roots and lately overwhelmed by environmental applications of the plant as such, as well as diverse industrial uses of aboveground plant parts (Chomchalow and Chapman 2003).

Types

Eleven species of *Vetiveria* are recorded, with distribution in tropical Asia (including Pacific Islands and Australia) and Africa. Vetiver is a diploid plant ($2n = 20$), broadly classified into two types, namely, the wild growing North Indian flowering

type and the cultivated South Indian nonflowering type. They are further distinguished by characteristic difference in the stem and root: former type has medium thick stem with more branching roots, while the latter has a thick stem and less branching roots; however, the former is more common (Weiss 1997).

Common Names

It is commonly known as khas-khas in India and Sri Lanka, khus-khus in Europe, and *akar wangi* in Indonesia. Tamil word *vetivern* is the origin of the name vetiver (Nair et al. 1979).

Morphology

Vetiver grass, *Vetiveria zizanioides* (L.) Nash, syn. *Chrysopogon zizanioides* (L.) Roberty, is a perennial grass belonging to Poaceae family (Joy 2009). The root being the most valuable part of the grass forms an intertwined network that stops erosion and contains the majority of the essential oils which has valuable aromatic and biological properties (Danh 2007). The roots of vetiver grass contain oil-producing cells, responsible for its characteristic fragrance (Peyron 1989). These secretory cells are localized inside hard-to-reach root tissues (Chomchalow 2001). Essential oil can be detected in the inner bark within the cortical layer, where lysigen lacunae are also observed, providing the true storage vessel for the essential oil of the vetiver root (Viano et al. 1991).

Habit

Vetiveria zizanioides is a densely tufted grass with the culms arising from an aromatic rhizome up to 2 m tall; the roots are stout, dense, and aromatic.

Leaves

Leaves are narrow, erect, keeled with scabrid margins.

Flowers

Inflorescence is a panicle, up to 15–45 cm long, with numerous slender racemes in whorls on a central axis, Spikelets are grey to purplish; 4–6 mm long; in pairs, one sessile and the other pedicelled; and two-flowered; the lower floret is reduced to a

lemma, upper bisexual in sessile, and male in the pedicelled spikelet; glumes are armed with stout, tubercle-based spines, lemmas awnless, and palea is minute (The Wealth of India: Raw materials 2003).

Geographical Distribution

Vetiver is native to India and is found in wild state throughout the Indian subcontinent encompassing temperate to tropical climate. The grass grows wild in Haryana, Uttar Pradesh, Rajasthan, Gujarat, Bihar, Orissa, Assam, and Madhya Pradesh and throughout South India. The yield from the cultivated crop, however, meets only a very small percentage of the requirements of khus in the country; the bulk of the roots used for cooling purposes and for the extraction of the oil is obtained from the wild formations. On the other hand, vetiver is grown globally, on a commercial basis, for oil in Java, the Seychelles, Rėunion Brazil, China, Japan, and Haiti but for other purposes in the USA, Central America, and West African countries. Haiti, Indonesia (only Java), and Reunion produce most of the world's vetiver oil (The Wealth of India: Raw materials 2003).

Essential Oil of Vetiver

The roots are the storehouse of the much valuable vetiver oil which exhibit a rich green-woody earthy and nut-like aroma and vary in color from light yellow to yellowish brown to reddish yellow. It is one of the most viscous of the essential oils and therefore has a low evaporation rate, and it is also soluble in alcohol (Lavania 2003). The oil is also known to differ in odor, i.e., the roseate note of Reunion (Bourbon) and Haitian oil is highly regarded in perfumery industry, while the vetiver (khus) oil obtained from wild "khus" roots in India is known for its woody balsamic note.

Phyto-constituents

The quality of the oil depends upon the age of the root and the time involved in distillation. Oil distilled from young roots exhibit a green, earthy, and somewhat harsh odor due to low specific gravity, a low optical rotation, and a poor solubility, while old roots yield oils possessing higher specific gravity, a higher optical rotation, higher content of sesquiterpene compounds, and better solubility. Vetiver oil contains a very specific unique note used in fragrance and flavor industry which comes from oxygenated sesquiterpenes formed from reaction of sesquiterpenes and their ketones, aldehydes, and alcohols with microbes and its bioconversion. Also there are many glycosides present in the roots of vetiver which, when reacting with

microbes, give rise to these primary sesquiterpenes. The odor in most essential oils varies widely with the natural elements and hence location (Dowthwaite and Rajani 2000). Like most essential oils, the composition of the vetiver essential oil is extremely complex; it is known to contain more than 100 sesquiterpene compounds and their derivatives (Lavania 2003). Vetiver oil is rich in C_{15} sesquiterpenoids which can boil at over 200 °C (Dowthwaite and Rajani 2000).

The main constituent of the vetiver essential oil includes (Lavania 2003):

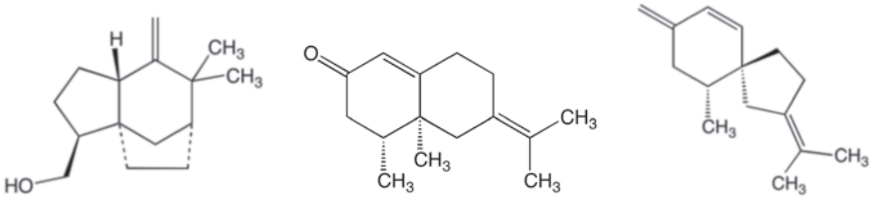
- Sesquiterpene hydrocarbons, e.g., cadenene, clovene, apomorphine, aromadendrin, junipene
- Sesquiterpene alcohol derivatives, e.g., vetiverols (45–80%) – khusimol (3.4–13.7%), epiglobulol, spathulenol, khusinol
- Sesquiterpene carbonyl derivatives (1.3–7.8%), e.g., α-vetivones, β-vetivone, khusimone
- Sesquiterpene ester derivatives, e.g., khusinol acetate

Besides these components, trace amounts of benzoic acid, vetivene, furfurol, khusemene, khusimone, ß-humulene, valencene, selinine, etc. are also present in the oil.

The three main odor-influencing constituents are known to be α-vetivone, β-vetivone, and khusimone (Bhatwadekar et al. 1982). α-Vetivone has a better odor and is considered the most important, while its major isomer nordihydro β-vetivone has a strong, rich, woody-peppery note. All these components individually and collectively contribute to the characteristic odor of the vetiver (Lavania 2003). Of course, α-vetivone, β-vetivone, and khusimol can be considered as the "fingerprint" of vetiver oil (Demole et al. 1995) (Fig. 1a–c).

Biosynthesis

As discussed earlier, vetiver oil is a complex sesquiterpene which is an outcome of cytosolic mevalonate-dependent pathway triggered by association of two acetyl coenzyme A units. The precursors in this pathway are isopentenyl diphosphate (IPP) and its allylic isomer dimethylallyl diphosphate (DMAPP). A concomitant



(a) Chemical Structure of Khusimol (b) Chemical Structure of α-vetivone (c) Chemical Structure of β-vetivone

Fig. 1 Chemical structures of three major odor-influencing components of essential oil vetiver

secondary metabolic pathway takes place in the plastids commonly known as MEP pathway. Although this subcellular compartmentalization allows both the pathways to operate independently in plants, there is evidence that they cooperate in the biosynthesis of certain metabolites (Laule et al. 2003; Kloer et al. 2006). According to Schalk and Deguerry (2013), the khusimol (an important constituent) results from oxidation of zizaene in the presence of cytochrome P450 reductase. Zizaene is apparently synthesized from farnesyl pyrophosphate (FPP) by the activity of zizaene synthase (Fig. 2). Since the biosynthetic pathway of vetiver EO is not yet clearly discussed, the present chapter unveils a plausible schematic representation of the secondary metabolic pathway of vetiver. The complexity of the EO stands as the major reason behind the tangled picture of its secondary metabolism and the mode of action of its active constituent.

Distillation

The efficiency of distillation is directly related to the age of the plant and stage of root harvest. The oil is obtained by distillation of either fresh or air-dried roots. The oil extracted from fresh roots has higher recovery of oil, while air-dried roots have higher quality of oil which can be attributed to natural evaporation of the undesirable nonpolar low boiling components of the oil. Three methods of distillation are traditionally practiced in India (Singh and Singh 1998). One of the three methods is *Bhapka* system popular in North India, while the other two methods make use of steam generating boilers and direct wood-fired distillers. For optimum recovery and economic productivity of oil, it is suggested that roots shall be harvested when the maximum day temperature is 25–27 °C. Roots should be distilled at the earliest after the harvest, and distillation should be performed just for about 15 h, with age of roots around 18 months (Aggarwal et al. 1998; Lavania 2003). The oil thus extracted is dehydrated either by anhydrous sodium sulfate or through natural evaporation by air-drying and allowed to mature by oxidation in amber bottle to improve quality and shelf life of the oil. The commercial distillation techniques include hydrodistillation, steam distillation, solvent extraction, and supercritical extraction. Among all, supercritical extraction is observed to be the most efficient (Martinez et al. 2004; Danh 2007; Danh et al. 2009).

Economics

The demand of vetiver oil is increasingly realized not only in India but world over. The unique base note of vetiver has grabbed the attention of flavor and fragrance industries in the recent times. Although Haiti contributes most of the oil to the world market, Indian vetiver oil still holds stronger grip because of its superlative quality. According to a report from CIMAP, North Indian vetiver oil is estimated to be best

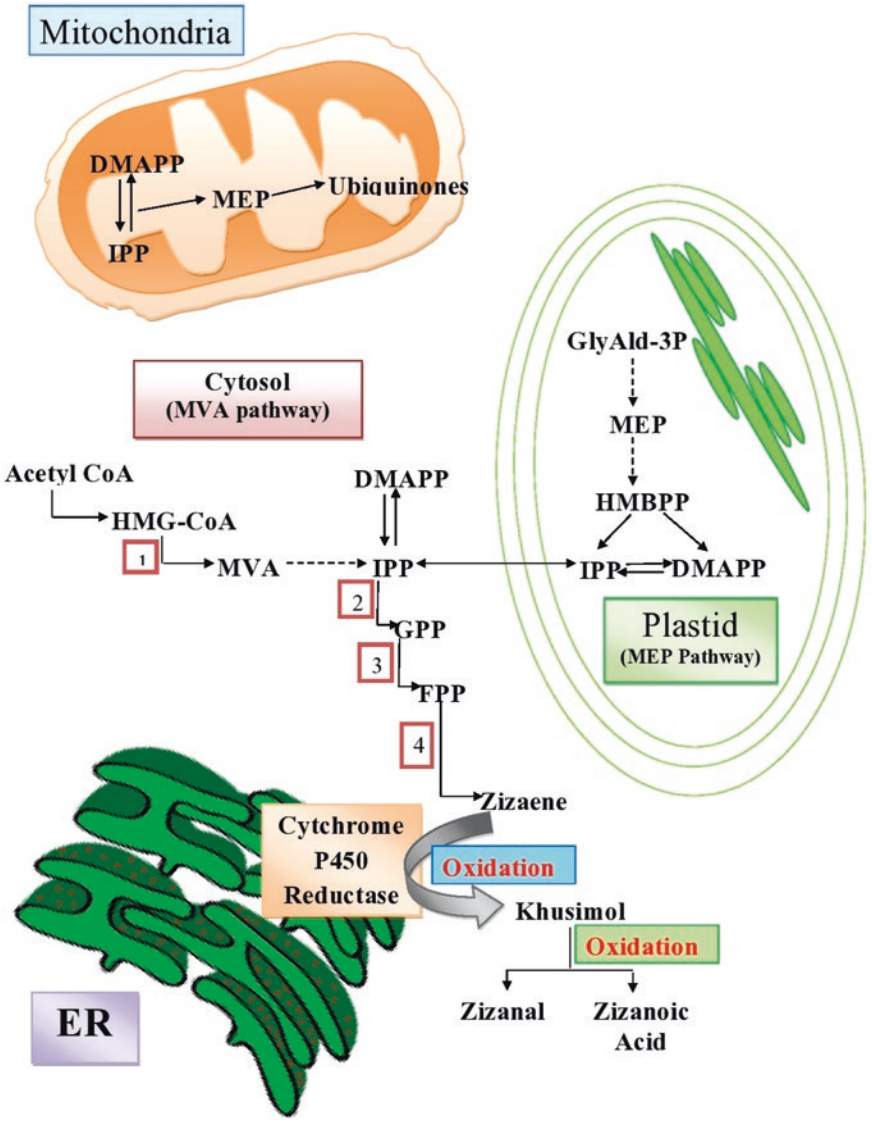


Fig. 2 Cellular compartmentalization of some of the important enzymes involved in the biosynthesis of some important sesquiterpenes in vetiver (dashed arrow means more than one step). After Laule et al. 2003 and Schalk and Deguerry 2013. DMAPP, dimethylallyl pyrophosphate; IPP, isopentenyl pyrophosphate; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; FPP, farnesyl pyrophosphate; GPP, geranyl pyrophosphate; HMBPP, 1-hydroxy-2-methyl-(E)-butenyl 4-diphosphate; MEP, methyl-D-erythritol 4-phosphate; MVA, mevalonic acid. Enzymes involved in sesquiterpene biosynthesis: 1, HMG-Co reductase; 2, geranyl pyrophosphate synthase; 3, farnesyl pyrophosphate synthase; 4, zizaene synthase

in quality and thus can be sold at Rs.13,000–17,000 per kilo, fairly costlier than South Indian type which is sold at only Rs.800–7000.00 per kilo. According to a latest report from Infodriveindia.com, during 27 July 2014 to 27 August 2014, India exported vetiver worth USD 7829 (473537.07 Indian rupees).

Ethnobotanical Uses

Not only the oil of vetiver finds its extensive use traditionally and commercially, but the rest of the plant parts too are found to be of prime importance. According to Chomchalow and Chapman (2003), vetiver can be utilized in two ways, i.e., either in the planted form or in the harvested form. The planted form has conventional as well as nonconventional uses. The conventional use mainly encircles its use in phytoremediation giving vetiver a gaining popularity in the recent times. The nonconventional uses, however, encompass the less popular uses such as livestock grazing, ornamentals, and barriers.

Due to wide ecological amplitude, vetiver is voluminously used in:

- Soil and water conservation
- Erosion control
- Slope stabilization
- Absorption of heavy metals (utilization of vetiver grass in stabilizing slime dams in the mining industry)
- Wastewater treatment

Harvested form of vetiver has been utilized both traditionally and commercially. Vetiver roots yield essential oil, while other plant parts serve different other purposes. Thus it implicates wide use of vetiver ranging from pharmaceuticals to handicrafts to flavor and fragrance industry.

Traditional Application

Vetiver has been popular among the tribes long before the utilities of vetiver drew its actuality before the world. They used different parts of the grass for many of their ailments such as mouth ulcer, fever, boil, epilepsy, burn, snakebite, scorpion sting, rheumatism, fever, headache, etc. (Rao and Suseela 2000).

Some traditional uses of *Vetiveria zizanioides*

Plant part	Tribe	Ailment
Root decoction	Santhals	As cooling in high fever, inflammation, sexual diseases, etc.

Plant part	Tribe	Ailment
Root paste	Lodhas	Headache, fever, Ayurvedic preparation "Brihat Kasturi," "Bhairava Rasa" for fever, diarrhea, chronic dysentery
Root ash	Oraons	Acidity
Root juice	Tribes of MP	Antihelmintic
Root vapor	Tribes of Varanasi	Malarial fever
Vetiver oil	Most tribes	Stimulant, diaphoretic, and refrigerant
Leaf paste	South Indian tribes	Rheumatism and sprain
Root and stem juice	South Indian tribes	Boil, burn, epilepsy, scorpion sting, snakebite, and mouth ulcer

Table courtesy: (Rao and Suseela 2000)

Nutraceutical Application

Extracts from the spent/waste part may find use as dietary/supplementary antioxidant in nutraceutical and/or cosmeceutical for protection against complications arising from the oxidative stress. The plants produce many types of scavenger molecules, mainly phenolic compounds; two new flavonoids from *V. zizanoides* and *V. nigritana* have recently been reported (Luqman et al. 2009).

Commercial Applications

The commercial applications of the vetiver grass reckon on the extraction of vetiver oil through distillation of the roots.

Agriculture-Related Uses

Manure

Vetiver grass system is becoming rapidly a global household name in soil conservation. It has been found to effectively enhance the soil quality of an eroded land when applied as vetiver grass strips, vetiver mulch, and composted vetiver prunes (veticompost) (Are et al. 2012).

Pesticide

Vetiver grass can also act as botanical pesticide since they are reported to have properties like insecticide, fungicide, and acaricide. They also exhibit allelopathy as vetiver extract contains in vetiver oil has allelopathic effect in inhibiting the germination of seeds of any plant growing in its vicinity (Balasankar et al. 2013).

Weed Control

When spread evenly on the ground, whole or desiccated vetiver leaves form a thick matt that suppresses weeds (Balasankar et al. 2013).

Flavoring Agent

Flavor industry uses vetiver oil widely for making flavored supari, pan masala, sharbat, and zarda (Balasankar et al. 2013).

Perfumery

Vetiver has marked its presence with its exclusive contribution to perfume industry in India and world over. It is a Gift of India (Morris 1983) to the world of perfumes, and its use in scents (*attar*) is known in India much before the world became familiar with rose scents. On account of its pleasing aroma and slow evaporation rate falling under the category of lower "base note," vetiver oil as such is a "perfume in its own right" for which no synthetic substitute is yet available. Vetiver oil is the basis of the Indian perfume "Majmua" and is the major ingredient in some 36% of all western perfumes (e.g., Caleche, Chanel No. 5, Dior essence, Parure, Opium) and 20% of all men's fragrances. In addition to its direct perfumery applications, vetiver oil in its diluted form is extensively used in aftershave lotions, air fresheners, and bathing purposes, as well as flavoring syrups, ice cream, cosmetic, and food preservation. Khus essence is used in cool drinks and for reducing pungency of chewing tobacco preparations, providing sweet note to other masticatories and incense sticks (Lavania 2003).

Aromatherapy

Vetiver oil owes several beauty benefits and emotional effects. It balances the activity of the sebaceous oil glands, has deodorizing properties, and helps normalize oily skin and clear acne. It replenishes moisture in dry and dehydrated skin and has a rejuvenation effect on mature skin, as well as cuts/wounds/irritated and inflamed skin. When used regularly during pregnancy, vetiver oil reportedly prevents stretch

marks. The oil strengthens the central nervous system and is helpful in overcoming depression, insomnia, anxiety, stress, tension, and nervousness (Wilson 1995).

Other Uses

Refrigerant

Vetiver roots are multifariously used for household and coolant purposes, viz., stuffed in the walls of desert coolers, as the tufted fibrous roots are endowed with high tensile strength, rich aroma, and refrigerant properties. Dried roots are also used to odorize linen and clothes (Lavania 2003).

Handicrafts

The vetiver roots have also been employed for making woven screens, mats, blinds (*chik*), hand fans, broom hangers, and baskets (Lavania 2003).

Construction

India being the indigenous user of vetiver roots exploits root mats to form *khus-tatti* (panels) to make huts and cabins for providing cooling effect during hot summers.

Dried culms are used as brooms and for thatching. Pulp of the plant is used for paper and straw board (Lavania 2003).

Textiles

Vetiver also suits to produce soft, durable fabric (Lavania 2003).

Medicinal Uses and Health Benefits

The vetiver oil extracted from roots proves highly beneficial to human health owing to its properties, like anti-inflammatory, antiseptic, aphrodisiac, cicatrisant, nervine, sedative, tonic, and vulnerary, for healing and calming. The vetiver oil is also known to possess several beauty benefits as it replenishes moisture in dry and dehydrated skin, rejuvenation of mature skin, as well as cuts wounds irritated and inflamed skin (Lavania 2003). The oil also improves the central nervous system, thereby combating depression, insomnia, anxiety, tension, stress, and nervousness. It makes a useful warming and pain-relieving rubbing oil, suitable for deep massage of muscular aches and pains, sprains, stiffness, rheumatism, and arthritis. It is also reported to act as antimicrobial and antifungal agent (Singh et al. 1978; Dikshit and Husain

1984). Moreover, vetiver oil has also been reported to have anticancer activity (Chen et al. 2003) and antioxidant activity (Kim et al. 2005).

An Update of Therapeutic Potentials of *Vetiveria zizanioides*

Like jungle terrain, vetiver also possesses therapeutic potentials, most of which still remain unexplored due to the complex nature of its essential oil, yet it is very rich in possibilities. Very few other remedies, aromatic or otherwise, exert the same depth of restorative action as vetiver, repairing body's four core systems: the nervous, endocrine, gastrointestinal, and immune.

Insecticidal Activity

Vetiver oil has great potential to be used as insecticide. Extracts of vetiver oil have repellent and toxicant properties toward ants, ticks, and cockroaches (Handerson et al. 2005). Earlier it was believed that tricyclic sesquiterpenoids, zizanal and epizizanal, isolated from vetiver oil were responsible for insect repellent activity (Jain et al. 1982). Later on it was observed that at least six compounds, i.e., α- and β-vetivone, khusimone, zizanal, epizizanal, and (C)-(1S,10R)-1,10-dimethylbicyclo[4,4,0]-dec-6-en-3-one, were found to be repellent to different insects (Babprasert and Karintayakit 1996).

Vetiver oil extracts showed repellent and anti-oviposition properties when applied to *Ceratitis capitata* (Mishra 2000). Flavonoids isolated from aqueous extracts of vetiver oil showed 80% insecticidal activity against lepidopterous stem borers on maize (Ndemah et al. 2002) in the humid tropics of Western Africa at a concentration of 0.07 mg/mL.

Termicidal Activity

The crude oil extracted from *V. zizanioides* significantly reduced the leaf damage caused by larvae of subterranean termite (Chomchalow et al. 1970). Three compounds, viz., nootkatone, zizanol, and bicyclovetivenol, isolated from vetiver oil showed repellent activity against Formosan subterranean termite (*Coptotermes formosanus*). Simple chemical modifications such as oxidation and reduction can be used to target different compounds present in vetiver oil so that the end product is directly used for pest control (Zhu et al. 2001; Nix et al. 2003). Vetiver oil in combination with nootkatone and disodium octaborate tetrahydrate acted as arrestant, repellent, and feeding deterrents against *C. formosanus*, and its symbiotic fauna

significantly decreases their tunneling activity, wood consumption, and survival (Maistrello et al. 2001; Maistrello et al. 2003; Ibrahim et al. 2004).

Pesticidal Activity

Vetiver oil also found to be effective against stored grain pests. Adults of *Tribolium castaneum* were repelled by contact with food medium treated with 2 and 5 g of vetiver grass oil dust/10 g flour (Vanden et al. 2000). Root extracts of vetiver in petroleum ether, ethyl acetate, acetone, and methanol showed insecticidal activity against XSM, SMC, SKS, and JTC strains of red rust flour beetle *T. castaneum*. In larval bioassay the highest toxicity was recorded for petroleum ether (LD50 = 0.051 g/cm^2) in XSM strain whereas the lowest toxicity using methanol extract (LD50 = 11.351 g/cm^2) in SMC strain (Sujatha 2010).

Similarly, nonpolar petroleum ether fractions of vetiver oil consisting mainly of four nonadjacent bis-tetrahydrofuranic acetogenins, named squamostatins B to E, showed insecticidal activity against *Sitophilus oryzae* infesting wheat seeds (Mishra 2000); whereas acetone extracts from fresh and stored leaves were toxic to adults of *Callosobruchus maculates* and ethanol extracts were found to be noninsecticidal (Pangnakorn 2009).

Anti-plasmodial (Antimalarial) and Larvicidal Activity

The oil also showed larvicidal and repellent activity against malarial vector, *Anopheles stephensi*, causing 85% mortality. The observed mortality rate suggested that the extracts can be used as biopesticides. The LC50 values of second, third, and fourth instar larvae of *A. stephensi* were 0.276, 0.285, and 0.305%, respectively (Aarthi and Murugan 2010).

Anti-tick Activity

Vetiver root extracts were able to control the growth of ticks during larval and adult stage including egg-laying stage of cow ticks *Boophilus microplus*. The ethanol extract had highest potential as compared to other extracts in controlling cow ticks (Korpraditkul et al. 1996).

The chemical analysis indicated that the sesquiterpene fraction is dominant in the composition of vetiver essential oils, though khusimol is the main active compound. Previous studies have demonstrated that monoterpenes and sesquiterpenes have acaricidal activity against *R. microplus* (Facey et al. 2005). These results indicate that the *C. zizanoides* essential oils are promising candidates as acaricidal

agents. In this study *C. zizanioides* essential oils reduced the production of eggs by female tick and the hatch of eggs, with consequent lower rates of tick reproductive efficiency. In addition, the rate of reduction of reproductive capacity observed in ticks treated with *C. zizanioides* essential oils was higher than that observed with the reference commercial products Natuneem (*A. indica* oil) and Butox P CE25 (deltamethrin). Concerning the activity of vetiver essential oils on tick larval stages, results here obtained showed that these essential oils have significant potential as acaricides for the control *A. cajennense* and *R. microplus* larvae if compared with the larvicidal activity of other plant-derived compounds.

Antibacterial Activity

Several studies have shown that vetiver oil possesses antibacterial activity against various bacterial strains like *Staphylococcus aureus*, *Streptococcus pyogenes*, *Escherichia coli*, and *Corynebacterium ovis*. The inhibition by pure oil was 60–70% more than penicillin (Gangrade et al. 1990). The hexane extracts of inflorescence, intact roots, and spent roots, upon hydrodistillation of the essential oil from two genotypes ("Gulabi" and "KS-1") of *V. zizanioides*, were evaluated for antibacterial activity against wild-type, drug-resistant strains of *Mycobacterium smegmatis* and *E. coli* using disk diffusion and micro-broth dilution methods. The extract showed antibacterial activity against both the strains (Luqman et al. 2005). The antibacterial activities of vetiver oil extracts (10 mg/mL) in polar solvents (methanol, chloroform) and nonpolar solvent (hexane) were tested against *S. aureus*. Chloroform extract showed antibacterial activity using cup borer method on the solid agar media (Sangeetha and Stella 2012; Barad et al. 2013).

The crude root extract of *V. zizanoides* (L.) Nash cultivar "Surat Thani" showed antimicrobial activity against four pathogenic bacteria. The alkaloid vetiverin (Khesorn et al. 2010) showed minimum inhibitory concentration of 1.626 mg/mL. Polar ethanolic extract showed better antibacterial activity against *S. aureus*, *E. coli*, *Pseudomonas aeruginosa*, and *Bacillus subtilis* as compared to aqueous extract. The phytochemical analysis revealed the presence of flavonoids, glycosides, phenol, tannins, saponins, and alkaloids (Dahiya et al. 2011).

Vetiver oil also showed antibacterial activity against various strains of bacteria like *Acinetobacter baumannii*, *Aeromonas veronii*, *Candida albicans*, *Enterococcus faecalis*, *E. coli*, *Klebsiella pneumoniae*, *P. aeruginosa*, *Salmonella enterica*, *Serratia marcescens*, and *S. aureus*. The minimum inhibitory concentration (Hammer et al. 1999) was found to be 0.008% (v/v).

The root extract showed larger zone of inhibition than leaf extract against two pathogenic bacteria *E. coli* (MTCC 443) and *S. aureus* (MTCC 737). All these results confirmed that the extracts of vetiver are pharmacologically important and could be used for human ailments (Jayashree et al. 2011).

Antifungal Activity

Vetiver oil showed a broad range of natural fungicidal effect against pathogens. Antifungal activity of transformed products of sesquiterpenoids present in vetiver oil was tested against two phytopathogenic fungi, i.e., *Alternaria alternata* (causing early blight of tomato and potato) and *Fusarium oxysporium* (causing wilt of tomato), using spore germination inhibition technique. Out of various compounds tested, khusinodiol monobrosylate was found to be effective antifungal agent against both the fungi (Dikshit and Husain 1984). Schiff bases of sesquiterpenoid, i.e., N-(Khusilidene)-p-methoxy aniline and N-(Khusilidene)-p-bromo-aniline, were synthesized by reaction with p-methoxy aniline and p-bromo-aniline. It was found that N-(Khusilidene)-p-methoxy aniline inhibited the growth of A. *alternata* up to 84.7%, whereas N-(Khusilidene)-p-bromo-aniline inhibited the growth of *F. oxysporium* up to 74.5% at 1 mg/mL level (Kaushal and Chahal 2008).

The antifungal potential of vetiver oil was also observed against *Rhizoctonia bataticola* and *Sclerotium rolfsii* (Sharma et al. 2009). Antifungal potential of root and shoot extracts of vetiver was studied against two potent pathogenic fungi, *Candida albicans* and *Cryptococcus neoformans*. The results of MIC of root and leaf fractions against pathogens (Jayashree et al. 2011) were 10 mg/mL, and IC50 of these fractions varied between 5 and 7.5 mg/mL.

Two types of Indian vetiver oils, namely, North and South Indian types, were evaluated and found to exhibit antifungal activity against *Rhizoctonia solani*. Fungal toxicity of South Indian vetiver oil was slightly higher than North Indian oil (Dubey et al. 2010).

The antifungal activity of vetiver oil was further screened against certain pathogenic microorganisms using fluconazole as positive control. Among the tested fungal cultures, *Aspergillus niger* exhibited a highest mean zone of inhibition (30 and 32 mm) against vetiver leaf and root extracts (Sangeetha and Stella 2012).

Herbicidal Activity

It was earlier hypothesized that certain substances excreted by the vetiver plant had allelopathic action due to inhibition of the growth of other plants. Now it has been confirmed that root and stem extracts of vetiver inhibited the germination of soybean seeds, and thus it could be applied for weed control (Techapinyawat 1994). Germination tests in Petri dishes were carried out to test the effect of vetiver oil and one of its minor components nootkatone on six common weed species: redroot pigweed, common lamb's quarters, giant ragweed, pitted morning glory, and velvet leaf. Vetiver oil inhibited germination of these weeds, in addition to inhibition of seedling expansion of redroot pigweed and common lamb's quarters acting as useful herbicide (Lixin et al. 2004). Vetiver oil and its component nootkatone also reduced plant growth in *Pisum sativum* L. (plant height, root length, dry weight) in

the laboratory and citrus trees under field conditions (Lixin et al. 2006). Allelopathic interaction of vetiver oil with two nonedible oil-yielding fence plants *Jatropha curcas* L. and *Ricinus communis* L. was tested and found that vetiver plant promoted the growth of jatropha seedlings and inhibited the growth of *R. communis* seedlings suggesting vetiver jatropha to be a suitable fence plant option for plant-plant interaction (Vimala et al. 2005).

Antioxidant Activity

Vetiver oil showed antioxidant and anti-inflammatory activities and can be used in medicine and perfumery (Chou et al. 2012). Antioxidant properties of vetiver oil were evaluated by two different in vitro assays: the DPPH• (1,1-diphenyl-2-picrylhydrazyl) free radical scavenging assay and the Fe^{2+} metal-chelating assay. Results revealed that the vetiver oil possessed a strong free radical scavenging activity as compared to standard antioxidants such as butylated hydroxytoluene (BHT) and alpha-tocopherol. Vetiver oil (0.01 mg/mL) dissolved in methanol exhibited 93 and 34% free radical scavenging activity in the DPPH• and Fe^{2+} chelating activity in the metal-chelating assay, respectively. In the crude vetiver oil, β-vetivenene, β-vetivone, and α-vetivone showed strong antioxidant activities (Kim et al. 2005).

Ferric reducing free radical scavenging and antioxidant activity of two genotypes, namely, "KS1" and "Gulabi" of vetiver oil, was investigated using in vitro assay, the ferric reducing antioxidant power (FRAP), DPPH, total phenolic content (TPC), total antioxidant capacity, and reducing power (RP). "KS1" genotypes showed higher FRAP values, DPPH inhibition, TPC, and RP potential as compared to "Gulabi." The antioxidant activity increased with the extract concentration (0.01–1 mg/mL) (Luqman et al. 2009).

The ethanolic and ethyl acetate extracts of roots of *V. zizanioides* showed free radical scavenging activity by DPPH model. The extracts showed significant dose-dependent free radical scavenging activity (Kumar et al. 2010).

Anticancer Activity

The aqueous extract of *Vetiveria zizanioides* root exhibited cytotoxic effect against human cancer cell line (MCF-7 human breast cancer cell line). The vetiver oil extract showed cytotoxic activity on the breast adenocarcinoma (MCF-7) cell line in a concentration-dependent manner. The extract on MCF-7 cell line produced a 50% of net killing (IC50) at the doses 37 μg and 31 μg/mL at 24 and 48 h, respectively (Chitra et al. 2014).

Previously, the study of Chen et al. (2003) showed that vetiver essential possessed anticancer activity. At 100 ppm in cancer cell lines, vetiver oil inhibited the

growth up to 89% of SiHa cervical cells, 88% of CaSki cervical cells, and 89% of MCF-7 breast cancer cells.

Sedative Activity

The sedative effects of vetiver oil upon inhalation in rats were observed in rearing motilities in the open field test (Thubthimthed et al. 2003). The results showed that vetiver oil decreased rearing motility when compared to the positive control lavender oil group.

Antidiabetic Activity

Studies showed that ethanol extract of *V. zizanioides* roots possessed antihyperglycemic activity (Karan et al. 2012). It was observed that ethanol extract of *V. zizanioides* (100, 250, 500, 750 mg/kg body weight) significantly reduced the blood glucose level at the end of 28 days in alloxan-induced diabetic rats.

Antidiuretic Activity

The sesquiterpene alcohol khusimol isolated from vetiver roots was found to competitively inhibit the binding of vasopressin to rat liver V1a receptors (Rao et al. 1994).

Anti-inflammatory Activity

VEO has shown strong anti-inflammatory activities in lipopolysaccharide (LPS)-induced RAW 264.7 macrophages by regulating the expression of the inflammation-related enzymes heme oxygenase-1, inducible nitric oxide synthase, and cyclooxygenase-2 (inducible cyclooxygenase) as well as the inflammatory cytokines tumor necrosis factor-α, interleukin-1β, and interferon-β (Chou et al. 2012).

Han and Parker (2017) studied the activity of vetiver essential oil (VEO) in a dermal fibroblast system, HDF3CGF, which features the microenvironment of inflamed human skin cells with boosted inflammatory and immune responses. Four concentrations of VEO (0.001, 0.00033, 0.00011, and 0.000037%, v/v in DMSO) were tested for cell viability. VEO showed significant antiproliferative activity in dermal fibroblasts. In addition, VEO significantly inhibited the production of collagen III, an extracellular matrix protein and fibrillar collagen found extensively in

connective tissues that is critically involved in the tissue remodeling process. This finding suggests that VEO might play a role in modulating the tissue remodeling process. Following intraperitoneal (i.p.) injection, EO at 50 and 100 mg/kg significantly reduced the number of writhes (51.9 and 64.9%, respectively) and the number of paw licks during phase 2 (56.7 and 86.2%, respectively) of a formalin model when compared to control group animals. However, EO-treated mice were ineffective at all doses in hotplate and rota-rod tests. The EO inhibited the carrageenan-induced leukocyte migration to the peritoneal cavity in a dose-dependent manner (34.7, 35.4, and 62.5% at doses of 25, 50, and 100 mg/kg, respectively). In the paw edema test, the EO (100 mg/kg) inhibited all three phases of the edema equally well, suggesting that the EO has a nonselective inhibitory effect on the release or actions of these mediators. Our results suggest possible antinociceptive and anti-inflammatory effects of the EO.

Conclusions

Unequivocally, the production of essential oils greatly underpins the fragrance and flavor industry, but the importance of these natural extracts is also increasing in pharmaceutical and natural cosmetic industry along with their use as nutraceutical ingredients in recent times which have opened up new vistas for industrial sector. Consequently, India is expected to play a dominant role in the production and processing of these natural extracts. Country's biodiversity coupled with competent scientific force makes India as the best choice to become a foremost leader in aroma business in the coming years. Going through the immense uses vetiver has, it's the need of the hour to accentuate its production to an extent where it can meet the demands of local as well as world market. India being the native country of vetiver holds back the originality and distinct note of the oil which cannot be reconstituted proving itself as a boon to commercial market. Presently, world demand of vetiver oil is 500 MT, and production in India is far too less. If, along with floriculture and horticulture, vetiver is taken as an intercrop, then we can easily reach the production capacity of 1000 MT, and there is enough market for the same. In the last 1½ years due to thrust area, 400% growth of essential oil is seen, and lot of vetiver will also come in due course of time and has good future.

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Evidence-Based Assessment of *Moringa oleifera* Used for the Treatment of Human Ailments

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Introduction

A wide range of several medicinal plants have been used traditionally over time, but it is also now widely becoming familiar in modern medicinal era for containing natural chemicals that may be used as an alternative against chemicals synthesized artificially (Verma et al. 2011; Abdelmalek et al. 2016). Knowledge can be gained from both scientific origins and traditionally old sources (García-Alvarado et al. 2011). Any collective body of information, traditional practices, and thoughts that have been passed down from generation to generation and different cultures can be called traditional knowledge (Berkes et al. 2003). Traditional medicine is based on medicinal plants that are readily available and is commercialized based on practiced traditional knowledge (Awas and Demissew 2009). Herbal medicine is popular as it is widely culturally recognized, is affordable, and is proven to be effective against some diseases in comparison to synthetic medicines. Indigenous groups all over the world have experiences in these kinds of plants and use their knowledge to classify plants and their various parts based on treating various conditions (Omoruyi et al. 2012). *Moringa oleifera* is one of the important medicinal plants commonly known as the "miracle tree," referring to the species being an important food crop and source of nutritious fruits and flowers in the developing countries or regions like

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Southeast Asia, African regions, and North and South America (D'souza and Kulkarni 1993; Anwar and Bhanger 2003; Anwar et al. 2005, Al-Asmari et al. 2015). Leaves, barks, seeds, flowers, and fruits of *Moringa oleifera* were widely used in traditional medicine (Stohs and Hartman 2015). The leaves contain large amounts of proteins, vitamins, minerals, and natural antioxidants like flavonoids, carotenoids, and phenolics that increase shelf life of fat- containing foods (Dillard and German 2000; Siddhuraju and Becker 2003a, b). *Moringa oleifera* pods and leaves are an abundant source of calcium, magnesium, potassium, manganese, phosphorous, zinc, sodium, copper, iron, etc. (Aslam et al. 2005). The mineral content varies throughout different geographical regions (Anjorin et al. 2010).

All parts of *Moringa oleifera* are edible and thus have been consumed by humans for a long time. Fuglie (1999) has stated that other uses of *Moringa oleifera* include biomass generation, animal fodder, biogas, traditional cleaning agent, production of blue dye, fertilizers, honey, gum, leaf juice, manure, use as a clarifier in sugarcane juice, use as fences, ornaments and pesticides, pulp production from wood and ropes from bark, tanning hides, as well as purifying water. In Western countries, *Moringa oleifera* seeds are powdered and used to remove contaminants from drinking water (Berger et al. 1984; Gassenschmidt et al. 1995; Olsen 1987) and also eaten raw or used to flavor curries and tea (Gassenschmidt et al. 1995). Since, Moringa *oleifera* is readily available and grows well in drought, relatively arid soils, and other difficult environmental conditions, it can be used in economic and medicinal health purposes in developing countries.

Botanical Description

Moringa oleifera belongs to the family of Moringaceae. *Moringa oleifera* leaves are imparipinnate, upper leaf surface colliculate, lower leaf surface tuberculate-reticulate, leaflet shape oboordate, symmetric base, emarginate apex, upper surface hairy, and lower one smooth. Seeds are round with tan "frilled" edges and brown and have rough texture. It has straight trunk, umbrella-shaped crown, and whitish bark. The pods are triangular in cross section which are legume-like in appearance, and the flowers are creamy white in color (Fig. 1).

Moringa oleifera, a well-known and majorly distributed species (Nadkarni 1976; Ramachandran et al. 1980), originates from Southeast Asia, Africa, and Arabian Peninsula and is now also distributed to North and South America, including the Caribbean nations (Somali et al. 1984; Mughal et al. 1999; Morton 1991).

Moringa oleifera grows well under hot climate conditions, being well-tolerant to drought. It grows with 250–1500 mm rainfall per year. The tree grows very rapidly, reaching a peak of 15 ft in a year. On average, the species grow up to 600 m; however, in some tropical areas, it is much higher, with a record growth at 2000 m. *Moringa oleifera* tree grows well in loam soil or well-drained sandy soil with a pH range of 5–9. *Moringa oleifera* grows easily from seeds and cuttings. It is suggested

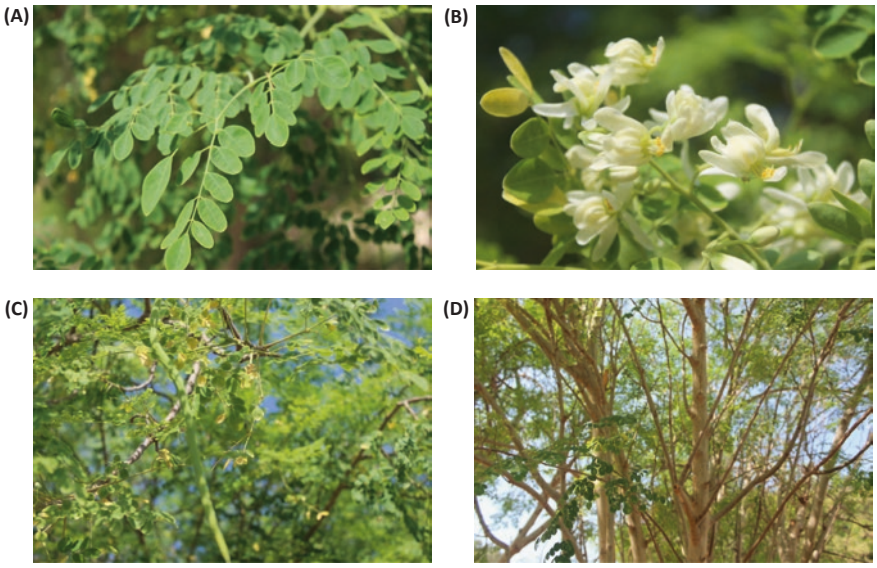


Fig. 1 Usable plant parts of *Moringa oleifera*. Leaves (**a**), flower (**b**), single pod (**c**), stem, and barks (**d**)

that the seeds are planted as deep as 2 cm so that they can germinate within 2 weeks. The best light conditions would be half shade for germination.

Nutrition Value

Moringa oleifera is a rich source of amino acids, antioxidants, and other antiaging and anti-inflammatory agents which makes it the most nutrient-rich plant (Table 1). The leaf of *Moringa oleifera* is a source of high nutrient contents (Zhang et al. 2017). Thus, the plant can be used to treat deficiency of nutrients instead of using imported food supplies (Johnson 2005; Manzoor et al. 2007; Sreelatha and Padma 2009). It contains a combination of all required nutrients for human beings and livestock animals (Fahey 2005). Reportedly, *Moringa oleifera* contains 7 times more vitamin A compared to oranges, 10 times more vitamin A compared to carrots, 15 times more potassium compared to bananas, 9 times more proteins compared to yogurt, 17 times more calcium compared to milk, and 25 times more amount of iron compared to spinach (Fuglie 1999). Other studies have noted the abundance of vitamin B complex and dietary elements like Cr, Cu, Mn, Mg, K, P, and Zn (Fuglie 2000, 2001). *Moringa oleifera* leaves are abundant in proteins and thus can be used to address the global malnutrition crisis (Thurber and Fahey 2009) (Table 2).

Table 1 Nutrition value of *Moringa oleifera. Moringa* leaves and pods have shown to contain the following per 100 g of edible portion adapted from Fuglie (1999)

Compounds	Leaves	Pods
Protein (g)	6.7	2.5
Carbohydrate (g)	13.4	3.7
Fiber (g)	0.9	4.8
Calories	92	26
Moisture	75	86.9
Minerals (g)	2.3	2
K (mg)	259	259
P (mg)	70	110
Mg (mg)	24	24
Cu (mg)	1.1	3.1
Fe (mg)	7	5.3
S (mg)	137	137
Ca (mg)	440	30
Vitamin A-B carotene (mg)	6.8	0.11
Vitamin B-choline (mg)	423	423
Vitamin B1-thiamin (mg)	0.21	0.05
Vitamin B2-riboflavin (mg)	0.05	0.07
Vitamin B3-nicotinic acid (mg)	0.8	0.2
Vitamin C-ascorbic acid (mg)	220	120
Arginine (mg)	402	90
Histidine (mg)	141	27.5
Lysine (mg)	288	37.5
Tryptophan (mg)	127	20
Phenylalanine (mg)	429	108
Methionine (mg)	134	35
Threonine (mg)	328	98
Leucine (mg)	623	163
Isoleucine (mg)	422	110
Valine (mg)	476	135

Medicinal Properties

Phytochemicals are molecules that can affect health and also the flavor, smell, and color of plants, but generally are not necessary for human nutrition. *Moringa* species have phytochemicals containing rhamnose, which is found abundantly in isothiocyanates and glucosinolate compounds, and also it has niazimicin, pterygospermin, and benzyl isothiocyanate which have shown antibacterial activity and activity against cancer and are helpful in lowering blood pressure (Faizi et al. 1998a, b, c; Fuglie 1999, 2000, 2001; Fahey et al. 2004; Costa-Lotufo et al. 2005; Al-Asmari et al. 2015). Several studies have shown other effects of treatment with *Moringa oleifera* phytochemicals, such as increase in detoxification and elevated levels of enzymes responsible for antioxidant activity. The phytochemicals have

Table 2 The therapeutic and prophylactic uses of *Moringa oleifera* adapted from Fahey (2005)

Medicinal use against the diseases	Plant parts	References
Typhoid	Gum	Fuglie (1999)
Syphilis	Gum	Fuglie (1999)
Infection	Leaves, flower	Fuglie (1999)
Urinary tract infection	Leaves	Shaw and Jana (1982)
HIV-AIDS	Leaves	Abrams et al. (1993), Prazuck et al. (1993)
Thrush	Oil	Fuglie (1999)
Bronchitis	Leaves	Fuglie (1999)
Thrush	Oil	
Earache	Gum	Fuglie (1999)
External sores	Leaves, flowers, roots, barks	Fuglie (1999)
Fever	Leaves, roots gum	Fuglie (1999)
Throat infection	Flowers	Fuglie (1999)
Antitumor	Leaves, flowers, seed, barks	Bharali et al. (2003), Costa-Lotufo et al. (2005), Fahey et al. (2004), Faizi et al. (1998a, b, c), Gupta et al. (1997), Hartwell (1971)
Prostate	Leaves	Fuglie (1999)
Skin	Pods	Bharali et al. (2003)
Antianemic	Leaves	Fuglie (1999), Quisumbing (1978)
Cardiotonic	Roots	Fuglie (1999)
Diabetes	Leaves, pods	Asres (1995), Faizi et al. (1998a, b, c), Kar et al. (1999), Makonnen et al. (1997)
Diuretic	Leaves, flowers, roots, gum	Asres (1995), Caceres et al. (1992), Nisa et al. (1998)
Thyroid	Leaves	
Hepatorenal	Leaves, roots	Mazumder et al. (1999), Pari and Kumar (2002)
Purgative	Oil	Fuglie (1999)
Snakebite	Barks	Fuglie (1999)
Scorpio bite	Barks	Fuglie (1999)
Colitis	Leaves, barks	Fuglie (1999)
Diarrhea	Leaves, roots	Fuglie (1999), Nisa et al. (1998)
Digestif	Barks	Fuglie (1999)
Dysentery	Leaves, gum	Fuglie (1999)
Flatulence	Roots	Fuglie (1999)
Ulcer/gastritis	Leaves, seeds	Pal et al. (1995), Ruckmani et al. (1998a, b)
Rheumatism	Leaves, flowers, seeds, pods, roots, gum	
Joint pain	Pods	Fuglie (1999)
Edema	Roots	Fuglie (1999)
Arthritis	Seeds	Fuglie (1999)

(continued)

Table 2 (continued)

Medicinal use against the diseases	Plant parts	References
Immune-stimulant	Seeds	Jayavardhanan et al. (1994)
Antispasmodic	Seeds, roots	Caceres et al. (1992), Gilani et al. (1994)
Hysteria	Flowers, roots, barks, oil	Fuglie (1999)
Headache	Leaves, roots, barks, gum	Fuglie (1999)
Abortifacient	Flowers, roots, barks, gum	Nath et al. (1992a, b), Nath et al. (1997), Tarafder (1983)
Aphrodisiac	Roots, barks	Fuglie (1999)
Birth control	Barks	Gilani et al. (1994), Faizi et al. (1998a, b, c)
Lactation enhancer	Leaves	Fuglie (1999)
Prostrate function	Oil	Fuglie (1999)
Antiseptic	Leaves	Fuglie (1999)
Astringent	Seeds	Fuglie (1999)
Pyoderma	Roots, gum	Caceres and Lopez (1991)
Vesicant	Roots	Fuglie (1999)
Bladder	Oil, seeds	Fuglie (1999)
Gout	Roots, oil	Fuglie (1999)
Hepatomegaly	Roots	Fuglie (1999)
Scurvy	Leaves, seeds, roots, bark	Fuglie (1999)
Tonic	Pods, seeds, oil	Fuglie (1999)

also shown effects on immunological response, antispasmodic activity, activity against ulcers, antimicrobial activity, effects of hypercholesterolemia (Talreja 2010), antihypertensive activity, and antiviral activity specifically against herpes simplex virus type 1 (Gilani et al. 1994; Hameed-Un-Nisa et al. 1998; Ghasi et al. 2000; Galan et al. 2004; Haristoy et al. 2005). The major role of antioxidants is to inhibit the free radicals so that the human body is protected against degenerative disorders and infections. *Moringa oleifera* oil was known to be used for skin beautification preparations since ancient Egyptian periods (Sairam 1999; Fuglie 2000; Monica 2005).

Antispasmodic, Antiulcer, and Hepatoprotective Activities

The roots of *Moringa oleifera* have shown to relieve involuntary muscle spasms (Caceres et al. 1992). Studies have shown that the ethanolic extracts of its leaves show antispasmodic effects, possibly via a mechanism that includes the calcium channel blockade (Gilani et al. 1992, 1994; Dangi et al. 2002).

4-[α-(L-Rhamnosyloxy) benzyl]- o-methyl thiocarbamate (trans) has been identified as the major compound responsible for antispasmodic activity; thus the leaves were traditionally used to treat diarrhea (Gilani et al. 1994). *Moringa oleifera* contains compounds that have reportedly shown spasmolytic activity, which explains why the plant is traditionally used to treat gastrointestinal motility problems (Gilani et al. 1994). Since aqueous leaf extracts have also exhibited activity against ulcer (Pal et al. 1995), this suggests that components responsible for such activity are abundant in *Moringa oleifera.* Its roots have shown a protective effect on the liver (Ruckmani et al. 1998a, b) as well as the aqueous and alcoholic extracts of flowers (Ruckmani et al. 1998a, b) which could possibly be due to quercetin, a flavonoid that has exhibited hepatoprotective activity (Gilani et al. 1997).

Antihypertensive, Diuretic, and Cholesterol-Lowering Activities

This plant is extremely useful for treating cardiovascular disorders as it contains compounds that can lower blood pressure and lipid levels and increase production of urine. Its leaf juice is used to stabilize blood pressure (Dahot 1988). *Moringa oleifera* leaves contain compounds that contain carbamate, thiocarbamate, nitrile, or full acetylated glycoside groups that are found rarely in nature (Faizi et al. 1995) but were found to be responsible for lowering blood pressure (Faizi et al. 1994a, b, 1995). Four pure compounds were isolated from the active ethanol leaf extract: niazinin A, niazinin B, niazimicin (Table 3, Fig. A), and niazinin (Faizi et al. 1995). Niaziminin is a thiocarbamate found in the *Moringa oleifera* leaves that has shown resistance against tumors induced as a result of Epstein-Barr virus infection. Niazimicin was suggested as an inhibitor of carcinogenesis (Guevara et al. 1999). These compounds displayed lowered blood pressure using a calcium antagonistic pathway in rats. The ethanolic and aqueous extracts of *Moringa oleifera* seeds showed more significant effect in lowering blood pressure, although results were different and comparable between water and ethanol extracts (Faizi et al. 1998a, b, c). Isothiocyanate and thiocarbamate glycosides isolated from the ethanolic extracts of *Moringa oleifera* pods show hypotensive effects (Faizi et al. 1995), as well as methyl p-hydroxybenzoate and β-sitosterol (Table 1, Fig. C) found in the pods (Faizi et al. 1998a, b, c). The roots, flowers, gum, and seeds of *Moringa oleifera* have shown to increase urine production (Morton 1991; Caceres et al. 1992). Makonnen et al. (1997) have reported that the leaves of *Moringa oleifera* contain compounds that can have potential antitumor effects. O-Ethyl-4-(α-L-rhamnosyloxy) benzyl carbamate, 4(α-L-rhamnosyloxy)-benzyl isothiocyanate, and 3-O-(6′-O-oleoyl-β-D-glucopyranosyl)-β-sitosterol are found in *Moringa* and were tested by using an in vitro assay in which the compounds have shown relevant amount of inhibition against the antigen of Epstein-Barr virus. Extracts of *Moringa oleifera* seeds have shown effects on enzymes that metabolize carcinogens in the liver, antioxidants, and cancerous growth in mice skin (Bharali et al. 2003). Ointment made

from *Moringa oleifera* seeds produced an inhibitory effect against *S. aureus* pyoderma in mice, like neomycin (Caceres and Lopez 1991). 4-[(4′-O-Acetyl-α-L-rhamnosyloxy) benzyl] is a naturally occurring isothiocyanate that has shown a significant reduction in tumors induced by Epstein-Barr virus activation, which implies that the structural isothiocyano group is crucial in the activity of inhibiting tumors (Murakami et al. 1998).

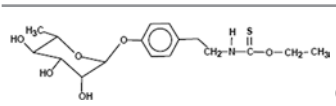
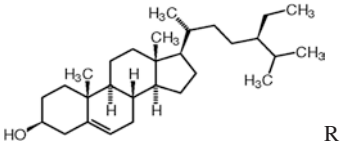
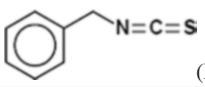
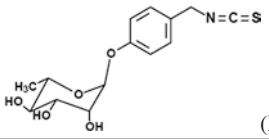
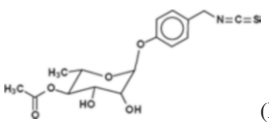
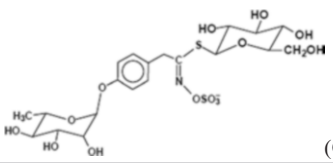
Antibacterial and Antifungal Activities

The plant parts of the *Moringa oleifera* plant have shown various antimicrobial effects in several studies. It contains many compounds such as benzyl isothiocyanates (D), 4-(4′-O-acetyl-α-L-rhamnopyranosyloxy) benzyl isothiocyanate (F), and 4-(α-L-rhamnopyranosyloxy) benzyl glucosinolates (G) which exhibit the antimicrobial activity (Table 3, Fig. D, F, G). The roots contain antimicrobial components that are responsible for antibacterial activity, e.g., pterygospermin (Table 3, Fig. B), which also acts against fungus growth (Ruckmani et al. 1998a, b). A compound showing similar effects to that of pterygospermin was found in *Moringa oleifera* flowers (Das et al. 1957). The antimicrobial activity in *Moringa oleifera* roots is possibly due to the existence of 4-α-L-rhamnosyloxy benzyl isothiocyanate (Eilert et al. 1981) (Table 3, Fig. E). The chloroform fraction of the ethanol extract of the root contains deoxy-niazimicin (*N*-benzyl, *S*-ethyl thioformate) which has aglycone group, thought to be responsible for the antifungal and antibacterial effects (Nikkon et al. 2003). The *Moringa oleifera* bark extract exhibited antifungal properties (Bhatnagar et al. 1961), and its juice showed activity against *S. aureus* (Mehta et al. 2003). Also, *Moringa oleifera* leaf juice was able to show inhibitory effects against *P. aeruginosa* and *S. aureus*, both of which are pathogenic.

Antidiabetic Activity

Moringa oleifera have also antidiabetic properties. Leaf extracts showed potential to decrease blood glucose levels in type 2 diabetes modeled rats (Ndong et al. 2007). One study showed that blood glucose lowers within 3 h after ingesting (Mittal et al. 2007). Dark chocolate polyphenols (Grassi et al. 2005) as well as other polyphenols are responsible for lowering blood glucose (Al-Awwadi et al. 2004; Moharram et al. 2003). Since *Moringa oleifera* leaves contain quercetin-3-glycoside and other polyphenols (Ndong et al. 2007), it holds the potential of possibly treating diabetes. With its wide availability, the antidiabetic property of *Moringa oleifera* can be commercialized by using proper technology.

Table 3 Major active constituent found in *Moringa oleifera*

Active constituent	Major application	References
 (**A**)	Anticancer Blood pressure Lowering effects	Guevara et al. (1999) Faizi et al. (1998a, b, c)
 (**B**)	Antibacterial and fungicidal effects	
 R = H (**C**)	Cholesterol Lowering effects	Ghasi et al. (2000)
 (**D**)	Antibacterial	Fahey (2005)
 (**E**)	Antibacterial	Fahey (2005)
 (**F**)	Antibacterial	Fahey (2005)
 (**G**)	Antibacterial	Fahey (2005)

Niazimicin (A), pterygospermin (B), β-sitosterol (C), benzyl isothiocyanates (D), 4-(-L-rhamnosyloxy) benzyl isothiocyanate (E), 4-(4′-O-acetyl-α-L-rhamnopyranosyloxy) benzyl isothiocyanate (F), 4-(α-L-rhamnopyranosyloxy) benzyl glucosinolates (G)

Antifertility Activity

The aqueous roots and bark extract of *Moringa oleifera* contain substances that show potential for antifertility activity (Prakash et al. 1987). It may induce estrogenic and progestational activities or activities against these to affect fertility (Shukla et al. 1988a, b, c, d). Another study showed that the leaf extracts of *Moringa*

oleifera with a dose of 175 mg/kg of starting dry material are fully effective in abortion (Nath et al. 1992a, b).

Antioxidant Activity

Moringa oleifera has high concentrations of antioxidants (Chumark et al. 2008), especially in the aqueous extracts of its leaves, seeds, and fruits (Singh et al. 2009). Reportedly, alcoholic extracts of freeze-dried *Moringa oleifera* leaves showed the most antioxidant activity (Lalas and Tsaknis 2002; Siddhuraju and Becker 2003a, b). Quercetin, kaempferol, and other phenolic compounds are suggested to be responsible for antioxidant properties (Bajpai et al. 2005; Siddhuraju and Becker 2003a, b). These compounds showed antioxidant activity against hepatocyte growth factor-induced methionine phosphorylation (Labbe et al. 2009). *Moringa oleifera* seed exhibits higher radical scavenging activity compared to palm oil (Ogbunugafor et al. 2011).

Anti-asthmatic Activity

Moringa oleifera plant alkaloid is similar to ephedrine in terms of action against asthma. Reportedly, moringine (benzylamine) helps bronchioles to relax (Kirtikar and Basu 1975). A study evaluating the safety and effectiveness of seeds to treat asthma patients showed that *Moringa oleifera* seed kernels have shown a relevant decrease in the symptoms of asthma and overall improvement in the respiratory system (Agrawal and Mehta 2008).

Anti-inflammatory Activity

Moringa oleifera root extracts helped to reduce inflammation significantly in rat paw edema induced by carrageenan (Ezeamuzie et al. 1996; Khare et al. 1997). The N-butanol extract of *Moringa oleifera* seeds has exhibited activity against airway inflammation induced by ovalbumin in guinea pigs (Mahajan et al. 2009). *Moringa oleifera* contains active compounds that have potential to reduce inflammation as a result of chronic diseases (Muangnoi et al. 2011).

Analgesic Activity

Several *Moringa* species have reportedly shown analgesic effects. Alcoholic extracts of *Moringa oleifera* fruits exhibited significant analgesic activity in animals (Rao et al. 2008). Hot plate and tail immersion method provided evidence that the alcoholic extracts of *Moringa oleifera* leaves and seeds show noteworthy analgesic activity (Sutar et al. 2008).

CNS Activity

Moringa oleifera root aqueous extracts were tested for their antiepileptic effects, against seizures induced by penicillin, movement, serotonin levels in the brain (5-HTT), neurotransmitters like dopamine, and hormones like norepinephrine, in albino rats (Rastogi et al. 2009). In the rat models of Alzheimer's disease, Ganguly and Guha (2008) tested the ethanol extracts of *Moringa oleifera* leaves and their effects on dopamine, norepinephrine, and serotonin levels and overall EEG wave pattern.

Anthelmintic Activity

The activity of *Moringa oleifera* against helminths was reported by Rastogi et al. (2009), where the ethanolic extracts of *Moringa oleifera* with various concentrations were used to measure inhibitory activity against *Pheretima posthuma* earthworms. The time of worm paralysis and death were recorded as results, with piperazine citrate (10 mg/mL) as the standard reference and control group being distilled water (Rastogi et al. 2009).

In Ocular Diseases

Moringa oleifera leaves, pods, and their extracts or powders are rich in vitamin A which could be used to treat vitamin A deficiency, since its deficiency can cause blindness and other eye problems. Studies have shown that the leaves can provide sufficient vitamin A to resist the formation of cataracts in eyes (Pullakhandam and Failla 2007). *Moringa oleifera* was suggested as a supplementary food due to its rich vitamin A source by the Integrated Child Development Scheme Supplementary Food (ICDS-SFP) (Nambiar et al. 2003).

Anticancer Activity

Moringa oleifera exhibited the anticancer activity against the breast and colorectal cancer cell lines (Al-Asmari et al. 2015). The leaves and barks of *Moringa oleifera* showed the notable characteristics against the cancer cell lines. Some active constituents were reported against the breast and colorectal cancer. These chemical compounds are eugenol, isopropyl isothiocyanate, D-allose, and hexadecanoic acid ethyl ester (Al-Asmari et al. 2015).

Future Prospect

Moringa oleifera has long been established as an abundant source of nutrients, particularly in the developing countries where the leaves are dried and milled for easy storage and added as a powder for daily dietary intake. The powder is also used to enrich food products. *Moringa oleifera* has sufficient amount of nutrients; this gives opportunities for developing countries to rely less on expensive imported vitamin and mineral dietary supplements which are generally used to treat malnutrition. Studies conducted on *Moringa oleifera* have shown that there are some properties of the various plant parts that could be useful for health. However, the preliminary studies were limited to animal models and cell lines; human trials are still on hold. However, human studies should be conducted since the plant is edible. Niazimicin, found in *Moringa oleifera* seeds, has been described as an antitumor promoter, although its mechanism of inhibiting carcinogenesis still needs further investigation. The mechanism of *Moringa oleifera* constituents acting against HSV-1 infection also needs to be further examined.

Conclusion

If *Moringa oleifera* plants are largely cultivated under favorable climate conditions, growth would be maximum; thus this would provide a large yield of its various parts. The different parts of the plant can then be used to isolate components that hold potential benefit for human health.

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Anticancer Mechanistic Insights of Epigallocatechin-3-Gallate, an Active Ingredient of Green Tea (*Camellia sinensis*)

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Introduction

The natural compounds derived from plants have been used since immemorial times by human. The health-promoting effect of certain natural compounds has fascinated the human toward natural phytocompounds for prevention and treatment of diseases. Currently scientists are doing a great deal of research in exploring the natural compounds and their photochemistry for promoting human health. The beauty of natural compounds is that they have minimum toxicity and highly effective in therapeutic action. Green tea is one of the natural product derived from *Camellia sinensis* plant and is being consumed globally next to water. Green tea is known for its health-boosting effects since ancient times. The health-boosting effect of green tea is due to its rich photochemistry. Polyphenols in green tea are mainly responsible for its health-promoting effect. The health-boosting compounds present in green tea are catechins, and among catechins the epigallocatechin gallate (EGCG) is one of the most common and effective types of catechin in green tea. The green tea catechins inhibit the carcinogenesis by modulation of different cellular signaling pathways which are key for the promotion of tumors (Khan et al. 2006). The anticancer mechanism of EGCG has been investigated in vitro in different cell culture system

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(Yang and Wang 2011). This is an amazing area of research to explore the exact mechanism involved in anticancer effects of green tea catechins in animals and humans. It has been recently found that EGCG inhibited mammary tumor induced in mouse model (Yanaga et al. 2002). Another study reported that EGCG reduced tumor mass in breast cancer xenograft model (Mineva et al. 2013). These studies summarized the significant role of EGCG present in green tea in chemoprevention. This review summarizes the effects of green tea catechins in different cancer signaling pathways and metabolism. EGCG is the main concern of this review to evaluate the anticancer properties of this molecule which has diverse action in different tumorigenesis.

Green Tea and Its Composition

Tea is the common consumable agent globally which serves as tasty beverage and exerts its health-boosting effect instantly. Tea is being consumed in the different forms like some people prefer green tea, some black tea, and some milk-added tea. Tea is obtained from a specific plant known as *Camellia sinensis*. The green tea beverage exhibits significant health-promoting effects in humans (Cabrera et al. 2006). The green tea has complex chemical composition. It contains certain important phytocompounds like polyphenols, flavonoids, flavonols, and other compounds such as proteins, lipids, polysaccharides, and vitamins. Polyphenols including flavonoids and phenolic acid account for 30% of dry weight of green tea (Balentine et al. 1997). The catechins are the main flavonoids present in green tea. The major catechins present in green tea include epigallocatechin, epicatechin, epicatechin-3-gallate, and EGCG (Hastak et al. 2003a). 2.5% of caffeine is also present in green tea as an active compound which acts as a key molecule in the prevention of skin and lung carcinogenesis in different animal models (Yang et al. 2009).

Bioavailability and Biotransformation of Green Tea Catechins

The green tea catechins are less absorbed from the gastrointestinal tract (GIT) due to polyphenolic structures which form large hydration shell in hydrogen bonding. Oral administrations of green tea catechins have less than 0.2% bioavailability in animal models and humans (Lambert and Yang 2003). Although EGCG is believed to enter into the cells by passive diffusion, certain specific transporter proteins likeIA2 and IB3 are involved in its transportation (Roth et al. 2011). After the oral consumption of two cups of green tea, the plasma concentration of green tea catechins reaches up to 0.5 μM (Lee et al. 2002). The administration of higher oral doses of EGCG leads the peak plasma concentration of 2–9 and 7.5 μM in animal model and humans, respectively (Yang et al. 2008). Green tea catechins are mostly metabolized in the liver and intestine. The various metabolic transformations involved in

biotransformation of green tea catechins include glucuronide conjugation, methylation, sulfotransferase-mediated sulfation, and ring fission (Sang et al. 2011). After the absorption of green tea, catechins from GIT are delivered to the liver where higher concentration of metabolic enzymes like glucuronyltransferases (Strassburg et al. 1999) and sulfotransferases (Matsui and Homma 1994) is present which properly metabolizes them and finally releases them into the blood circulation system.

Anticancer Mechanism of Action of Green Tea

The therapeutic effect of green tea catechins for wide range of diseases including cancers has been reported by various researchers. The probable anticancer mechanism involved in green tea catechin supplementation is briefly described as follows and in Fig. 1.

1. Green tea prevents certain enzymes and proinflammatory cytokines like cyclooxygenase, lipoxygenases, and TNF-α and different interleukin pathways and lastly impedes the tumor promotion.

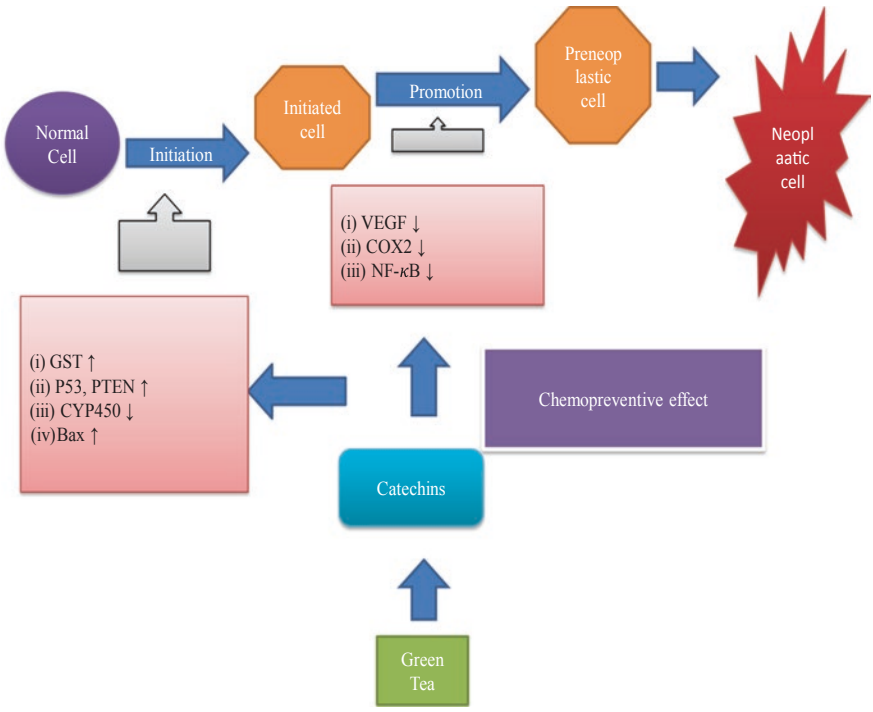

Fig. 1 Anticancer mechanism of green tea catechins

2. Green tea stimulates the p53 gene activation that is involved in tumor suppression and inhibits the activity of transcriptional factors such as NFκB.
3. The potent antioxidant effect of green tea leads to inhibition of carcinogenesis development by scavenging free radicals and neutralization of oxidants (Osada et al. 2001).
4. The key anticancer mechanism involved in supplementation of green tea catechins is the modulation of certain specific gene activity which is involved in promotion, development, and progression of carcinogenesis.

Anticancer Effect of Green Tea via Modulation of Signaling Pathway

Cancer is uncontrolled growth of cells and is a multifactorial imperfection including genetic alterations, metabolic alterations, and physical alterations. The currently available anticancer allopathic drugs are full of side effects, costly, and less effective. The plant-derived natural compounds have potent anticancer effects and are economical and easily available, and the most important thing is that they have minimum side effects. Various natural compounds derived from plants are showing anticancer property via modulation of different cellular signaling pathways (Rahmani et al. 2014). The anticancer effect of green tea by modulation of certain cell signaling and metabolic pathways is discussed as under.

Effects of Green Tea Catechins on Tumor Suppressor and Proliferator Gene Modulation

Tumor suppressor gene is responsible for regulation and suppression of genes involved in tumorigenesis. Mutation or inactivation of tumor suppressor gene leads to the development of tumor. Green tea plays a noteworthy role in p53 gene activation. The rate of transcription and acetylation activity involved in p53 gene is augmented by EGCG and GTP by impeding class 1 deacetylase activity (Thakur et al. 2012a). One more study reported that EGCG present in green tea leads to elimination of cancer cells by promoting apoptosis through inducing p53 gene-dependent pathways of apoptosis (Hastak et al. 2003a). It has been studied that EGCG the major constituent of green tea strongly activates p53 and Bax protein expression in breast cancer cells (Roy et al. 2005). Other studies revealed that EGCG at 10–20 μmol/L leads to p53-dependent programmed cell death of JB6 cells by mitochondrial dysfunction pathway. Epigallocatechin-3-gallate is also causing alteration in p16 methylation pattern from the methylated state to unmethylated state due to depletion of folic acid. Green tea catechins inhibit the growth of melanomas by significant suppression of histone deacetylase (HDAC) genes and augmentation of

histone acetyltransferase (HAT) activity (Prasad and Santosh 2015). In human colorectal cancer, the downregulation of transcriptional activity of the proliferative gene NUDT6 is mediated by supplementation of green tea catechins (Sukhthankar et al. 2010).

Effects of Green Tea Catechins on Proteasome Inhibitory Activity

Proteasome targeting has become a famous approach in the treatment of different carcinomas as most of the intracellular tumor-associated proteins are tarnished by ubiquitin proteasome pathways (Ciechanover et al. 2000). Proteasome-mediated protein degradation not only serves as wastebin for useless and aged proteins but also controls the cellular signaling pathways by activation or inhibition of cell cycle, apoptosis, and transcription factors (Naujokat and Hoffmann 2002), since cancer cell proliferation and resistance of cancer cell against anticancer drugs essentially depend on proteasome activity (Hideshima et al. 2001). Therefore pathways mediated by proteasomal degradation are being considered as effective targets for carcinogenesis promotion. Commonly used proteasome inhibitor is imatinib which mediates its anticancer action by inhibition of proteasome (Kane et al. 2006). EGCG has been reported to inhibit chymotrypsin activity of proteasome (Nam et al. 2001). EGCG has potential to inhibit the proteasome of whole cell resulting in accumulation of the higher concentration of substrates of proteasome like p27 and IκB-α which in turn arrest the cell growth in G phase (Yang and Wang 2011). EGCG does not affect the proteasome activity of normal cells, but it only alters proteasomal activity in cancerous cells (Kuhn et al. 2005). Therefore, the anticancerous effect of green tea is attributed to its ability to inhibit the proteasomal activity by its phytocompound EGCG which is going to act as an effective anticancer agent in the future.

Effects of Green Tea Catechins on Prolylcis/Trans Isomerase (Pin1) Modulation

Peptidyl prolyl cis/trans isomerase (Pin1) is responsible for isomerization of the peptide bond of some residues of specific phosphorylated serine or threonine amino acids and preceding the amino acid proline in various proteins which are specifically involved in various cellular functions including transcription, mitosis, differentiation, and response to the DNA damage (Wulf et al. 2005). Epigallocatechin-3-gallate (EGCG) is one of the most important and active anticancer compounds present in green tea and exhibits its anticancer effect by modulation of different cell signaling pathways. Pin1 plays an essential role in complete activation of various cellular signal transduction pathways including NF-κB (Lee et al.

2010), AP-1 (Dong et al. 1997), and β-catenin (Ryo et al. 2001). Pin1 exhibits its key role in various signaling pathways of oncogenesis (Yang and Wang 1993) and is significantly upregulated in different tumors including breast (Wulf et al. 2001), colorectal (Kim et al. 2005), prostate (Ayala et al. 2003), and thyroid (Nakashima et al. 2004). Pin1 inhibition in tumor cells exerts apoptosis and inhibits their phenotypic transformation (Ryo et al. 2002). It has been reported that EGCG inhibits the activation of HER-2/neu activation signal downstreaming in certain cancer cells including head and breast (Masuda et al. 2003). Neu/ras oncogenic signaling is downstreamed by Pin1 expression (Wulf et al. 2004). Therefore, we summarized that EGCG may act directly to target Pin1 in order to inhibit the lethal progression of oncogenic signaling pathways and neoplastic cell transformation. The effective inhibitors of Pin1 may acts as valuable agents for cancer development in future.

Effect of Green Tea Catechins on Apoptosis

Apoptosis is a programmed cell death or physiological death of cells. However there is imbalance between cell division and cell death in cancer cells. Cancer cells show resistance against apoptosis. Epigallocatechin-3-gallate (EGCG) has been reported to reduce the tumor proliferation by the promoting apoptotic pathways in cancer cells by downregulation of NFκB activity which in turn stimulates cell for apoptosis (Yang and Wang 1993). It has been recently studied that polyphenols present in green tea enhance the transcriptional activity of p21/waf1 and Bax genes responsible for apoptosis and also accelerate the proteasomal degradation of class HDACs and prompt the Histone H3 acetylation which in turn leads to inhibition of cell cycle and apoptosis in the prostate cancer cells (Thakur et al. 2012b). EGCG has been reported to induce apoptosis of cancer cell by blocking cell division at G1 phase subsequently followed by enhanced expression of P53 tumor suppressor gene and upregulation of proapoptotic proteins (Kuo and Lin 2003). Other studies also reported that EGCG shows dose- and time-dependent inhibition in proliferation of cancer cells and effective apoptotic induction in cancer cells (Qin et al. 2007). EGCG has exerted its antiapoptotic effect in human hepatocellular cancer cells but not in normal hepatocytes by increasing the membrane potential of mitochondrial membrane and arresting of cell cycle at G0/G1 phase, increased expression of caspase-3, and downregulation of NFκB (Zhang et al. 2015) (Fig. 2).

Effect of Green Tea Catechins on Angiogenesis

Angiogenesis is the recruitment of new blood vessels to the tumor cells in order to provide efficient nutrition and route to enter the blood circulation for metastasis. The state of vascularity of tumor indicates its potential of metastasis. Tumors with

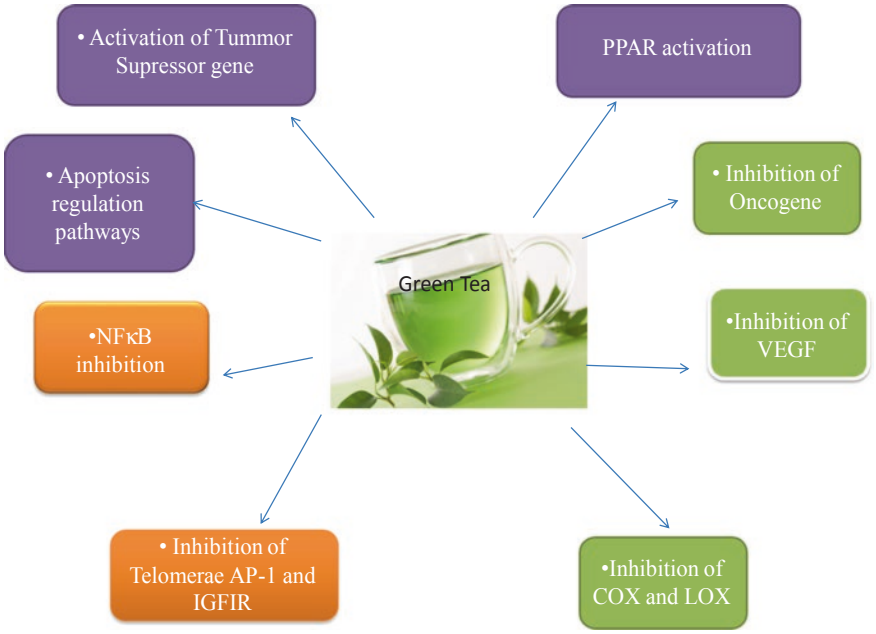

Fig. 2 Modulation of various pathways involved in initiation, promotion, and progression of carcinogenesis by green tea catechins

higher degree of vascularity are their metastatic potential. Angiogenesis inhibition plays a significant role in tumor prevention and its metastasis. Studies have reported that epigallocatechin-3-gallate has strong ability to inhibit vascularity and proliferation of tumor in nude mice (Jung et al. 2001). EGCG has also been reported to suppress the growth, angiogenesis, and cancer cell proliferation of breast tumor by inhibiting redox-sensitive NFκB pathways. Previously it was reported that EGCG inhibits angiogenesis process by blocking Erk-1 signaling pathways and activation of Erk-2 and VEGF expression (Jung et al. 2001). FOXO transcriptional factor plays a major role in tissue homeostasis and in certain lethal disease like cancer and diabetes. EGCG inhibits the angiogenesis of tumor cells by increasing the transcriptional activity of FOXO (Shankar et al. 2008).

Effect of Green Tea Catechins on NFκB

NFκB is a redox-sensitive nuclear transcription factor present mainly in cytoplasm of cell and is transported to nucleus upon activation due to cellular responses to certain stimuli such as free radicals, stress, and bacterial and viral antigens. NFκB in cytoplasm is regulated in inactivated state by IκB (inhibitory protein). NFκB activation results in increased expression of antiapoptotic genes and forces the cell

to take away the physiological mechanism of cell death and triggers the release of inflammatory cytokines (TNF-α, IL1, IL6, and IL8) (Guttridge et al. 1999). NFκB signaling facilitates the metastasis of cancer cell by regulating epithelial to mesenchymal transition of cells increasing the activity of metalloproteinases which in turn triggers the weakening of extracellular matrix for an erosion of cancer cells (Huber et al. 2004). Finally NFκB promotes the tumor proliferation by increasing vascularity of tumors through upregulation of VEGF (vascular endothelial growth factor) and its respective receptors (Xie et al. 2010). EGCG which is the major polyphenolic constituent of green tea has been reported to suppress the transcriptional activity of gene encoding NFκB and accompanied by reduction in antiapoptotic protein (Bcl-2) levels and augmentation of Bax proteins via P53 gene activation (Kedar et al. 2003). Reactive oxygen species play a key role in activation of NFκB signaling pathways, and inhibition of the NFκB activation by EGCG supplementation is attributed to its potent antioxidant and free radical scavenging activity (Lee et al. 2004). Another study reported that EGCG has potent ability in inhibiting the overexpression of HIF-1α and NFκB in breast cancer cells (Gu et al. 2013).

Effect of Green Tea Catechins on Androgen Receptors

In different kinds of tumors, it has been seen that there is overexpression of androgen receptors. Natural compound having effective chemopreventive properties has been found to downregulate the cancer-triggering genes including suppression of androgen. EGCG in green tea have been found to suppress proliferation of cells, reduction in expression of prostate-specific antigen (PSA), and transcriptional activity of androgen receptors in various types of cell lines (Chuu et al. 2009a). Androgen-regulated PSA have been found to be inhibited by EGCG.

Effect of Green Tea Catechins on Telomerase

Telomeres are specific structural entity mostly found at the end of chromosomes. Telomeres play a significant role in aging, and their overexpression has been found in most of cancers. It has been found that green tea is quite effective in management of telomerase activity and inhibits the pathogenesis of tumor formation. Epigallocatechin in green tea has been found to reduce 50–60% of telomerase activity in drug-sensitive and drug-resistant lung cancer cell line incubated for 24 h (Sadava et al. 2007). Earlier studies reported that epigallocatechin inhibits the telomerase activity in tumor-inducing human papillomavirus type 18- (HPV 18-), certain immortalized endocervical cell (HEN-18), and cells of ectocervical cell line (HEC-18) (Yokoyama et al. 2004). The beauty of EGCG lies in its strong ability in inhibiting telomerase activity among different catechins (Naasani et al. 1998).

Effect of Green Tea Catechins on Wnt Signaling

The canonical Wnt signaling pathway leads to the accumulation of β-catenin inside cytoplasm and facilitates its translocation into the nucleus to activate transcriptional activity of certain proto-oncogenes (Cyclin D1, Cmyc, and COX-2) and transcription factors (Ju et al. 2005). Supplementation of EGCG in Apcmin/+ mice has inhibited the Wnt signaling (Ju et al. 2005). Nuclear levels of β-catenin in human colorectal cell line were significantly reduced following the treatment with EGCG (Singh and Katiyar 2013). The phosphorylation of β-catenin is subsequently followed by downregulation of prostaglandins (PGE-2) which induced higher levels of MMP2 and MMP9 following the treatment of cancer cells with EGCG (Singh and Katiyar 2013). EGCG was reported to inhibit the tumorigenesis in hepatocytes by inhibiting the Wnt signaling pathway (Godeke et al. 2013).

Effect of Green Tea Catechins on MicroRNA

MicroRNA are noncoding, small endogenous RNAs that regulate the expression and stability of genes. The dysregulation of miRNA affects the hallmarks of carcinogenesis such as increased proliferative signaling, evasion of growth suppressors, angiogenesis, and metastasis of cancer cells (Bartel 2009). EGCG supplementation in human hepatocellular leads to the alteration in miRNA levels which in turn triggered the changes in different cancer signaling pathways. EGCG in human HepG2 cancer cells leads to the upregulation of miR-16 which in turn facilitated apoptosis by downregulation of bcl-2 proteins (Tsang and Kwok 2010). Recently it was reported that EGCG supplementation has upregulated the miR-16 expression in breast cancer cell line 4TI (Jang et al. 2013). EGCG also upregulated the expression of miR-210 in human and animal lung cancer cells which in turn prevents the proteasomal degradation of HIF-1α (Wang et al. 2011).

Effect of Green Tea Catechins on Different Carcinomas

Effect of Green Tea Catechins on Chronic Myeloid Leukemia

Chronic myeloid leukemia (CML) is nowadays a common hematopoietic cancer characterized by uncontrolled proliferation of myeloid cells. Millions of people around the world are suffering from this devastating and dreadful disease. The currently used drugs against the CML are tyrosine kinase inhibitors like imatinib. BCR-ABL tyrosine kinase acts as the novel molecular target in CML therapy (Druker et al. 2001). But it has been observed that some CML patients show low sensitivity and low therapeutic response for tyrosine kinase inhibitors (Perl and

Carroll 2011). These studies indicate the need of finding new and effective therapeutic interventions in CML. EGCG in green tea have been recently reported as an effective therapeutic agent against CML. The mechanism responsible for inhibiting myeloid cell proliferation is that EGCG induces clustering of lipid rafts in human CML cells and cell death through the sGC/ASM pathway (Huang et al. 2015).

Effect of Green Tea Catechins on Breast Cancer

Breast cancer is one of the major malignancies and fatal diseases in women around the world. The pathogenesis of breast cancer is a complicated process which finally results uncontrolled proliferation of cells due to loss of regulatory mechanism. It has been reported that small set of specialized cells known as cancer stem cells (CSC) are responsible for initiating tumorigenesis within tumor (Rathone and Wang 2013). These cells are self-differentiating and resistant to chemotherapy. The effective resistance mechanism of CSC cells against the conventional chemotherapy forces the researchers to find a novel and effective agent against CSC in breast cancer patients. The biomolecule commonly known as signal transducer and activator of transcription 3 (STAT3) plays a major role in maintaining the self-renewal and resistance property of CSC. Constant activation of STAT3 in different human malignancies leads to profound tumorigenesis and very weak prognosis (Turkson and Jove 2000). Current studies have observed that STAT3 activation plays a key role in breast carcinogenesis (Azare et al. 2011). EGCG in green tea have been recently found as an effective compound to reduce CSC cells in breast carcinogenesis by inhibiting STAT3 phosphorylation which in turn inhibited the translocation of STAT3 in nucleus. As a result, the gene expression responsible for tumorigenesis of CSC cells is downregulated. Thus EGCG reduced the CSC cells in breast cancer by modulating STAT3 pathways (Chung and Vadgama 2015).

Effect of Green Tea Catechins on Digestive Tract Carcinomas

Digestive tract like the esophagus, stomach, and intestine is susceptible to tumorigenesis, and the gastric and colon cancers are common nowadays globally. The lining of esophagus and stomach and intestine are quite often exposed to different kinds of carcinogenic compounds. Green tea catechin supplementation prevents tumorigenesis in the intestine of rats by elevating the E-cadherin proteins in the plasma membrane and subsequently decreasing the levels of c-Myc, nuclear β-catenin, and phospho-AKT (Ju et al. 2005). The aberrant crypt foci (ACF) formation in intestine induced by azoxymethane (AOM) in mice was reversed by supplementation of green tea extract in mice (Ju et al. 2003). Shimadazo et al. reported that green tea catechins have strong potential to inhibit the ACF formation in the colonic mucosa by inhibiting the insulin growth factor (IGF) signaling pathway (Shimizu

et al. 2008a). Liver cancer is the sixth most commonly occurring carcinoma and the second leading cause of death due to tumors all over the world (Ferlay et al. 2015). EGCG of green tea has been reported to have the protective effect against the liver carcinogenesis (Sakata et al. 2004).

Effect of Green Tea Catechins on Prostate Cancer

Prostate cancer is the most common cause of mortality in males in the USA. This cancer is taking the life of more than 29 000 people annually. Green tea catechins inhibited the propagation of prostate cancer cells and expression of prostate-specific antigen (PSA) (Chuu et al. 2009b). It was reported that catechins of green tea promote DNA methylation and acetylation of histone proteins in cancer cells and inhibit their progression (Balasubramanian et al. 2010). Green tea catechins have been reported to inhibit the development of prostate adenocarcinoma in mice by increasing the apoptosis, reducing the IGF1 levels, and restoring the levels of IGF-binding protein-3 (Gupta et al. 2001; Adhami et al. 2004). The suppression of tumor growth in prostate cells by catechins of green tea is also associated with the alleviating levels of phosphatidylinositol 3-kinase (PI3K) phosphorylated forms of AKT and ERK1/2 (Adhami et al. 2004).

Effect of Green Tea Catechins on Cervical Cancers

Cervical cancer is the leading and second most common cause of death in women. EGCG of green tea exerts inhibitory effect on cervical cancer proliferation. Green tea catechins promote the apoptosis of cervical cancer cells by markedly enhancing the expression of P53 and P21 genes and reducing the expression of HPV-E7 proteins (Zou et al. 2010; Singh et al. 2010). The proliferation of cancer cells is inhibited by green tea catechins through amplification of G-2 phase in mitotic cell cycle. The main mechanism behind the apoptosis of cervical cancer cells is the profound reduction in membrane potential of mitochondria and augmentation of phosphatidylserine residues of mitochondrial membrane (Al-Hazzani and Alshatwi 2011).

Synergistic Anticancer Activity of Green Tea Catechins with Different Allopathic Anticancer Drugs

It has been observed that EGCG in combination with various drugs has shown positive correlation in countering the tumorigenesis of different kinds of tumors both in in vivo and in vitro studies. EGCG combined with sulindac (NASID) increased the

Table 1 List of antitumor agents that have exhibited synergistic anticancer therapeutic effects with EGCG

Cancerous tissue	Anticancer agent used in experiment	References
Head, neck, and lung	Curcumin celecoxib, luteolin erlotinib, 5-fluorouracil, tamoxifen sulindac	(Qiao et al. 2011a; Stearns et al. 2010)
Breast	Raloxifene, curcumin, resveratrol, tocotrienol, 4-hydroxytamoxifen, trichostatin A, tamoxifen	(Liang et al. 2010a; Farabegoli et al. 2010a)
Prostate	Docetaxel, bortezomib, sulforaphane, resveratrol, doxorubicin, genistein, NS398 quercetin, paclitaxel	(Chuu et al. 2009b; Siddiqui et al. 2011)
Ovaries	Sulforaphane, cisplatin, trans-palladiums	(Chan et al. 2006)
Leukemia	Curcumin, benzyl isothiocyanate, cytosine, celastrol, arabinoside, H_2O_2	(Lee et al. 2011)
Pancreas	TRAIL, celecoxib, thymoquinone	(Tang et al. 2012)
Colon	Sulforaphane, sodium butyrate	(Qiao et al. 2011b)

expression of growth arrest and DNA damage-inducible gene 153 by 12 times as compared to the alone treatment with EGCG (Fujiki et al. 2002). Similarly in case of intestinal neoplasia of mice, EGCG in combination with sulindac showed prominent reduction in tumor growth from 72.3 to 32%, whereas EGCG alone was found to be less effective (Suganuma et al. 2001). Paclitaxel and EGCG combination completely eradicated human prostate cancer development in xenograft mouse model (Stearns and Wang 2011a). Tables 1 and 2 show the combinations of compounds which show synergistic action with EGCG against various tumors.

Clinical Trials

The limited data is available for anticancer effects of green tea catechins in human trials. Human trials are going on globally by researchers to study the chemopreventive effects of EGCG in humans. Although the EGCG and other catechins of green tea are not available in purified form in market, the green tea is globally available which is quite economical and can be administered orally (Sartippour et al. 2002). EGCG has exhibited potent clinical results as compared to traditional anticancer drugs. Green tea catechins target the specific biochemical and molecular signaling pathways involved in tumor cell proliferation (Fujiki et al. 1999). The currently used conventional anticancer drugs exert their toxic insults, but the EGCG supplementation in clinical trials till date was reported to have acceptable safety outline (Hastak et al. 2003b). It was reported in prospective cohort study of 8000 individuals that daily supplementation of green tea resulted delayed cancer progression. The delivery of EGCG in capsulated forms (200 mg p.o.) for 12 weeks improved the health status of patients suffering from human papilloma virus (Ahn et al. 2003). It has recently been reported first time that 90% of prostate cancer prevention can be obtained by green tea catechins supplementation in persons which are susceptible to

Table 2 Modulation of anticancer drug activity by epigallocatechin-3-gallate in experimental studies

Drug name	Type of cancer	Mechanism of action	Effects of combination of drugs with EGCG	Events induced by EGCG
Doxorubicin (Liang et al. 2010b) 5-fluorouracil5 Tamoxifen (Stearns et al. 2010)	Prostate cancer (Stearns et al. 2010) Colon cancer Bladder cancer Stomach cancer Chemo-resistant Hepatocellular Carcinoma	Modification in pharmacokinetics of drug	Bioavailability of anticancer drug consumed orally is increased The concentration of anticancer drug in tumor cells is enhanced Acceleration of chemo sensitization to cancer cells	Retention of anticancer drug by blocking its efflux (Kuo and Lin 2003) Modification in enzyme activity involved in drug metabolism Reduction in activity of YP-gP efflux pump of tumor cells (Liang et al. 2010b; Farabegoli et al. 2010b) Enhanced upregulation of multidrug resistance gene 1 (MDR1) (Stearns et al. 2010) Decrease in expression of BCRP
Tamoxifen (Farabegoli et al. 2010b) TSA (Li et al. 2010)		Binding and interaction with hormone receptor complex	Proliferation of cancer cells responsive to estrogen is decreased Cancer cells are sensitized to anticancer drugs through steroid receptors Proliferation of prostate cancer cells are inhibited	Leads to binding of Estrogen receptors (Tu et al. 2011) Downregulation of androgen binding in prostate cancer cells (Chuu et al. 2009c)
Taxane (Stearns and Wang 2011b) Gemcitabine Vorinostat SU-541611 Celecoxib13	Prostate cancer (Stearns and Wang 2011b) Melanoma (Nihal et al. 2010) Neuroblastoma (Mohan et al. 2011) Pancreas Urothelial carcinoma (Huang et al. 2012)	Alteration in signaling of cell cycle, angiogenesis, and apoptosis	Arrest of cell cycle and induction of apoptosis Inhibition of tumor cell growth Inhibition of angiogenesis by synergistic action blocked the metastasis of tumor cells	Acceleration in activity of proapoptotic genes (Nihal et al. 2010) Modification in activity of bcl_2 proteins Alteration in signaling pathways involved in cell cycle and apoptosis pathways (Mohan et al. 2011) Reduction in proteins that trigger angiogenesis and metastasis

develop prostate cancer (Bettuzzi et al. 2006). Another study revealed that human colorectal cancers can be prevented significantly by daily consumption of green tea (Shimizu et al. 2008b).

Conclusion

This chapter summarizes the mechanisms of green tea catechins in inhibiting the proliferation of different tumors. The multiple mechanisms include potent scavenging free radical ability, modulation of different signaling pathways, methylation, DNA damage, and enhancement of activity of conventional anticancer drugs through synergistic action. The anticancer properties of green tea catechins are attributed to its prooxidant action which directly interacts with proteins and phospholipids of membrane and regulates the signal transduction pathways, activation of caspases, inhibition of transcriptional factors activity, and release of cytochrome c (Kim et al. 2014). The beauty of EGCG is that it induces apoptosis only in cancer cell lines but not in normal cells. Limited human trials have been conducted to investigate the anticancer action of EGCG and other catechins of green tea, but in animal models, its potent anticancer action is well documented. Therefore the catechins of green tea generally and EGCG in particular could be developed as an advanced and future anticancer drugs. Taking abovementioned anticancer potential of green tea into account, it is highly advisable to have green tea consumption regularly in our diets.

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Bioactive Profile of Edible Ripened Split Beans of Three Wild Landraces of Coastal *Canavalia*

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Introduction

Protein-energy malnutrition is one of the major problems in developing countries owing to dependence largely on monocarbohydrate diet (e.g., maize or rice), which has insufficient quantities of essential nutrients (proteins, fats, vitamin A, iodine, zinc, and iron) (Boye et al. 2010). Edible legumes (peas, lentils, and beans) as well as wild legumes (winged bean, cluster bean, and velvet bean) serve as inexpensive sources of protein-energy against limited supply of meat and animal products (Singh et al. 2007; Boye et al. 2010). About 30 wild legumes growing in different parts of India are consumed by the rural or tribal population in India as nutraceutical sources (Arora et al. 1980; Gunjatkar and Vartak 1982; Mohan and Janardhanan 1995; Viswanathan et al. 1999, 2001; Vadivel and Janardhanan 2005; Narayanan and Kumar 2007; Sridhar et al. 2016). Utilization of such wild legumes has received more attention in the recent past owing to their nutritional as well as health benefits.

The vast and long coastal region of the Indian subcontinent is endowed with several underexplored and economically valuable wild legumes (e.g., *Alysicarpus*, *Canavalia*, *Sesbania*, *Tephrosia*, and *Vigna*) (Arun et al. 1999; Rao and Suresh 2001; Rao and Sherieff 2002; Sridhar and Bhagya 2007). In the coastal sand dunes and mangroves, two landraces of *Canavalia cathartica* are widely distributed (Arun et al. 1999; Seena et al. 2007). Similarly, another wild legume *Canavalia maritima* on the coastal sand dunes received little attention although widely distributed in the pantropical region (Nakanishi 1988). These three landraces possess valuable agrobotanical traits especially fast growth, large seeds, high quantity of seed yield, and tolerance to adverse conditions (temperature, salinity, burial, and pest attack) (Arun et al. 2003; Seena and

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Fig. 1 Horizontal spread of *Canavalia cathartica* on the coastal sand dunes of the Southwest India (**a**) with ripened pods (**b**), ripened beans (**c**), and split beans (**d**); horizontal spread of *C. maritima* on the coastal sand dune of Southwest India (**e**) with ripened pods (**f**), ripened beans (**g**), and split beans (**h**)

Sridhar 2006; Sridhar and Seena 2006; Seena et al. 2007). During the survey of ethnic knowledge of coastal inhabitants of the southwest coast of India, it was realized that *Canavalia* spp. also provide nutritional and health benefits (Bhagya and Sridhar 2009). The fishermen community usually employs many traditional approaches to consume the ripened beans similar to common green and leafy vegetables. They gather the ripened beans of *Canavalia* spp. and eliminate the seed coat along with testa followed by cooking like other vegetables for consumption. Nutritional qualities of ripened split beans (devoid of seed coat and testa) of *Canavalia* spp. have been evaluated recently and ascertained their adequacy for human consumption (Sridhar et al. 2016). Therefore, the present study envisaged to expand further by evaluation of bioactive components and antioxidant potential of uncooked and cooked ripened split beans (devoid of seed coat and testa) to project their nutraceutical or medicinal qualities.

Ripened Beans and Processing

Greenish yellow ripened pods of *Canavalia cathartica* were collected from plants grown on five independent coastal sand dunes of Someshwara (12°47′N, 74°52′E) and Nethravathi mangrove (12°50′N, 74°51′E) of Southwest India (post-monsoon season) (Fig. 1). The ripened beans were separated from the pods, seed coat along

with testa was removed, and cotyledons (split beans) were divided into two groups in five replicates. The first group was sun-dried 2–3 days, powdered (Wiley Mill, mesh # 30), and preserved in airtight glass containers. The second group was pressure-cooked (6.5 L, Deluxe stainless steel; *TTK* Prestige™, Prestige Ltd., India) with freshwater (1:3 v/v) followed by sun drying and milling.

Assessment of Bioactive Components

Total Phenolics

The total phenolics of the split bean flours were determined after extracting twice with methanol (50%) in a water bath (95 ± 1 °C, 10 min) (Rosset et al. 1982). The pooled extract was made up to 10 mL; the extract (0.5 mL) was mixed with equal quantity of distilled water and treated with Na_2CO_3 (5 mL in NaOH, 0.1 N). After incubation (10 min), Folin-Ciocalteu's reagent (0.5 mL) (diluted 1:2 v/v) was added, and OD was read (725 nm; UV-VIS spectrophotometer-118, SYSTRONICS, Ahmedabad, Gujarat, India). Tannic acid served as a standard.

Orthodihydric Phenols

Orthodihydric phenol content of split bean flours was estimated according to Mahadevan and Sridhar (1985). A 250 μL of ethanol extract (100 mg, 30 mL 80% ethanol) was mixed with HCl (0.05 N, 1 mL); Arnow's reagent (1 mL) (sodium nitrate, 10 g; sodium molybdate, 10 g; distilled water, 100 mL), distilled water (10 mL), and NaOH (1 N, 2 mL) were added; and the OD was measured (515 nm). Catechol (Sisco Research Laboratories, Mumbai, India; purity, 98%) served as standard (20–100 μL).

Tannins

Tannin content of split bean flours was determined by vanillin-HCl method (Burns 1971). The flour (1 g) was treated with methanol (10 mL, 28 °C, 12 h), vortexed, and decanted. The process was repeated with the precipitate. The pooled supernatant was made up to 25 mL. The extract (1 mL) was treated with reagent mixture (5 mL) (1:1, 4% vanillin in methanol and 8% concentrated HCl in methanol). After 20 min the color developed was read at 500 nm with catechin (Sigma-Aldrich, 98% HPLC grade, USA) (50–250 μg) as standard.

Canavanine

To estimate canavanine of split bean flours, ammonium disodium pentacyanoamminoferrate dihydrate reagent (PCAF) ($Na_2NH_4[Fe(CN)_5NH_3]\cdot 2H_2O$) (1% w/v) (Sigma-Aldrich, # 09710, USA) (100 mg) was dissolved in double-distilled water (10 mL) in a volumetric flask, exposed to light and air for an hour to undergo spontaneous oxidation, and preserved (4 °C, can be preserved up to 1 month in a brown glass bottle). Phosphate buffer (pH, 7) [29.54 mL 0.2 M NaOH (solution A) and 50 mL 0.2 M KH_2PO_4 (solution B)] was made up to 500 mL with distilled water and stored at low temperature. The pH was set to 7 for solution A followed by addition of solution B, and final pH was adjusted to 7. Aqueous solution of canavanine derived from *Canavalia ensiformis* (Sigma-Aldrich, # C 1625, purity ≥98%) (2 mg/10 mL) was prepared as standard and preserved (4 °C). As observed by Fearon and Bell (1954), addition of PCAF (5–10 drops) to the standard or sample (5 mL, pH 7) within 10 min resulted in an appearance of a red to purple color, which indicated positive reaction, and the OD was read (462 nm). The flour sample (50 mg) was homogenized with phosphate buffer (10 mL). The suspension was centrifuged (3500 rpm, 10 min) at room temperature. The supernatant was filtered through Whatman (# 1) filter paper and made up to 10 mL. 1 mL of the suspension was diluted to 5 mL with distilled water, and five drops of PCAF reagent was added. The OD was read (462 nm) using phosphate buffer as blank.

Vitamin C

With minor modifications, vitamin C content of split bean flours was estimated according to Roe (1954). The sample (1 g) was extracted in TCA (5%, 10 mL). An aliquot (0.2 mL) was made up to 1 mL in TCA (5%), and 2,4-dinitrophenylhydrazine (DNPH) (1 mL) was added. The reaction mixture was boiled (10 min), cooled to room temperature; sulfuric acid (65%, 4 mL) was added, incubated at room temperature (30 min); and the OD was measured (540 nm). Ascorbic acid (Sisco Research Laboratories, Mumbai, India; purity, 99.8%) served as standard.

Trypsin Inhibition Assay

Trypsin inhibition activity of the split bean flour was determined by enzymatic assay as outlined by Kakade et al. (1974). Freshly ground flour (1 g) was extracted with NaOH (0.01 N, 50 mL), the suspension was made up to 2 mL with distilled water, and 2 mL trypsin solution (4 mg in 200 mL 0.001 M HCl) was added to each test tube and incubated in water bath (37 °C, 10 min). To each tube, 5 mL BAPNA [(40 mg N-a-benzoyl-DL-arginine p-nitroanilide hydrochloride) (Aldrich, 85711–4; purity, 99%) in dimethyl sulfoxide (1 mL) diluted (to 100 mL) with tris buffer at 37 °C)] was

added; after incubation (10 min) the reaction was terminated by addition of acetic acid (30%, 1 mL), thoroughly mixed and filtered; and absorbance of the filtrate was measured (410 nm) against reagent blank (1 mL 30% acetic acid containing 2 mL each trypsin and distilled water +5 mL BAPNA solution). One unit of trypsin activity (TIu/mg) is defined as 1 μM of p-nitroanilide released per min by the enzyme.

Hemagglutinin Assay

The hemagglutinin activity was assessed based on Occenã et al. (2007). Split bean flour (2 g) was suspended in NaCl (0.9%, 20 mL), shaken vigorously (1 min), and allowed to stand (1 h) followed by centrifugation (2000 g, 10 min) to obtain clear solution, which was filtered and used as crude agglutinin extract. Heparinized blood samples (5 mL) of different groups (A, B, O) were collected, and RBCs were separated from the whole blood suspension by centrifugation (2000 g, 10 min). One volume of RBCs was diluted with four volumes of cold saline (0.9%) and centrifuged (2000 g, 10 min), and the supernatant was discarded. The pellet was washed with saline thrice until the supernatant became colorless. Washed erythrocytes (4 mL) were suspended in phosphate buffer (0.0006 M, pH 7.4) (100 mL). Trypsin solution (2%, 1 mL) was added to washed erythrocytes (10 mL), mixed, and incubated (37 °C, 1 h). The trypsinized erythrocytes were washed (4–5 times in saline) to remove traces of trypsin. The packed cells (1.2–1.5 mL) obtained were suspended in saline (100 mL).

Hemagglutinin activity of split bean flour was assessed by microtiter plate method. Microtiter plate (8 rows of 12 wells) was used. The first well served as the test in which the crude agglutinin extract was added. The 12th well served as the control in which the crude agglutinin extract was not present. The saline (0.3 mL) was dispensed to each well (# 2 to # 12). Serial dilution was made from the well # 2 to well # 11. The suspension of trypsinized RBC (in saline 2%, 0.3 mL) was dispensed to wells # 1 to # 12. The contents were mixed and incubated at room temperature (4 h). The hemagglutination pattern in each well was noted, and the hemagglutinating unit per gram (Hu/g) was calculated: $Hu/g = (Da \times Db \times S) \div V$ (where Da, dilution factor of extract in well # 1, is the crude agglutinin extract where it remains as 1 if the original extract is not diluted; Db, dilution factor of well containing 1 Hu is the well in which hemagglutination is first seen; S, mL original extract/g split bean flour; V, volume of extract in well # 1).

Antioxidant Assessment

To prepare the split bean flour extract for antioxidant assay, the sample (10 mg) was extracted in methanol (10 mL, w/v) and centrifuged (4500 rpm, 15 min). A known amount of the supernatant was used for the antioxidant assays.

Total Antioxidant Assay

To determine the total antioxidant activity, to split bean extract (0.1 mL), reagent mixture (1 mL) was added (sulfuric acid, 0.6 M + sodium phosphate, 28 mM + ammonium molybdate, 4 mM) (Prieto et al. 1999). The samples were incubated (95 °C, 90 min) and cooled to room temperature followed by OD measurement of phosphomolybdenum complex (695 nm) with methanol (0.1 mL) as blank. The antioxidant activity was expressed as the number of gram equivalents of ascorbic acid per gram (μM AAEs/g).

Reducing Power Assay

Reducing activity of split bean flours was determined employing the method outlined by Oyaizu (1986) with a slight modification. Different concentrations (200–1000 μg) of the sample extracted in methanol were taken; phosphate buffer (0.2 M, pH 6.6, 2.5 mL) was added followed by addition of potassium ferricyanide ($K_3Fe(CN)_6$) (1%, 2.5 mL). The contents were mixed well and incubated (50 °C, 20 min). After incubation, TCA (10%, 2.5 mL) was added and centrifuged (3000 rpm, 10 min); to the supernatant (2.5 mL), equal volume of distilled water was added and mixed. To this mixture, ferric chloride (0.1%, 0.5 mL) was added and the OD was measured (700 nm). Higher absorbance of the reaction mixture indicated greater reducing power. The assay was carried out in triplicates to express the mean values.

Data Analysis

Variations in total phenolics, tannins, orthodihydric phenols, canavanine, vitamin C, total antioxidant activity, and reducing power between uncooked and cooked beans were assessed by *t*-test using Statistica version 8.0 (StatSoft Inc. 2008). Three-way ANOVA (followed by Holm-Sidak's method) was employed to assess the interaction between three landraces (mangrove *Canavalia cathartica*, dune *C. cathartica*, and dune *C. maritima*), four bioactive components (total phenolics, tannins, canavanine, and vitamin C), and two processes (uncooked and cooked) (SigmaPlot, version # 11, Systat Inc., San Jose, USA).

Bioactive Components

The total phenolic and tannin contents were significantly higher in uncooked than in cooked split beans of all landraces ($p < 0.05$) (Fig. 2). Canavanine content was significantly higher in uncooked than in cooked split beans of *C. cathartica* and *C.*

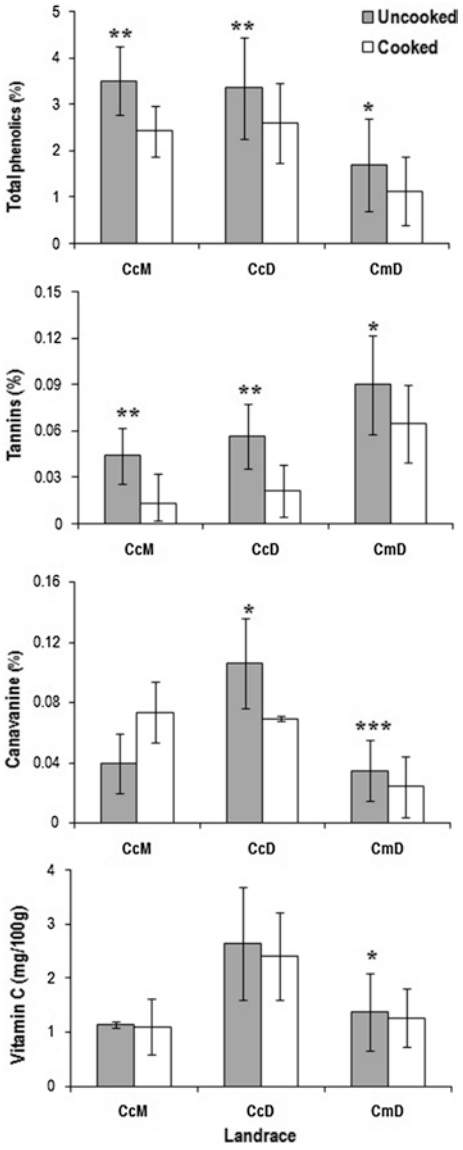

Fig. 2 Total phenolics, tannins, canavanine, and vitamin C of uncooked and cooked split beans of three *Canavalia* landraces of the Southwest India (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$)

maritima of coastal sand dunes ($p < 0.05$), while vitamin C content was significantly higher in uncooked split beans of only *C. maritima* of coastal sand dunes ($p < 0.05$). The content of orthodihydric phenols was higher in uncooked than in cooked split beans of *C. cathartica* landraces ($p < 0.001$), while it was not detectable in *C. maritima* of coastal sand dunes (Table 1). Split beans of all the three landraces were devoid of trypsin inhibition activity. The hemagglutinin activity was eliminated in cooked split beans of *C. cathartica* landraces, while only O^+ blood group retained one-third of hemagglutinin activity in cooked *C. maritima* of coastal sand dunes.

Table 1 Orthodihydric phenols, trypsin inhibition activity, and hemagglutinin activity of uncooked and cooked split beans of three landraces of *Canavalia* (orthodihydric phenols: $n = 5$, mean ± SD)

	Mangrove		Coastal sand dune			
	Canavalia cathartica		*Canavalia cathartica*		*Canavalia maritima*	
	Uncooked	Cooked	Uncooked	Cooked	Uncooked	Cooked
Orthodihydric phenols (%)	0.019 ± 0.001*	0.007 ± 0.002	0.026 ± 0.001*	0.011 ± 0.001	ND	ND
Trypsin inhibition activity	ND	ND	ND	ND	ND	ND
Hemagglutinin activity (Hu/g)						
A + ve	120	NA	120	NA	120	0
B + ve	80	NA	80	NA	140	0
O + ve	80	NA	80	NA	120	40

Asterisks across the cooked and uncooked samples are significantly different (*t*-test: $*p < 0.001$)
ND not detectable, *NA* no agglutination

Phenolic compounds are the most important groups of secondary metabolites in plants, which are abundantly present in the seed coats of legumes, and are responsible for antinutritional properties. Lowered phenolics and tannins in cooked split beans are due to removal of seed coat and testa. Total phenolics in uncooked split beans were lesser than ripened seeds (Bhagya et al. 2006, 2007, 2009); it further decreased significantly on cooking. As the tannins and orthodihydric phenols lowered in cooked split beans, they serve as bioactive compounds rather than potential antinutrients.

Low canavanine in uncooked split beans may be due to removal of seed coat as canavanine is known to accumulate in seed coat (Williams and Hunt 1967). Canavanine content of split beans was lower than ripened beans (D'Cunha and Sridhar 2010). Canavanine content of the uncooked split beans decreased significantly on cooking. As seen in phenolics, canavanine is known to accumulate in seed coat, and low canavanine content in uncooked split beans was mainly due to removal of seed coat and testa (Williams and Hunt 1967). Similar to canavanine, the lectin "con A" is also present in seed coat as well as cotyledons of *Canavalia ensiformis*, which prevents insect herbivory (Oliveira et al. 1999; Niveditha and Sridhar 2012). The ripened split beans devoid of seed coat and low accumulation of lectin in cotyledons in ripened stage are responsible for lack of hemagglutinin activity against human erythrocytes.

Although hemagglutinin activity was substantially high in uncooked split beans (80–120 Hu/g), on cooking it was completely eliminated, which was supported by significant increased in vitro protein digestibility on cooking denoting safe levels of antinutritional components to support their edibility (Sridhar et al. 2016). The hemagglutination activity in legumes is commonly linked with the presence of lectins (e.g., concanavalin), which interferes absorption of nutrients by binding to the brush border mucosa (Liener 1980; Niveditha and Sridhar 2012). Although cooked dry seeds, sprouted seeds, and ripened beans of *C. maritima* are potential source of protein, its bioavailability is limited due to presence of lectin "con M." However, the hemagglutinin activity was completely knocked off in cooked split beans except for human erythrocytes O^+ blood group in *C. maritima* of the coastal sand dune (decreased to one-third). Decrease in globulin fraction of uncooked split beans on cooking might be responsible for overall quality improvement especially the in vitro protein digestibility (Sridhar et al. 2016), elimination of hemagglutinin activity, and moderate antioxidant activities. The split beans of three landraces were devoid of trypsin inhibition activity as seen in ripened beans (Bhagya et al. 2006, 2007) which is another advantage of elimination of antinutritional potential of split beans.

Bioactive Potential

The total antioxidant activity of uncooked split beans of *C. cathartica* was higher in mangrove than in coastal sand dune landrace as well as *C. maritima* of coastal sand dunes, which significantly decreased on cooking. The reducing power of

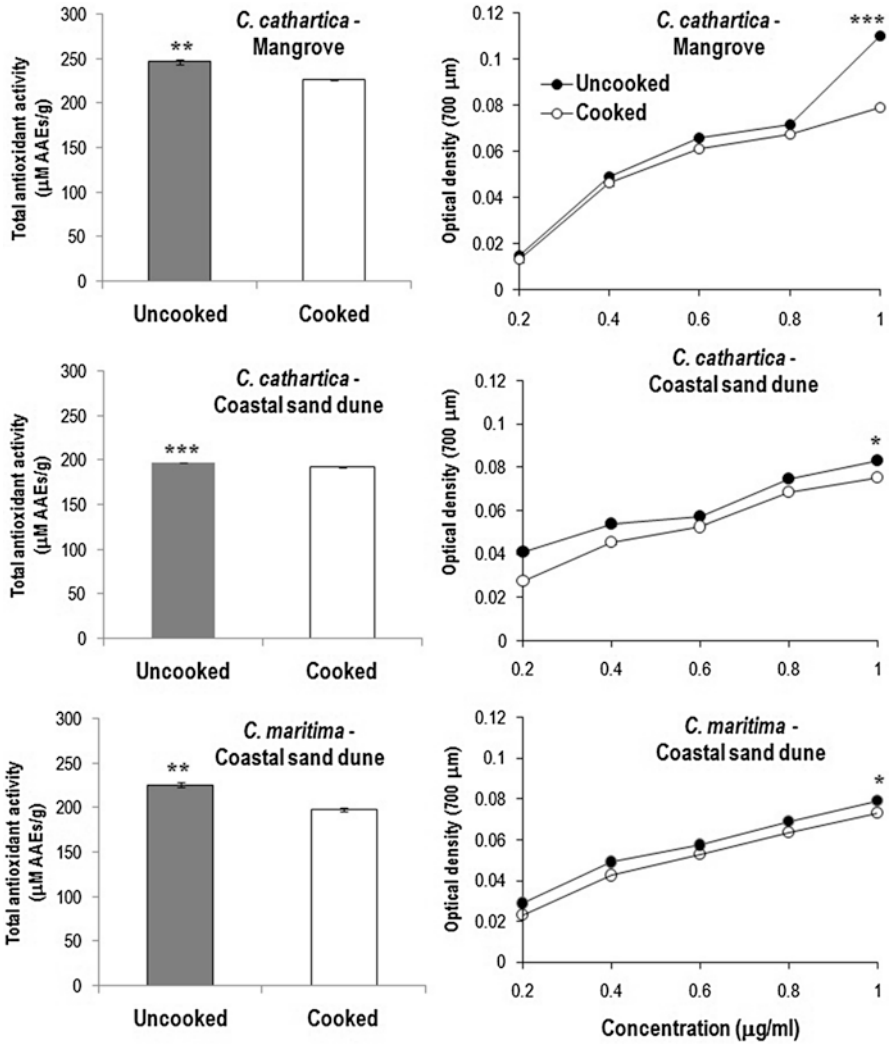

Fig. 3 Total antioxidant activity and reducing power of uncooked and cooked split beans of three *Canavalia* landraces of the Southwest India (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$)

split beans was at higher range in the mangrove *C. cathartica* than the coastal sand dune *C. cathartica* as well as *C. maritima* (Fig. 3). In all landraces, uncooked split beans showed significantly higher reducing power compared to cooked split beans ($p < 0.05$).

Phenolic compounds are known to function synergistically to promote human health by enhancing antioxidant activity, impacting cellular processes associated with apoptosis, platelet aggregation, blood vessel dilation, enzyme activities (associated with starch, protein, and/or lipid digestion), carcinogen activation, and detoxification (Shahidi and Wanasundara 1992; Tapiero et al. 2002; McDougall

Table 2 Three-way ANOVA of the interaction between the landraces (mangrove *Canavalia cathartica*, dune *C. cathartica*, and dune *C. maritima*), bioactive components (total phenolics, tannins, canavanine, and vitamin C), and processes (without cooking and cooking)

Treatment	df	F	*p*
Landrace	2	343.025	<0.001
Bioactive component	3	7451.633	<0.001
Process	1	221.863	<0.001
Landrace × bioactive component	6	343.708	<0.001
Landrace × process	2	6.159	0.004
Bioactive component × process	3	198.115	<0.001
Landrace × bioactive component × process	6	7.998	<0.001

and Stewart 2005). Canavanine content in split beans is comparable or lower than domesticated *Vicia* spp. (0.07–0.1 vs. 0.05–0.3%), and such concentration seems to be safe or advantageous in controlling colon tumors in rat model (Thomas et al. 1986; Enneking and Wink 2000). Canavanine administration at 1% in the diet consisting of protein greater than 15.7% is shown to improve the longevity of BALB/c mice (Brown 2005). There seems to be an interaction between level of dietary protein and canavanine with respect to lifespan in mice. The hemagglutination activity in legume seeds has been linked with the presence of lectins (concanavalin), as it interferes with the absorption of nutrients by adhering to the brush borders (Liener 1980). Hemagglutinin activity against human erythrocytes was completely eliminated in cooked split beans and serves as a potential source of human nutrition. Vitamin C content in split beans of both landraces was higher than ripened seeds (Bhagya et al. 2006, 2007) and not significantly decreased on cooking. The presence of vitamin C and other antioxidants is reflected in total antioxidant activity of uncooked and cooked split beans.

Various methods of elimination of antinutritional components of seeds of wild legumes have been reviewed by Bhat and Karim (2009). However, small quantities of several so-called antinutritional components possess health-promoting capabilities (e.g., antioxidants, phenolics, phytic acid, and selenium; anticarcinogenic, phytic acid and saponins; hypocholesterolemic, saponins; hypoglycemic, phytic acid) (Koratkar and Rao 1997; Shamsuddin et al. 1997; Combs and Gray 1998; Cardador-Martinez et al. 2002). Considerable protein digestibility of split beans and decrease in antinutritional components to low levels on cooking show the edible quality of all landraces of coastal *Canavalia* (Sridhar et al. 2016). Three-way ANOVA revealed highly significant difference between bioactive components (total phenolics, tannins, canavanine, and vitamin C), landraces (mangrove and coastal sand dunes), and processes (uncooked and cooked) ($p < 0.001$) (Table 2). Holm-Sidak's method showed highly significant difference between landrace: (1) *C. cathartica* of mangrove vs. *C. maritima* of coastal sand dunes ($p < 0.001$); (2) between *C. cathartica* vs. *C. maritima* of coastal sand dunes ($p < 0.001$); (3) between total phenolics vs. tannic acid ($p < 0.001$), vs. canavanine ($p < 0.05$), vs. vitamin C ($p < 0.001$); tannic acid vs. vitamin C ($p < 0.05$); canavanine vs. vitamin C ($p < 0.05$); between total phenolics vs.

tannic acid, vs. canavanine, vs. vitamin C of all three landraces ($p < 0.001$). Based on the ANOVA, among the bioactive components, the total phenolics of all landraces attained optimum level without affecting the nutritional value of split beans, while its component was also at optimum to serve as antioxidant to protect the human health. Recently, attempt to preserve the dried ripened split beans of *C. maritima* has been attempted using electron beam irradiation to prevent fungal infestation (Supriya et al. 2014). It has resulted in fungal decontamination (decrease in fungal incidence and elimination of mycotoxins) and improvement of shelf life of dry split beans (at least up to 6 months) could be achieved at 10 kGy.

Conclusions

Removal of seed coat and testa of the ripened beans of three landraces of coastal region of Southwest India eliminated several antinutritional factors. The contents of total phenolics, tannins, orthodihydric phenols, canavanine, and vitamin C in ripened split beans of all landraces were lowered on cooking; they attained optimum level as nutraceuticals to promote human health. Lack of trypsin inhibition as well as hemagglutinin activities is an additional advantage for human consumption. Besides, decrease in antinutritional components and optimum level of bioactive components is responsible for antioxidant activity as well as reducing power. To conclude, the ripened split beans of three landraces of coastal wild legume *Canavalia* are endowed with nutritional components with adequate bioactive components to serve as future nutraceutical and health-promoting indigenous food commodity.

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Modern Molecular Biology Technologies and Higher Usability of Ancient Knowledge of Medicinal Plants for Treatment of Human Diseases

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Abbreviations

AKR1C3	*Aldo-keto reductase family 1 member C3*
BH-FDR	Benjamini and Hochberg False Discovery Rate
BLOSUM	BLOcks SUbstitution Matrix
BONF	Bonferroni correction
DASH	Dynamic allele-specific hybridization
DNA	Deoxyribonucleic acid
dNDPs	Deoxynucleotide diphosphates
dNTPs	Deoxynucleotide triphosphates
FASTA	FAST-All
gDNA	Genomic DNA
GeCKO	Genome-scale CRISPR knock-out
GWAS	Genome-wide association studies
HDR	Homology-directed repair
HOXB8	*Homeobox B8*
HWE	Hardy-Weinberg equilibrium
IBS	Identical by state
LD	Linkage disequilibrium
MAFFT	Multiple alignment using fast Fourier transform
ME	Minimum evolution
ML	Maximum likelihood
MP	Maximum parsimony
mRNA	Messenger ribonucleic acid
NJ	Neighbour joining
NHER	Non-homologous end joining
PAM	Point accepted mutations
PCa	Prostate cancer
PSA	Prostate-specific antigen
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
RNAi	RNA interference
sgRNA	Single guide RNA
shRNA	Short hairpin RNA
SNP	Single nucleotide polymorphism
TALEN	Transcription activator-like effector nuclease
UPGMA	Unweighted pair group method with arithmetic means
ZFN	Zinc finger nuclease

Introduction

The process of treating the sick and/or injured with medicinal plants and/or plant products is very old, perhaps as old as the lithographic history of humankind itself. The link between humans and the quest for naturally obtained substances to be used against various discomforts to well-being dates from ages ago. Interestingly, the naturally obtained substances used for treating the sick and/or injured did provide comfort to the ill, as is well documented in the various evidences such as clay slabs and various books (Petrovska 2012), even holy books such as the Vedas (from India) (Tucakov 1971).

Knowledge of medicinal plants' usage is the result of a number of years of struggles against illnesses and deaths (Petrovska 2012). Some investigators have used different plants such as barks of trees, seeds, and fruit bodies and various other parts of the plants such as roots, leaves, and even flowers (Petrovska 2012) and even fungi (Kao et al. 2013). One of the most ancient written proofs of the preparation of drugs using medicinal plants has been identified on a Sumerian clay slab found in Nagpur, Maharashtra, India, and is estimated to be around 5000 years old (Kelly 2009). It comprised 12 recipes for drug preparation referring to over 250 various plants, some of them alkaloid such as poppy, henbane, and mandrake (Kelly 2009). Modern-day science has also acknowledged the bioactive components from various plants, and their actions thus are included in pharmacotherapy even till date (Petrovska 2012).

A wide range of drugs of plant origin, known to us since ancient times, has been in use throughout the last millennium (Pan et al. 2013), yet the exact number of users and frequency of usage of medicinal plants are still unknown (Smith-Hall et al. 2012). Since the knowledge and scope of medicinal plants are crucial in both developing and developed countries (Smith-Hall et al. 2012), and yet, it finds a limited scope in modern healthcare system in the post-genomic area, as mentioned by Pan et al. (2013), we have discussed some modern technologies that are used in molecular medicine, knowledge of which, we believe, will improve the overall impact of medicinal plants in treating human diseases in today's world.

We believe that the various molecular biology technologies discussed in this chapter bind to form a very strong story and aid in identifying the actual cause of any diseases and target the gene pathways and/or genes, thereby rendering the treatment with medicinal plants much more effective, as shown in Fig. 1. This modern approach to an age-old practice is also a one of its kind thought process. Here we have discussed a systematic approach to molecular diagnostics of human diseases by first identifying a gene (or genes) of interest pertaining to any particular pathway and then checking if there are single nucleotide polymorphisms (SNPs) in the gene(s) of interest. The SNPs are then genotyped, following which a series of analyses are done to identify the SNP and gene of interest for the population in concern. This helps build a very tight story towards the identification of specific targets for medicinal plant and/or plant products to attack. It is then crucial to validate the proposed gene and SNP of interest by knocking it down and/or out by genome editing, which shall be discussed in details further.

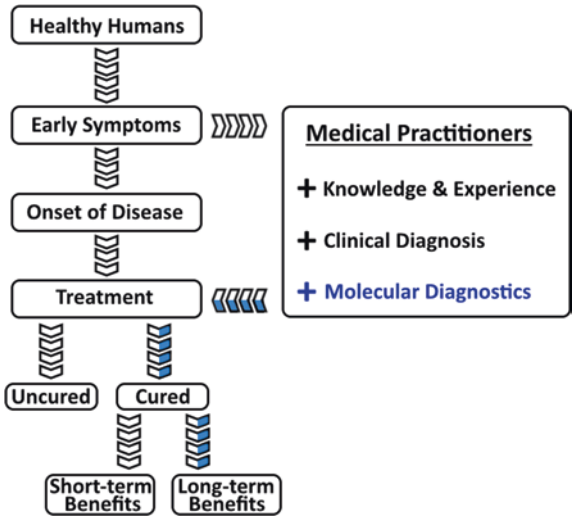

Fig. 1 A systematic order of the event with and without the implementation of molecular diagnostics by medical practitioners. After the identification of early symptoms of any disease, any individual normally visit a medical practitioner who, using his/her knowledge and experience along with the understanding of the patient's discomfort and clinical diagnosis, treats the established diseases (in most cases, by then). The treatment may or may not cure the disease. Moreover, the cure may be for a short-term or for a long-term. However, if molecular diagnostics is included in the battery of analyses (marked in blue), the treatment will eventually lead to cure, which will be for long-term, as it will predominantly be pertaining to the alteration of gene function

SNP Genotyping and Identification of Gene and SNP of Interest

Genetic variations are a major factor that contributes to the differences in various diseases susceptibly amongst individuals even belonging to the same family (Tweardy and Belmont 2009). SNPs are the variations in deoxyribonucleic acid (DNA) sequence that occur when only a single nucleotide in the genome alters and is retained to be transferred to the subsequent progeny or progenies. SNPs are the most commonly occurring variations in the human genome. Functional SNPs with non-synonymous alterations could contribute to changes in protein structure(s) leading to different phenotypic characteristics (Anderson et al. 2006). Sometimes, even if the protein produced as a function of SNP is similar to the wild-type protein, still there could be a destabilization of the amino acid interactions and hydrogen-bonding networks, which may eventually lead to varied gene function (Saleh et al. 2016).

Whole-genome association analyses pertaining to complex human diseases are useful in understanding the way of these diseases (Hu et al. 2005). A number of research groups working on different human diseases have identified that there is an association between disease susceptibility and SNPs (Ciampa et al. 2011; Liu et al.

2011; Orr and Chanock 2008; Pomerantz et al. 2011), which are hence being increasingly used as potential biomarkers for various diseases including cancers such as of the prostate (Vaidyanathan et al. 2017).

The SNP genotyping methods can be classified based on their chemical properties and differential hybridization and the enzyme-based methods, although some genotyping methods rely on both bases (Ding and Jin 2009). The differential hybridization method of SNP genotyping involves the thermodynamic differences between hybridization of the target and matched probes against the target and mismatched probes (Ding and Jin 2009). The probes used, however, must clearly distinguish between the matched and the mismatched target(s); thus, the application of SNPs is highly dependent on their flanking sequences (Ding and Jin 2009). Examples of this kind of genotyping include Affymetrix array and dynamic allele-specific hybridization (DASH) (Bichenkova et al. 2011; Ding and Jin 2009). One of the earliest methods for the identification of SNPs used an enzyme-based method (Todd et al. 2001). Restriction fragment length polymorphism (RFLP) employs certain endonuclease enzymes which can recognize and cleave specific DNA sequences and was thus a very useful technique in identifying SNPs (Todd et al. 2001). TaqMan assay combines the advantage of both differential hybridization and enzymes for SNP genotyping (Shen et al. 2009). TaqMan utilizes allele-specific probes with fluorophores linked to their 5′ end, which provides fluorescent signals after the correct probes bind to the SNP target, due to the 5′ nuclease enzyme cleavage and release of fluorophore (Shen et al. 2009). Selection of the most appropriate SNP genotyping methods depends on the amount of samples and SNPs to be screened due to cost-effectiveness. Sequenom MassARRAY iPLEX is a highly sensitive and specific differential hybridization method. It is designed to distinguish genotypes rapidly by combining iPLEX Gold chemistry with matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF MS). The MassEXTEND primer extension chemistries along with the high-density SpectroCHIP arrays allow for a high-throughput analysis of up to 40 SNPs in a single reaction (Gabriel et al. 2009; Wright et al. 2008).

Owing to the extremely complex and heterogeneous nature of most life-threatening diseases, SNPs from any one particular gene are highly unlikely to have a strong predictive value for the risk and the subsequent prognosis. Therefore, using multiple functional SNPs of genes that are associated with prostate cancer (PCa) pathology would have a better predictive value of PCa risk and aggressiveness than a single gene SNP can offer (Anderson et al. 2006). Since Sequenom MassARRAY iPLEX is a cost-effective genotyping tool (Syrmis et al. 2011) and one of the most commonly used methods to genotype SNPs, we will be discussing this in details in this chapter.

The most important aspect of this exercise, however, is to understand the results obtained by using SNP genotyping. PLINK is an unrestricted, open-source whole-genome association study package created to conduct a set of standard, large-scale investigation in a computationally systematic way (Purcell et al. 2007). PLINK has various constructive attributes for managing and inspecting genetic information. It has various benefits such as reading data in numerous formats, concatenating two or

more files, extricating subsets (SNPs), and compressing information in a binary file format to name some (Purcell et al. 2007).

Phylogenetic Tree

Phylogenetic study is the means of deducing or determining evolutionary associations or relationships (Brinkman and Leipe 2002). Phylogeny is the evolutionary background and lineage of a species (Brown 2002). The evolutionary history deduced from phylogenetic study is usually represented as branching, treelike figures that illustrate an evaluated ancestry of the inherited associations amid organisms, molecules, or both (Brown 2002). Phylogenetics is occasionally known as a cladistics in view of the fact that the term "clade", a group of descendants from a particular pedigree, is obtained from the Greek term for branch. Nevertheless, cladistics is a specific approach of hypothesizing in relation to evolutionary associations (Brinkman and Leipe 2002).

The main purpose of phylogenetic trees (or phylogenetics) is to reassemble the correct linealogical links amongst associated biological sequences. The biological sequences typically are nucleic acid (DNA) or amino acid (protein) sequences. The other purpose is to determine the time of separation amid them (sequences) from the time they last shared a common predecessor in family (ancestor) (Brown 2002).

The use of phylogenetics is important to understand how closely are certain genes related and can be used to better understand the function(s) of the genes too.

Genetic Engineering Techniques to Study the Genes of Interest

Traditionally, validation of the involvement of a gene in the mechanism of a drug of interest was primarily performed by pharmacological approaches, wherein certain chemical compounds were used to inhibit the action of the gene products, and thus concludes about the function of the drug (Reddy and Zhang 2013). However, one of the main limitations of this approach is that the pharmacological compounds often have more than one target, which makes it difficult to distinguish between the genuine effects of the gene of interest and the off-target actions of the compounds (Reddy and Zhang 2013). To avoid this, in recent times, certain developments in gene technology have led to the emergence of novel methods for validating gene functions in drug discovery studies.

Genetic engineering was born in the late 1970s, drawing examples from the processes occurring in nature already (Fig. 2), when exogenous DNA could be taken up or randomly integrated into genome by yeast or bacteria was shown (Griffiths et al. 2000), using restriction enzymes (Fig. 3). Later on, in the late 1970s, Mario Capecchi

Fig. 2 Schematic diagram of principle of natural homologous recombination. Damage to one strand by either internal or external factors would result in the recruitment of damage control machinery of cell, which would take help from its homologous strand to repair its damaged sister strand by the process called homologous recombination

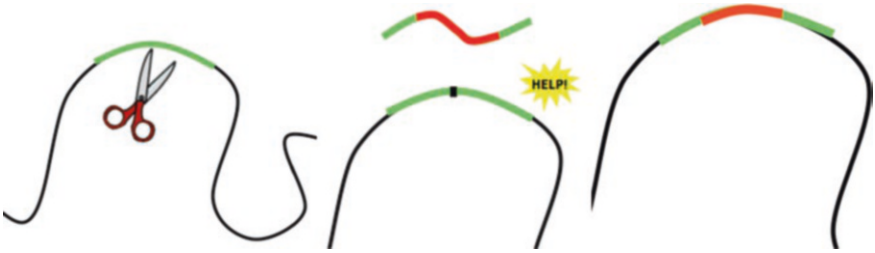

Fig. 3 Schematic diagram of increase of homologous recombination efficiency by exogenous DNA donor template

showed gene targeting by DNA microinjection into a cell's nucleus would stimulate cellular homologous recombination (Capecchi 1980). This made it possible for the first time to modify the expression of specific gene in the genome of mammals (Capecchi 1980). In 1989, Mario Capecchi, Martin Evans, and Oliver Smithies created the world's first knock-out (KO) mouse for which they got Nobel Prize in 2007 (Hansen 2007). This has been permitting scientists to study the role of specific genes in different disciplines of biology including development, physiology, and pathology.

In the last few decades, scientists were able to use "reverse genetics" as an approach to understand gene function and linking up genotype to phenotype (Gilchrist and Haughn 2010). Genetic modification using KO and knock-in (KI) or RNA interference (RNAi) has been successfully used to study gene function (Gilchrist and Haughn 2010). In the pursuit for genome editing tool development and transposon-mediated modification of endogenous gene, site-specific recombinase technology, such as Cre/loxP (Ledbetter et al. 2014) and FLP/FRT systems (Park et al. 2011), has been developed. However, the efficiency of exploration of gene function in vivo was affected by random insertion of transposons into genome, temporary knock-down (KD) effect and less efficiency of RNAi, and labour-intensive recombinase technologies (Gottumukkala et al. 2013).

Instead of indirect assessment on gene products, modern gene function validating methods directly reduce the level of gene expression (KD) by targeting its corresponding messenger ribonucleic acid (mRNA) (Witkos et al. 2011). More recently, clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated (Cas) gene (CRISPR/Cas9)-based approaches allow direct intervention on the gene of interest and permanently interfere with its function (KO) (Boettcher and McManus 2015). Latest development in CRISPR/Cas9-based methods not only allows KOofa single/multiple gene(s) but also at genome-wide scale (Boettcher and McManus 2015).

Gene Knock-Down

RNA Interference (RNAi)

Inhibition of mRNA translation by short interfering RNA is the most frequently used RNAi method. RNAi is a phenomenon of mRNA degradation in which short double-stranded RNA (dsRNA) is the initiating factor for post-transcriptional gene silencing (PTGS) (Boettcher and McManus 2015; Fire et al. 1998) and is a conserved biological response to double-stranded RNA that mediates resistance to both endogenous parasites and exogenous pathogenic nucleic acids (Hannon 2002). RNAi plays a vital role in triggering a cascade of events that lead to the degradation or blocking of the corresponding mRNA in a sequence-specific manner (Hayafune et al. 2006) and subsequently to a reduction in the levels of the corresponding protein (Hannon 2002). In addition, RNAi has also emerged as an important regulator for the expression of endogenous transcripts (Hannon 2002). The power and utility of RNAi and its recent advances have driven its incredibly rapid adaption as a tool for the stable silencing of genes in a variety of different animal species including mammals (Kelly 2009).

RNAi is triggered by dsRNAs in the form of invading RNA viruses or by using artificial dsRNA molecules (Boettcher and McManus 2015; Shen et al. 2009). The dsRNAs homologous to the target mRNA are converted into 21–23 nt siRNA duplexes by RNase III enzyme called dicer in the cytoplasm (Meister and Tuschl 2004), and these siRNA duplexes are assembled into a multi-protein RNA-induced silencing complex (RISC) in the cytoplasm. Functional RISC has helicase, exonuclease, and endonuclease activities and also comprises homology-searching domains (Nykanen et al. 2001). Within RISC, the small interfering RNA (siRNA) molecule becomes separated, and the “sense” strand is degraded. RISC uses the unwound antisense strand as a guide strand to base pair with the target mRNA to disrupt the function of the specific gene (Elbashir et al. 2001). To protect from the invading virus dsRNAs, host organisms utilize the RNAi pathway to destroy cognate RNAs.

On the other hand, microRNA(s) (miRNA) can be produced from intergenic regions of genome that regulates endogenous gene expression or artificially intro-

duced molecules such as short hairpin RNA (shRNA) or miRNA (sh/miRNA). The hairpin RNAs are first transcribed in the cell nucleus as long primary transcripts that possess a 5′ cap and a poly-A tail (Lee et al. 2002; Murchison and Hannon 2004). The transcribed primary transcript is cleaved in the nucleus by Drosha enzyme and produces a 70 nt hairpin transcript (pre-miRNA) (Lee et al. 2003; Zeng and Cullen 2004). The pre-sh/miRNA transcripts are transported to the cytoplasm by exportin 5 through nuclear pores. In the cytoplasm, the pre-miRNA transcripts are cleaved by dicer enzyme, and the small ds-transcripts are loaded into RISC complex (Meister and Tuschl 2004). Hairpin-based RNAi pathway mediates either cleavage or translation termination. A perfect match of mRNA and antisense strand in RISC induces cleavage of target mRNA (most plant miRNAs) (Bartel 2004) (Fig. 4).

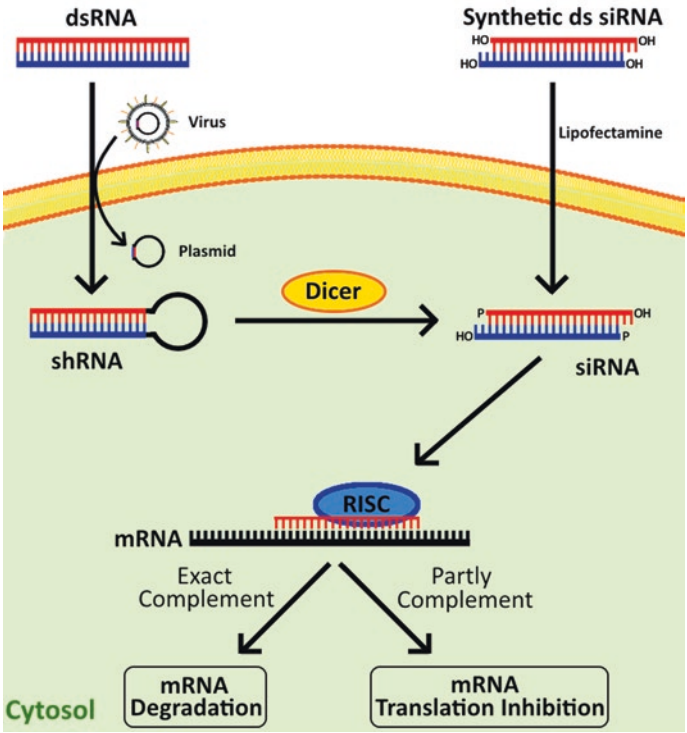

Fig. 4 RNAi pathways. Hairpin-based RNAi pathway: Long dsRNA molecules are cleaved by Drosha to produce shRNA/miRNA inside the nucleus. Hairpin RNA molecules are then transported to the cell cytoplasm and again processed into siRNA molecules by dicer. The siRNA molecules are then incorporated into RISC. The duplex RNA is unwound leaving the antisense strand to guide RISC to complementary mRNA for subsequent translational inhibition. RNAi pathway: Long dsRNA molecules are cleaved to produce siRNA molecules by dicer. The siRNA molecules are then incorporated into RISC. The duplex RNA is unwound leaving the antisense strand to guide RISC to complementary mRNA for subsequent mRNA degradation

Gene Knock-Out

In recent years, there were a number of major breakthroughs in gene targeting technology developing tools that can precisely, cheaply, and efficiently edit genome such as zinc finger nuclease (ZFN), transcription activator-like effector nuclease (TALEN), and CRISPR/Cas9 (Boettcher and McManus 2015). Principally, all three new genome editing techniques (i.e. ZFN, TALEN, and CRISPR/Cas9) apply double-strand breaks (DSB), followed by DSBs being corrected by error-prone non-homologous end joining (NHEJ) and/or homologous recombination (HR) (Boettcher and McManus 2015; Chen et al. 2016).

These reagents can be used to precisely alter the genomes of higher organisms by exploiting endogenous DNA repair machinery.

Zinc Finger Nuclease (ZFNs)

ZFNs represent the first artificial restriction enzymes engineered by fusing a DNA-binding domain to a DNA cleavage domain. Zinc finger domains can be engineered with a series of zinc fingers to target unique sequences within a specific genomic locus, subsequently fused to FokI nuclease (Boettcher and McManus 2015). Paired ZFNs recognizing two adjacent DNA sites and the FokI cleavage domain must dimerize to cleave DNA initiating either non-homologous end joining (NHER) or homology-directed repair (HDR) (Figs. 5 and 8), resulting in the disruption or change of function of the targeted gene. The utility of ZFNs is limited by their long synthesis time, high cost, and nonmodular assembly process (Boettcher and McManus 2015; Chen et al. 2016).

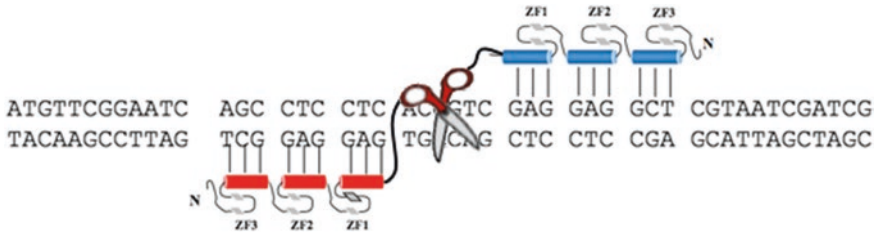

Fig. 5 A genomic double-strand break, illustrated for ZFN cleavage. A pair of three-finger ZFNs is shown at the top in association with the gene of interest. If a homologous donor DNA construct is provided, repair mechanism can proceed by HDR using the DNA construct as a donor template. Otherwise, the double-strand break can be repaired by NHEJ pathway, leading to mismatch bases at the cleavage site. These may be deletions, insertions, and base substitutions

Fig. 6 Schematic diagram of TALEN action. Heterodimeric TALENs bind to target DNA sequences to direct a fok1 nuclease domain to generate DSB between binding sites. These engineered nucleases frequently induce breaks that can be repaired via either error-prone NHEJ or HDR (Figs. 6 and 8)

Transcription Activator-Like Effector Nuclease (TALENs)

TALENs, a type of restriction enzymes, represented a huge step forward to introduce double-strand cuts on specific sequences of DNA—like ZFNs. TALENs are made by fusing transcription activator-like effectors, which can be engineered to bind to target gene sequence of interest and a FokI nuclease that cut at specific locations in a non-sequence-specific manner (Fig. 6) (Boettcher and McManus 2015; Chen et al. 2016).

CRISPR/Cas9

Clustered regularly interspaced short palindromic repeats, or CRISPR, are genomic loci originally observed in *Escherichia coli* composed of short DNA repeats with spacers interspersed. CRISPR loci have been found in a variety of Archaea and bacterial genomes. This relatively novel family of repeats was initially discovered in 1987. CRISPR loci include repeats, spacers, a leader sequence, and CRISPR-associated (Cas) genes.

Mechanistically, CRISPR-Cas system-driven immunity in bacteria works in three major steps (Boettcher and McManus 2015):

1. Acquisition, uptake of foreign DNA sequence, and integration as new CRISPR spacers into CRISPR locus
2. Expression, where Cas proteins are produced and the CRISPR RNA (crRNA) targeting sequences are transcribed from DNA sequences known as protospacers
3. Interference, where crRNA-Cas ribonucleoprotein complexes mediate cleavage of DNA in a sequence-specific manner upstream of the protospacer adjacent motif (PAM) in any genomic location (Fig. 7)

The CRISPR loci in bacterial systems include Cas genes, a leader sequence, and several spacer sequences (in red, blue, and yellow) derived from engineered or foreign DNA that are separated by short direct-repeat sequences (in blue) (Fig. 7). Individual CRISPR RNAs (crRNAs) are generated by processing of the CRISPR

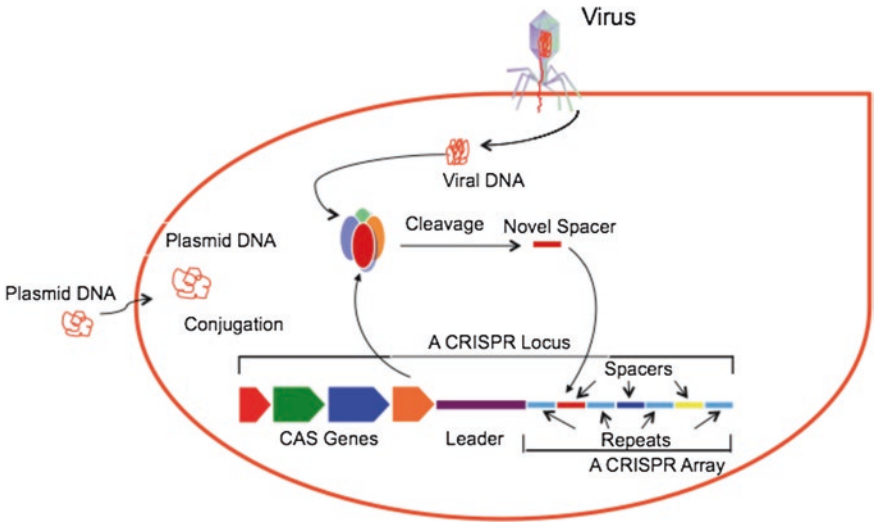

Fig. 7 Bacterial CRISPR/CAS immunity system

transcripts (van der Oost et al. 2014). Cas proteins and the crRNA make an effector complex that recognizes a target DNA sequence in a sequence-specific manner. Cleavage of the target sequence occurs and is followed by DNA repair by the endogenous cellular repair machinery or using exogenous donor template known as KI (Nurnberg et al. 2016).

The bacterial CRISPR-Cas system can be exploited for genome editing in mammalian system. Once the CRISPR components (Cas9 protein and single guide RNA (sgRNA), equivalent of bacterial crRNA) are delivered inside the mammalian cells, the expression, if CRISPR components are delivered through plasmid system, and interference steps are similar to bacterial system. Site-specific homologous sequences can also be delivered inside the cells in the form of a single-stranded DNA or as part of a plasmid to aid the specific homologous recombination event, i.e. HDR. When the cleavage at the specific loci takes place, it can be either repaired by NHEJ or HDR. The common pathway of CRISPR/ZFN/TALENs exploited for genome editing in higher organisms is shown in Fig. 8.

Genome-Scale CRISPR/Cas9 Knock-Out (GeCKO)

One of the challenges in identifying molecular mechanisms of novel compounds is that their mechanisms often relate to a group of genes rather than just a single gene. To validate such a group of genes by using single KO would therefore require a high cost and a large amount of time. The latest development in CRISPR/Cas9 technology in the last 3 years has enabled the KO of nearly 20,000 genes at the same time using GeCKO (Konermann et al. 2015).

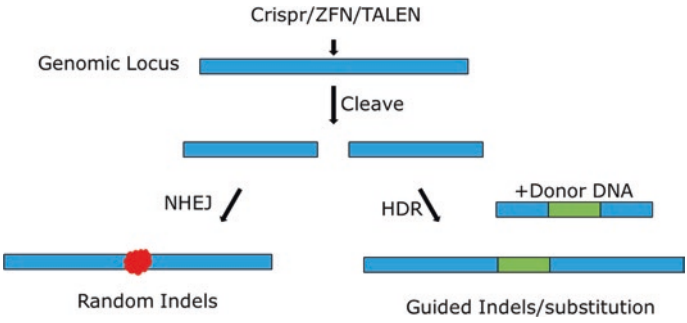

Fig. 8 Manipulation of CRISPR/ZFN/TALENs for genome editing in higher organisms: CRISPR/ZFN/TALENs are targeted to genomic locus of interest. Due to nuclease activity, once the DNA is cleaved at specific locus, the locus can be repaired by either NHEJ leading to random insertion or deletions (indels) or HDR leading to guided indels or substitutions

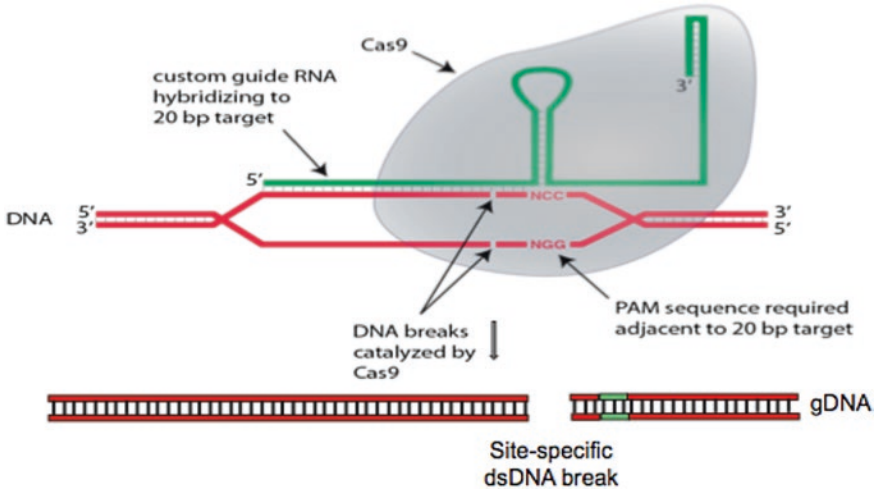

Fig. 9 CRISPR Loci and targeting of DNA sequence in mammalian system

Similar to single CRISPR/Cas9 KO, GeCKO also involves two major parts, including CRISPR/Cas9 and sgRNA, as is shown in Fig. 9. However, instead of using only one sgRNA each time, GeCKO utilizes a library containing more than 100,000 sgRNAs targeting nearly 20,000 different genes (Konermann et al. 2015).

The result of this process is a mixed population of cells, which contains different subgroups of cells, each of which has one certain gene KO, as is shown in Fig. 10. On these cells, the selection process is applied either in the form of a novel compound or a known drug to enrich for specific cells surviving those selections. Analysing the survived cell clones allows us to identify which genes are important for the sensitivity and resistance of the drug in the target cells (Konermann et al. 2015).

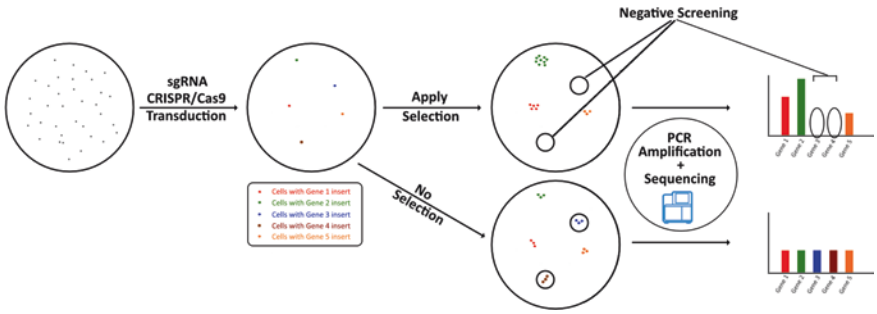

Fig. 10 A schematic plan of Genome-scale CRISPR/Cas9 KO

Animal Studies

The ultimate aim of all these modern-day technologies is to hasten the process of discovering either the novel or more efficient or sustainable way to treat the ailments chiefly in human beings (Chen et al. 2016). Before any novel treatment can be tried directly in human beings, it needs to undergo a kind of quality control in the form of animal studies.

In general, there are two set criteria for any animal studies:

1. To show that the novel treatment could work in humans
2. To show that it does not elicit adverse reactions

Quite often, most of the animal studies fail even after passing the crucial first criteria due to their potential harmful effect on human beings. The major reason for this is due to the novel treatment discovered being synthetic (man-made), which might not have undergone any selection process in the evolution (Stock 2008). In case of the treatments from natural sources, such as medicinal plants, it is more likely to pass the second criteria as both ancestors of humans and plants have sustained millions of years of common natural selection process during evolution (Stock 2008).

To begin with, to show any treatment works in animal model, we require the laboratory animal to mimic the similar human ailments that we want the treatment for. The common animals that are used for animal studies are rodents for obvious reasons, and most often, the ailment that we want to treat in humans does not manifest itself either in great numbers, which we require for statistical reasons, or is completely absent (Abubakar et al. 2016). So, to generate these humanized disease models in animals, in the past, we greatly relied on breeding for mutations, which could take decades, or the embryonic stem cells, which could easily take around 2–5 years.

However, thanks to the advent of the most recent cutting-edge gene editing technologies, as discussed in above sections, now we could create the human disease models we want in rodents at brisk rate. One technology that is revolutionizing the modification of genes in laboratory animals, and is worthy of mention, is CRISPR/

Cas9 (Chen et al. 2016). Just to exemplify this, now the intended disease model(s) can be created with good success rate in a short time span of time, sometimes within just 3 months. The mechanism of CRISPR/Cas9 has already been explained in preceding sections. The use of these technologies to generate human disease models in rodents or any other animals is outside the purview of this chapter and thus not discussed in here.

Steps to Decode the Function of Genes in Human Diseases and Role of Medicinal Plants

It is crucial, we believe, for anybody prescribing medicinal plants to patients to know the working structure of a few molecular biology diagnostic technologies to understand the mechanism of human diseases better. Here, we have given examples of various methods such as SNP genotyping, its associated precursor steps, and the data analyses and gene KD and KO to validate the gene of interest associated with any disease. We have used the example of PCa in this chapter, as we have done extensive work with regard to this major.

Selection of SNPs of Interest

SNPs in the intronic and exonic regions and also in intergenic regions should be considered for analysis when understanding the effect of SNPs on various human diseases (Vaidyanathan et al. 2017). SNPs can be identified by a thorough literature search of the published genome-wide association studies (GWAS) or SNP genotyping pertaining to the diseases to be treated or pathways associated in disease outcome (Vaidyanathan et al. 2017).

Normally research articles published on or after the year 2000 are considered for SNP genotyping studies to maintain the current trend of research (Vaidyanathan et al. 2017). Care should be taken to identify studies from the same region or comprising individuals of the same ethnicities, as certain genotypes work dissimilar across different regions and/or ethnicities (Vaidyanathan et al. 2017).

Collection of Blood Samples and DNA Extraction

Blood samples from each volunteer (patient and healthy control) need to be collected in Vacutainer® tubes (Becton Dickinson) containing ethylenediaminetetraacetic acid (EDTA). EDTA works as an anti-coagulating agent. An aliquot of the sample collected must then be used for the genomic DNA extraction.

Each individual's DNA can be extracted using a genomic DNA extraction kit (commercially available) following the manufacturers' protocol either with the aid of a fully automated machine or manually. The extracted DNA samples then need to be tested for purity and concentration. The DNA templates need to be highly pure as the following polymerase chain reaction (PCR) process could be affected by RNA and protein contaminants present. The ratio of absorbance reading at 260 nm and 280 nm wavelength of UV light NanoDrop® ND-1000 Spectrophotometer (Thermo Fisher Scientific) should be between 1.7 and 2.0 (A260/A280 = 1.7–2.0). This represents highly pure DNA.

SNP Genotyping of Candidate Genes

SNP Genotyping of Candidate Genes by iPLEX MassARRAY

MassARRAY iPLEX Gold (Sequenom®) utilizes just a single-base extension of the dideoxynucleotide triphosphates (ddNTPs) at the allele of interest (SEQUENOM 2006). The basic working principle of this protocol is that two possible alleles in a SNP can be distinguished by the different masses of ddNTPs using mass spectrometry. Different SNPs comprise of extension primers of different length and mass. This variation in the mass of the extension primers allows for easy distinction of different SNPs in the same MassARRAY assay. Each extension primer should be approximately 15–30 nucleotides long and should produce products with masses in the range of 4500 Da and 9000 Da (SEQUENOM 2006). A single MassARRAY experiment can accommodate as many as 40 SNPs at the same time. Detection of the mass-specific products can be achieved by using MALDI-TOF MS. The mass spectrometry data can then be translated into genotype calls with known ddNTPs and extension primer mass via MassARRAY® Typer 4.0.2 software for analysis (SEQUENOM 2006).

Chronologically, after extracting genomic DNA from the blood sample, the next step of an iPLEX MassARRAY is PCR amplification of sequences around the SNPs of interest. Setting up a PCR reaction involves the dissolution of 10x reaction buffer (usually provided with PCR amplification kits); MgCl2, which acts as a cofactor for DNA polymerase; deoxynucleotide triphosphates (dNTPs); forward and reverse primers; the genomic DNA; and Taq DNA polymerase in nuclease-free water. The forward and reverse primers are designed to bind to the DNA sequence at around 200 bps upstream and downstream of the SNP of interest. HotStar Taq DNA Polymerase (Sequenom®) is activated at 95 °C, and the condition is maintained for 5 min in a thermocycler. This step of maintaining 95 °C ensures that the double strand of the DNA is denatured, and the forward and reverse primers can bind at the specific regions, at a temperature which suits Taq DNA polymerase to function well, as the next few repetitive steps are completely dependent on this step. Next action in a standard PCR cycle involves a repeat of three steps over a number of times. These three steps are a short denaturation step (95 °C for around 30–45 s), a step to allow the primers to anneal (55 °C for around 45–60 s), and an extension step to

allow the dNTPs to bind, while the Taq DNA polymerase aids in the process of duplication of the DNA strand with the SNP of interest (72 °C around 120 s). These steps are carried out to denature the double-stranded DNA, help in the forward and reverse primers to anneal, and extend or multiply the gene of interest using dNTPs and Taq DNA polymerase. Following these three steps is another major step of around 7 min—final extension at about 72 °C.

Once the PCR is completed, the remaining dNTPs need to be removed using shrimp alkaline phosphatase (SAP) (SEQUENOM 2006). SAP enzymes dephosphorylate dNTPs to deoxynucleotide diphosphates (dNDPs) rendering them unavailable for the following iPLEX extension reaction. Next, during the iPLEX extension reaction, the extension primers anneal to the amplified PCR products specifically to near the SNP (SEQUENOM 2006). The extension primers were extended by one nucleotide with mass-modified ddNTPs complementary to the SNP sites. The ddNTPs terminate the extension of primers.

After the PCR amplification of the gene with the SNP of interest is carried out, resin desalting is required for optimization of mass spectrometric analysis (SEQUENOM 2006). Since sodium (22 Da) and potassium (38 Da) ions have a similar mass to A/C (24 Da) and C/G (40 Da), certain SNPs can be difficult to be discriminated if these ions are present in solutes. Resin forms a complex with the free ions thus retaining and removing them from mass spectrometry analysis. Mass spectrometry analysis is carried out using a SEQUENOM® machine (SEQUENOM 2006).

The overall flow chart of the iPLEX process of SNP genotyping is provided in Fig. 11.

Genotype calling was performed using the standard post-processing calling parameters in SEQUENOM Type 4.0 software (SEQUENOM 2006). Each 384-well plate prepared for genotyping contained known HAPMAP control samples, negative controls (water), and repeats of samples used in different locations in the 384-well plate for validation of the genotyping procedure.

An example result plate using SNP rs6162 and PCa samples is shown in Fig. 12. The green-coloured wells depict the wells in which the entire procedure of SNP genotyping and subsequent result calling was successful, and the light green- and light yellow-coloured wells are also indicators of lesser quality successful results, whereas the red-coloured wells depict failures. These may be due to the presence of blanks or negative controls in the well.

SNP Genotyping of Candidate Genes by TaqMan Assay

SNPs can be genotyped using the Applied Biosystems (ABI) TaqMan MGB diallelic discrimination system. TaqMan SNP genotyping assays can be obtained either predesigned (assay-on-demand) or custom-made through Assay-by-Design service by ABI. The reactions can be prepared using 20x Primer (forward and reverse primers) and TaqMan probe labelled dye mix (FAM, VIC), 2x TaqMan Genotyping Master Mix, DNase-free water, and 10 ng/μL sample DNA as well

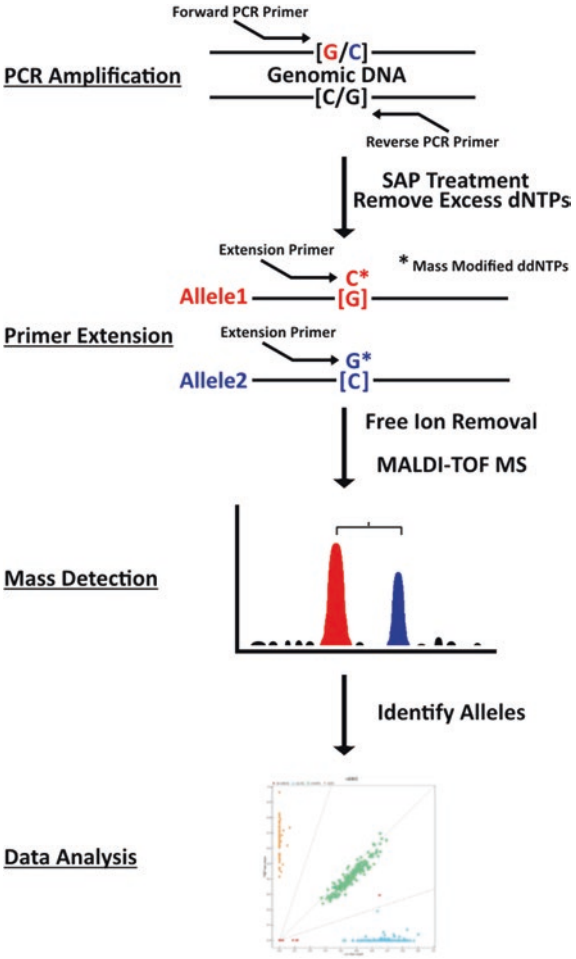

Fig. 11 iPLEX flow chart

as no-template controls (blank) in a total well volume of 5 µL. The PCR amplification can be performed using the ABI Prism 7900 HT sequence-detector machine using the following conditions, as an example: 95 °C for 10 min, followed by 40 cycles at 92 °C for 15 s, and 60 °C for 1 min.

After PCR amplification, the allelic discrimination results can be determined by performing an endpoint read. They can be analysed using the SDS 2.1 software program. The software program "calls" the genotypes depending on the fluorescence signal given through PCR amplification which exceeds the level of background.

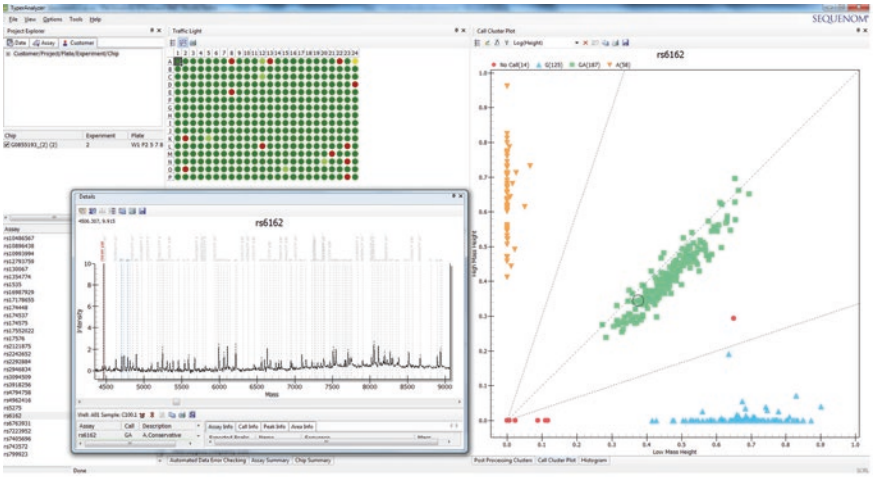

Fig. 12 An example of result plate after calling the alleles in a SNP

Statistical Analysis

SNP Data Cleaning

Before going ahead with analysing the data obtained after the Sequenom® procedure, checking the genotypes for compliance with Hardy-Weinberg Equilibrium (HWE) and for linkage disequilibrium using PLINK is a good practice (Purcell et al. 2007).

Data Analysis

A structured association approach similar to the one proposed by Vaidyanathan et al. in 2017 or PCa can be followed. This provides a simple but powerful method, to detect population stratification, and is implemented in PLINK (Vaidyanathan et al. 2017).

PLINK's clustering approach is based on the genome-wide average proportion of alleles shared identical by state (IBS) between two individuals SNPs, i.e. pairing up the SNPs based on genetic identity (Purcell et al. 2007). The IBS clustering is used to test whether the SNPs of two individuals belong to the same population. Following the stratification analysis, we performed a standard case-control association test using a Cochran-Mantel-Haenszel statistic (1df (degree of freedom)) that tests for SNP-disease association conditional on the clustering. This accounts for stratification effects, as has been reported by Vaidyanathan et al. (2017).

To avoid the possibility of false positives with multiple SNP testing, statistical significance can be restricted by the most conservative Bonferroni correction (BONF) along with the less conservative Benjamini and Hochberg false discovery

rate (BH-FDR) for multiple testing corrections. However, as in most cases, the tested SNPs are already shown to be statistically significantly associated with risk for most diseases by other researchers; variations that demonstrated significant association to risk of PCa before BONF and BH-FDR may also be considered for analyses (Vaidyanathan et al. 2017).

Association Analysis

To assess if the genes that were identified using a literature search and the SNPs present in them have an association with the samples begotten from a particular cohort, the data generated by SNP genotyping is first assessed for association analysis. To perform association analysis using PLINK, one needs genotype data in two text files, i.e. a pedigree file (.ped) and a map file (.map).

The command to perform an association analysis with multiple corrections is as follows:

```
plink --file mydata --assoc –adjust
```

The above command outputs two files: one for the association (plink.assoc) which gives the results for the association analysis and the other for adjustment (plink.assoc.adjusted) which gives the results for the association analysis with multiple adjustments. Figure 13 shows the two input files (.map and .ped), a command prompt console of the completed association analysis, and the two output files (plink.assoc and plink.assoc.adjusted) for PCa data (as an example) with 138 SNPs for 566 samples between two groups, i.e. between aggressive cancer patients and control patients from a New Zealand population as explained in Vaidyanathan et al. (2017).

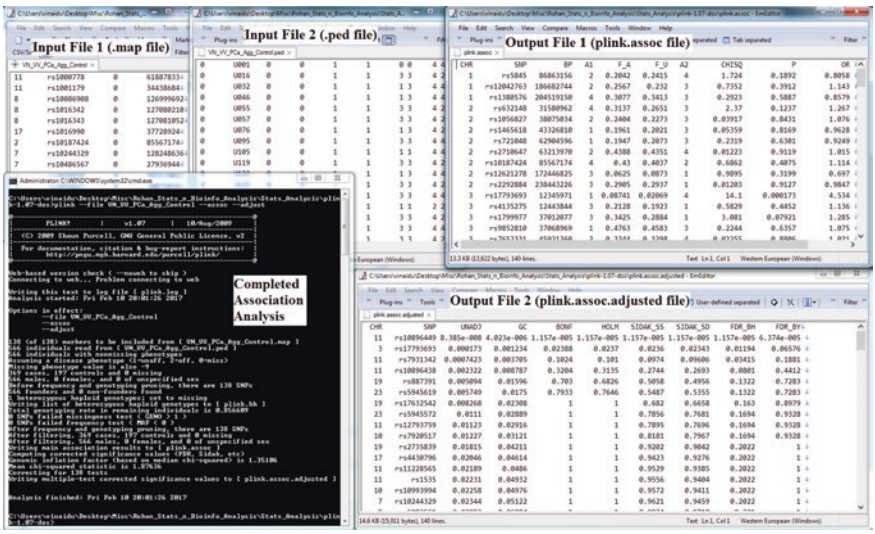

Fig. 13 Association analysis using PLINK showing the two input files, completed association analysis on a command prompt console and the two output files

The association analysis generates data to analyse and understand the effect of gene x environment interactions with regard to the disease of interest. However, the total genes of interest that were first considered before carrying out SNP genotyping should further be analysed for gene-only effect as well in the cohort. This is carried out by an interaction analysis, after adjusting for the demographic factors which are statistically identified to have a significant association with the patients having the disease(s) to be treated by using medicinal plants and/or plant products.

Interaction Analysis

It is possible that certain diseases may be driven by external or environmental factors rather the genes have a main say. In order to identify the gene-specific cause of any particular human disease, it is important to analyse the data generated by SNP genotyping by reducing the statistical influence of the environmental factors on the disease (identified by using simple student t-test). Once the association of certain genes is established, it is easier to target it in various populations.

To perform logistic interaction analysis using PLINK, one needs genotype data in three binary files, i.e. a BED file (.bed), a BIM file (.bim), and a FAM file (.fam) and a covariate file (.text) containing the covariates (environmental factors).

The command to perform a logistic interaction analysis with multiple corrections is as follows:

```
plink --bfile mydata --logistic --genotypic --covar covariates.txt --covar-name C1,C2 --adjust
```

The above command outputs two files: one for the association logistics (plink.assoc.logistic) which gives the results for the association logistic analysis and the other for adjustment (plink.assoc.logistic.adjusted) which gives the results for the association logistic analysis with multiple adjustments. Figure 14, shows the three input files (.bed, .bim and .fam), a covariate file (.text), a command prompt console of the completed interaction analysis, and the two output files (plink.assoc.logistic and plink.assoc.logistic.adjusted) for PCa data with 138 SNPs and four covariates (i.e. age, C1; BMI, C2; smoking habits, C3; and alcohol consumption, C4) for 566 samples between two groups, i.e. between aggressive cancer patients and control patients from a New Zealand population. Similar strategy can be applied for a linear model as explained in Vaidyanathan et al. (2017).

Epistasis Analysis

Once the SNPs have been genotyped, it is also crucial to see the effect of SNP x SNP interaction on the progression of that particular disease of interest. This is carried out by performing epistasis analysis using PLINK; one needs genotype data in two text files, i.e. a pedigree file (.ped) and a map file (.map).

The command to perform an epistasis analysis is as follows:

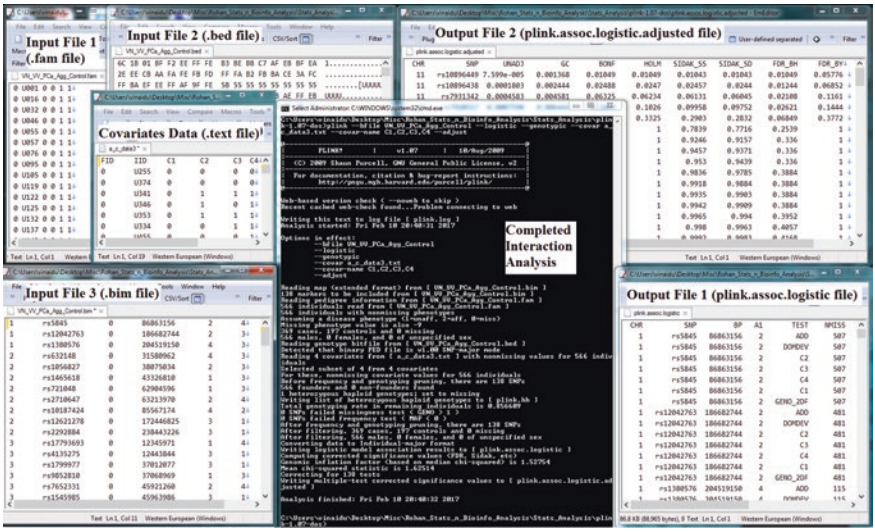

Fig. 14 Interaction analysis using PLINK showing the three input files, covariate file, completed interaction analysis on a command prompt console, and the two output files

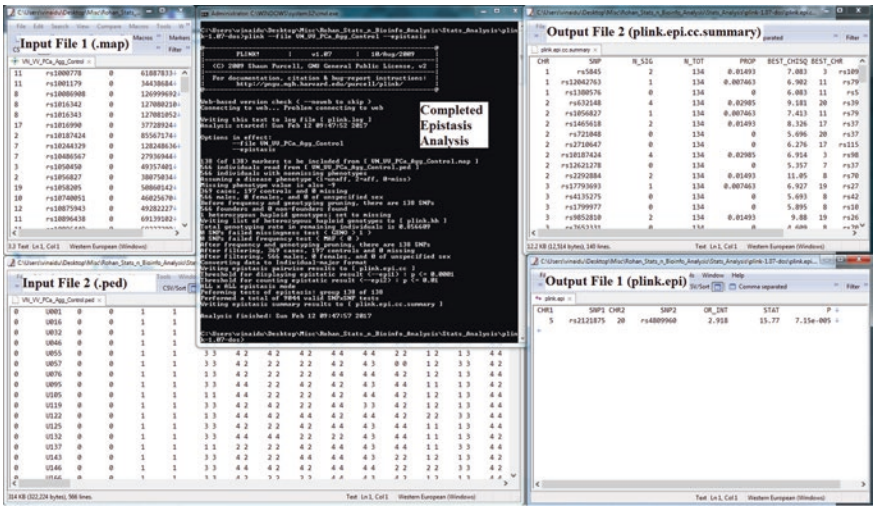

Fig. 15 Epistasis analysis using PLINK showing the two input files, completed epistasis analysis on a command prompt console and the two output files

plink --file mydata --epistasis

The above command outputs two files: one for the epistasis (plink.epi) which gives the results for the best epistasis analysis and the other for summary (plink.epi.cc.summary) which gives the results for all the epistasis analysis that were performed. Figure 15, for example, shows the two input files (.map and .ped), a com-

mand prompt console of the completed epistasis analysis, and the two output files (plink.epi and plink.epi.cc.summary) for PCa data with 138 SNPs for 566 samples between two groups, i.e. between aggressive cancer patients and control patients from a New Zealand population as explained in Vaidyanathan et al. (2017). We identified a unique SNP x SNP interaction by this scheme.

Phylogenetic Tree Generation: Four Steps

Once a gene of interest is identified after undergoing the aforementioned analyses, it is important to see if the genes have a shared evolutionary history. This aids in tackling the gene much better. There are four basic steps to generate a phylogenetic tree as shown in Fig. 16:

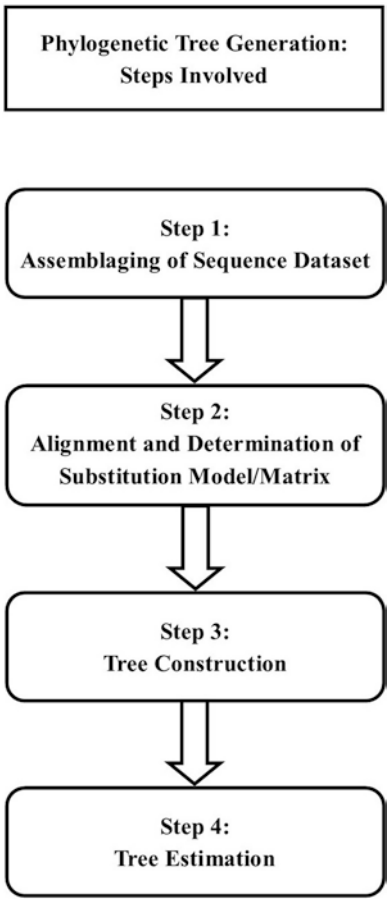

Fig. 16 The four standard steps for generating a phylogenetic tree

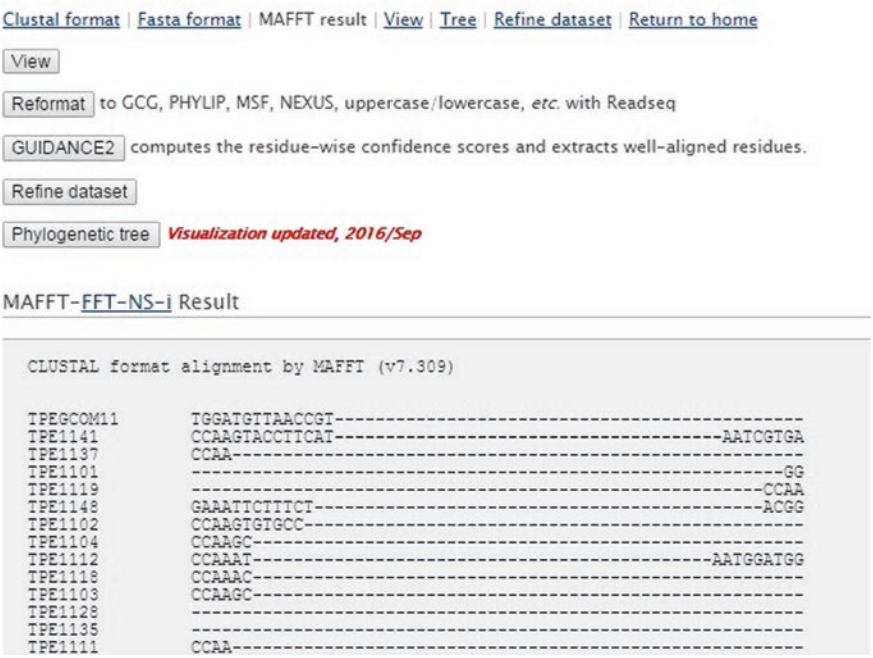

Fig. 17 Output of a multiple sequence alignment approach using MAFFT (Katoh et al. 2002; Katoh and Standley 2013). Figure 18 represents the Jalview (Waterhouse et al. 2009) output of a multiple sequence alignment using MAFFT

1. **Assemblaging of sequence dataset:** In this step, a group of homologous sequence dataset (either DNA or protein sequences) is compiled in either FASTA or Clustal format.
2. **Alignment and determination of substitution model/matrix:** In this step, the sequence dataset is aligned using openly available alignment programmes. Multiple sequence alignment approach will be used in this step to align the homologous sequence dataset. It is very crucial to choose a proper substitution matrix for a proper meaningful alignment. It is standard practice to use either BLOSUM or JTT matrix for amino acid sequences and PAM matrix for nucleic acid sequences. Figure 17 shows the output of a multiple sequence alignment using a freely available online tool known as MAFFT (Katoh et al. 2002; Katoh and Standley 2013).
3. **Tree construction:** In this step, a phylogenetic (or an evolutionary) tree is constructed from the aligned sequences. There are three different types of trees that can be constructed in this step depending on the circumstances and as discussed in the types of phylogenetics/phylogenetic trees (Table 1). Figure 19 shows a circular tree of 51 sequences, as an example, belonging to the TridenT Polymorphic Engine (TPE) virus (and its 50 variants) after representing their hexadecimal sequences into biological sequences (in this case—DNA).

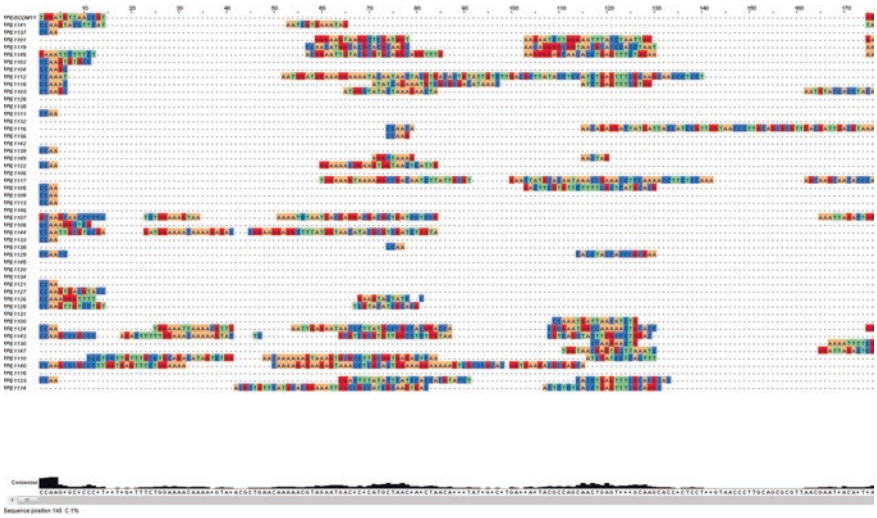

Fig. 18 Jalview (Waterhouse et al. 2009) output of a multiple sequence alignment approach using MAFFT (Katoh et al. 2002; Katoh and Standley 2013)

Table 1 Comparative analysis of the three different types of phylogenetic trees

Feature	Cladistics	Phenetics	Evolutionary systematics
Homology	Of prime significance	Not employed	Essential
Evolutionary similarity	None employed except apomorphies	All types employed (i.e. apomorphies, convergences, evolutionary reversals, parallelisms, and plesiomorphies)	All types employed (i.e. apomorphies, convergences, evolutionary reversals, parallelisms, and plesiomorphies)
Fossils	Possibly employed, but of no better significance than living species	Not employed	Possibly very essential
Association interpreted by classification or tree	Genealogy	Complete similarity or dissimilarity	Both complete similarity or dissimilarity and genealogy
Estimates (rates) of evolution	Not employed	Not employed	Very essential
Character weighting	Not employed	Not employed	Employed
Evolutionary and ecological information	Employed but occasionally	Not employed	Possibly very essential
Conversion of tree into classification	Classification literally represents branching framework (pattern) on cladogram	No complete rules; random grades of complete similarity/ dissimilarity selected to determine taxa	Classification indicates both degree of dissimilarity amongst taxa and branching framework

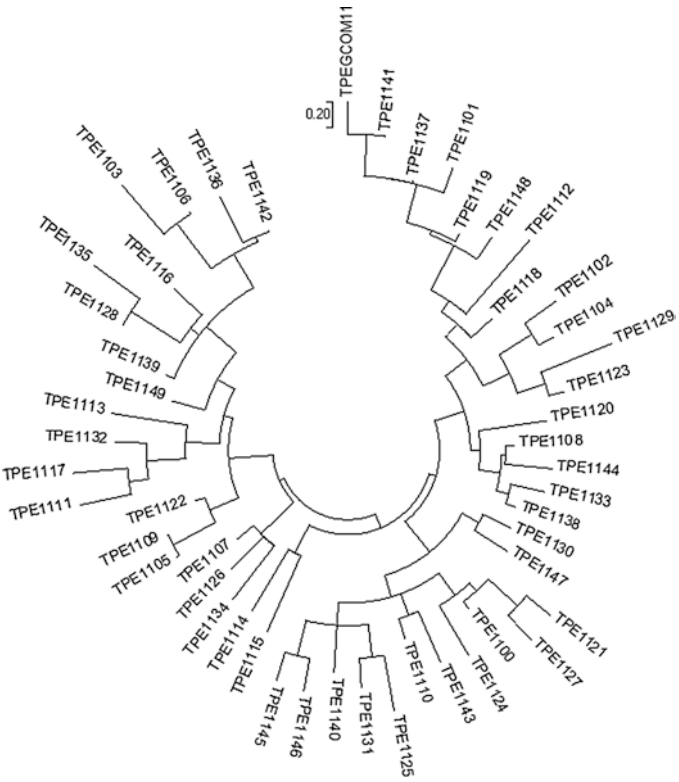

Fig. 19 Circular phylogenetic tree of 51 sequences

4. **Tree estimation:** In this step, a tree is estimated with the help of traditional statistical analysis in such a way as to evidently indicate the suitable information. Distance-based approaches such as unweighted pair group method with arithmetic means (UPGMA), neighbour joining (NJ), and minimum-evolution (ME) or discrete-based methods such as maximum parsimony (MP) and maximum likelihood (ML) can be employed to construct a significant tree. UPGMA and NJ are faster methods that use clustering algorithm, whereas ME, MP, and ML are slower methods that use optimality criterion.

Comparative analysis of different statistical analysis approaches used to estimate a tree is shown in Table 2.

Gene Knock-Down and Knock-Out

Gene Knock-Down

The following steps are carried out to KD the gene(s) of interest:

Table 2 Comparative analysis of different types of statistical analysis methods employed to construct a tree

Distance-based method	**Features**	**Limitations**
Unweighted pair group method with arithmetic means (UPGMA)	Faster and uses clustering algorithm	Loss of information
Neighbour joining (NJ)	Faster and uses clustering algorithm	Loss of information
Minimum evolution (ME)	Faster and uses optimality criterion	Loss of information
Discrete (or character)-based method	**Features**	**Limitations**
Maximum parsimony (MP)	Fast, powerful, and uses optimality criterion	Frequent statistical power depletion
Maximum likelihood (ML)	Powerful and uses optimality criterion	Sometimes slower and frequent statistical power depletion

Step 1: Designing miRNA—Selection of miRNA sequences for targeting gene of interest miRNA duplexes can be designed (gene sequence can be retrieved from the NCBI database) using online tools like BLOCK-iT™ RNAi designer tool (e.g. Invitrogen, USA). The BLOCK-iT™ RNAi designer tool utilizes proprietary algorithm to design miRNA sequences.

Step 2: Generation of single miRNA expression constructs—Both lyophilized single-stranded sense and antisense miRNA oligos are diluted in Tris-EDTA (TE) buffer to a final concentration of 200 μM. Each of the above oligos was mixed to the final concentration of 50 μM. The single-strand oligos are annealed according to the manufacturer's procedure at room temperature in a 0.5 mL sterile microcentrifuge tube.

Step 3: Identification of single miRNA inserts—Screening of single miRNA inserts is done by the plasmid DNA preparation using available DNA miniprep kits and quantification of DNA followed by restriction enzyme digest of the plasmid DNA. The correct size of miRNA inserts after restriction digestion can be visualized by gel electrophoresis.

Step 4: Transfection of mammalian cells—For transfection of cells, 2×10^5 cells are cultured in 24-well tissue culture plate containing 0.5 mL of fresh medium containing 10% FBS. Cells are cultured at 37 °C in a CO_2 incubator 12–16 h prior to transfection (to reach approximately 70–80% confluence). In order to express miRNAs transiently in cells, transfection efficiency of cells can be optimized using different transfection reagents available such as Lipofectamine 2000, Lipofectamine LTX, and FuGENE 6. The mixture of DNA and lipid is added to the cell well, mixed gently by rocking the plate back and forth, and allowed to grow for 48 h at 37 °C in a CO_2 incubator. After 4–6 h of transfection, the medium is replaced with fresh medium with 10% FBS and penicillin-streptomycin antibiotics. To increase transfection efficiency of cells, cells are subjected to shock using 10% glycerol for 3 min after 20 min of transfection period. This is then followed by gentle PBS wash to get rid of residues of glycerol.

Step 5: In vitro knock-down analysis by Western blot—For in vitro analysis of gene KD, transfected cells are lysed using lysis buffer (100 mM Tris (pH 8), 5 mM EDTA, 0.1% SDS, and 200 mM NaCl). The protein concentration of the lysates can be measured by BCA protein assay kit in accordance to the manufacturer's guidelines. Total protein is subjected to SDS-PAGE along with Benchmark™ pre-stained protein ladder (e.g. Invitrogen, USA). A transfer sandwich is set up and placed on the iBlot gel transfer device (e.g. Invitrogen, USA).

Protein transfer is done in 7 min, and the efficiency of transfer is checked by Ponceau staining. The membrane is then blocked with blocking buffer for 2 h at room temperature. Primary antibody is added to the membrane for overnight incubation at room temperature. A secondary antibody with HRP conjugate is added to the membrane and incubated at room temperature for 2 h. To visualize the target protein bands, the membrane is treated with enzyme-linked chemiluminescence detection reagents (A and B) from Amersham, for example.

Gene Knock-Out (CRISPR/Cas 9 System)

The following steps are carried out to KO the gene(s) of interest:

Step 1: Procedure for generating sgRNA expression vectors—Targeting sgRNAs are designed based on the rules outlined in Mali et al. (2013). The bicistronic pX335 (Addgene) expression vector expressing Cas9 nickase or wild-type Cas 9 system (pX458) is digested with BbsI and phosphorylates with T4 PNK (e.g. NEB). The linearized vector is gel purified, and a pair of DNA oligos for each targeting site is annealed and ligated to the linearized vector.

Step 2: Cell culture and transfection—Cell lines are cultured in growth medium supplemented with 10% foetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mmol/L L-glutamine in a 37 °C humidified incubator with 5% CO_2. For transfection, cells are co-transfected with two plasmids expressing mammalian codon-optimized Cas9 and sgRNA (single targeting), along with plasmid expressing GFP (e.g. Addgene) to check for transfection efficiency, using Lipofectamine 2000 (e.g. Life Technologies) following the manufacturer's protocol.

Step 3: Surveyor nuclease assay and RFLP analysis for genome modification—Transfected cells are incubated for 72 h prior to genomic DNA extraction. Genomic DNA is extracted from cells using PureLink genomic DNA (gDNA) mini kit (e.g. from Life Technologies) following the manufacturer's protocol. The genomic region flanking the Cas9 target sites is PCR amplified followed by the QIAquick spin column (e.g. QIAGEN) purification. Purified PCR products are subjected to a reannealing process by heat and cold to enable heteroduplex formation. The products are treated with Surveyor mutation detection kit (e.g. Transgenomics) and run on DNA gel. PCR products are also used for RELF analysis. PCR products are digested with specific restriction enzymes and separated on DNA gel. Finally, in order to confirm indels, amplified DNA sequence is cloned into a TOPO TA vector (e.g. Invitrogen) followed by Sanger sequencing.

Step 4: Protein analysis—KO cell clonal lines are cultured until 80% confluency. The consequence of gene KO is determined by Western blot analysis.

Gene Knock-Out (GeCKO)

The following steps are carried out to KO the gene(s) of interest:

Step 1: The first step of a genome-scale CRISPR/Cas9 KO (GeCKO) screen involves transducing cells with a genome-wide library of sgRNAs. To date, there are two approaches that have been proposed for these steps. In one-vector model by Shalem et al. (2014), cells are transduced with the Cas9-containing vector and sgRNAs at the same time. To increase the efficiency of the transduction, Sanjana et al. (2014) proposed to use a two-vector approach, in which cells are first transduced with blasticidin-Cas9 to select a permanent Cas9-expressing cell population. A second transduction is then performed with a library of puromycin sgRNAs with such a low multiplicity of infection that each cell is likely to be transduced with a maximum of one single gRNA.

Step 2: In the second step of a GeCKO screen, cell libraries are further selected by the treatment of compounds of interest. The control library and the survival cells after the treatment selection are then subjected to DNA extraction and deep sequencing in the third step(s).

Step 3: In the bioinformatics analysis step, sequencing data files are demultiplexed to FASTQ files. The output files are further trimmed to select only 20 bp of the sgRNA amplicons, which are then aligned and then exported into sam and bam files for the sorting steps by either one or combination of three analysis packages, which have been introduced, including RIGER, MAGECK, and MAGECK-MLE. Analysis results are then further analysed by gene enrichment tools to identify genes and pathways responsible for the drug sensitivity and resistance.

Once a gene of interest is identified after the aforementioned methods, we can screen the medicinal plants which can affect the expression of the same gene for better patient outcome. This will help save a lot of revenue as well, and the scheme of plans will be very strict, thereby reducing the chances of human errors by a trial and error approach.

Discussion

Molecular diagnostics is the future for human healthcare system (Green 2005). There are a number of advantages of using molecular technologies in diagnosing and treating human diseases (Green 2005; Vaidyanathan et al. 2017). There are a number of approaches that can be taken to identify the genic cause of health issues in humans. There are many aspects, in turn, which affect the expression of those genic factors too, as was explained in Vaidyanathan et al. in 2017 with PCa being the disease studied in a population of New Zealand men (Vaidyanathan et al. 2017).

In this chapter, we have proposed that molecular diagnostics, including the validation of results using animal studies, will be highly beneficiary for using the efficiency of medicinal plant(s) and/or plant product(s) in combatting various human diseases, including and not limited to various cancers. To further our point, we would draw an example from the findings of our group while working on aggressive PCa.

One of our recent research publications identified that the G allele of the SNP rs12529 in the gene *Aldo-Keto Reductase family 1 member C3* (*AKR1C3*) was significantly associated with lower prostate-specific antigen (PSA) levels (a test for clinical diagnosis of PCa) in PCa and benign urology disease groups compared to healthy controls (Karunasinghe et al. 2013). The SNP rs12529 does not appear in the GWAS pertaining to PCa, probably due to the pro-cancer modulation effects brought to the forefront only after the interaction(s) with confounding factors (Karunasinghe et al. 2016). It is even more interesting because an isoquinoline alkaloid found in a Chinese traditional medicinal plant, berberine (2,3-methylenedioxy-9,10-dimenthoxyproto-berberine chloride), was recently demonstrated to inhibit the AKR1C3 enzyme without having much influence on the transcription and eventually protein levels (Tian et al. 2016). According to Tian et al. (2016), berberine can also suppress cell proliferation as well as testosterone production in 22Rv1 PCa cell line (Tian et al. 2016). This is a perfect demonstration of how the knowledge of molecular diagnostics can benefit the use of medicinal plants for treating human diseases and vice versa.

There are a number of other examples; medicinal plants and/or plant product(s) have been used for various diseases, but an in-depth knowledge of molecular mechanism of diseases, especially the ones pertaining to life-threatening conditions such as various cancers, can lead to a better patient outcome. With a bulk of the developed and developing countries relying heavily on medicinal plants (Petrovska 2012) and most modern pharmaceutical companies too relying on identifying the active bio-component in medicinal plants, it is absolutely vital that this ever-advancing field of study—molecular biology—be employed to prepare better sustainable drug(s) to combat a number of ailments.

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EST (Expressed Sequence Tag): A Technique for Identification of Plant Secondary Metabolite Genes

Aruna G. Joshi and Ashutosh R. Pathak

Introduction

Plants are the major sources of various secondary metabolites as over 2,00,000 are produced by them (Dixon and Strack 2003; Kutchan and Dixon 2005). However, plants synthesize them as a defense mechanism to sustain in wild environment. These metabolites also have medicinal value and are used to treat infections, health disorders, and illness in humans (Wyk and Wink 2004). They are routinely used by pharmaceutical, herbal, flavor, and aroma industries for various purposes. Pharmaceuticals have a high market value, and about 25% of drugs contain at least one active ingredient of plant origin (Rischer et al. 2006). Synthesis of these metabolites is under the control of different genes, and they are expressed in a particular tissue or cell type that varies with time, physiology, and environmental conditions of the plant (Pichersky and Gang 2000). Plant genomes are known to contain approximately 20,000–60,000 genes, out of which 15–25% help in biosynthesis of secondary metabolites (Bevan et al. 1998; Somerville and Somerville 1999). To unravel the biosynthetic pathways of different secondary metabolites, identification and sequencing of different gene involved for the same is essential, which can be used in metabolic engineering of plants. Whole genome sequencing is laborious and expensive, for organisms with large genome sizes. There may be genome expansion, due to retrotransposon repeats, which makes it less attractive for higher plants (Bennetzen 2002).

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Plant Genome Analysis Utilizing ESTs

Functional genomics are powerful tools for identifying expressed genes. One of the popular and relatively cheap methods in transcriptome analysis is generation and analysis of expressed sequence tags (ESTs). They are typically unedited and automatically processed small sequences (200–800 bp), generated by single pass sequencing of randomly selected cDNA clones. It provides information of the genes being expressed in particular tissue at that particular time (Hatey et al. 1998). These cDNA libraries contain tens of thousands of clones and sequencing of the 5′ and 3′ ends from these libraries allows the identification of different transcripts. It is useful for the purposes of gene identification and/or confirmation of gene functions in plants whose genome sequences are not available (Parkinson and Blaxter 2009). In this technique, sequencing of cDNAs is done followed by comparison with databases which are the repository of genes. By comparing the sequences, putative functions for cDNA clones may be determined (Velculescu et al. 1995). Hence it is an important tool by which one can understand plant genome structure, gene expression, and its functions (Lopez et al. 2005). Identification of gene sequences helps in functional analyses of genes involved in secondary metabolite biosynthesis, which in turn has led to understand the metabolic systems of plants (Yonekura-Sakakibara and Saito 2009). First use of this technique dates back in the 1980s when it was used for sequencing inserts from 178 clones derived from a rabbit muscle cDNA library (Putney et al. 1983). Then a decade later, this technique was accepted as an alternative method for genome sequencing projects (Adams et al. 1991). In case of plants, EST was first used in *A. thaliana* (Hofte et al. 1994); it helps in identification of significant portions of an organism's gene content. Hence it is a key step toward genome sequencing projects (van der Hoeven et al. 2002). On 1 January 2013, a record number of 74,186,692 public entries were listed in dbEST (www.ncbi.nlm.nih.gov/genbank/dbest/dbest_summary/). The highest number of ESTs was for humans (87,04,790), whereas in case of plants, the highest number is for *Zea mays* (20,19,137). Table 1 summarizes the list of the top 10 plant species for which highest number of ESTs was submitted in dbEST.

Table 1 Plant species with highest number of ESTs in dbEST database

Plant	No. of ESTs
Zea mays	20,19,137
Arabidopsis thaliana	15,29,700
Glycine max	14,61,722
Triticum aestivum	12,86,372
Oryza sativa	12,53,557
Panicum virgatum	7,20,590
Brassica napus	6,43,881
Hordeum vulgare + subsp. *Vulgare*	5,01,838
Vitis vinifera	4,46,664
Phaseolus coccineus	3,91,150

Due to cost-effectiveness, it is routinely used for isolation and expression of several genes responsible for the biosynthesis of secondary metabolites (Ohlrogge and Benning 2000; Brandle et al. 2002; Lopez et al. 2005). In addition to gene discovery, this technique is also useful in providing information on gene functions and clarifying structural gene annotation and for development of molecular markers (Yonekura-Sakakibara and Saito 2009; Kalia et al. 2011), construction of genome maps (Paterson et al. 2000), confirming the coding regions of genome (Adams et al. 1991), analyzing phylogenetic relationships between the species (Nishiyama et al. 2003), helping in interpretation of transcriptome activity (Ewing et al. 1999; Ogihara et al. 2003) as well as providing the basis for development of DNA chips (Schena et al. 1995; Chen et al. 1998). One of the first uses of EST collection was in identification of genes involved in ricinoleic acid biosynthetic pathway (van de Loo et al. 1995). Till date many genes of different plant secondary metabolite pathways have been identified through EST analysis; however, due to random selection of cDNAs, there are chances to miss the rare transcripts, and only 60% of genes can be analyzed (Bonaldo et al. 1996). This can be overcome by next-generation sequencing technologies like Roche-454 GS FLX, ABI SOLiD, Illumina GAII, HeliScope, etc. which reduce the cost and complexity of sequencing, rendering large-scale EST analyses that are more feasible (Morozova et al. 2009; Simon et al. 2009). Earlier the sequencing was done using Sanger method which could generate very less ESTs as compared to high-throughput technique like GS FLX which generated a vast number of ESTs (McCombie et al. 1992; Bainbridge et al. 2006). GS FLX technique is being widely used for de novo sequencing and EST analyses in plants by many researchers (Wang et al. 2009; Luo et al. 2010a, b).

Development and processing of ESTs involve several steps, and finally the sequences have to be compared with the available sequences using various databases.

Expressed Sequence Tag (EST)

Methodology

The mRNAs are isolated from the tissue/cell, and cDNAs are synthesized to generate ESTs (Fig. 1).

After generation and sequencing of cDNA clones, different steps of processing are carried out to avoid contamination in the final result. Once the assembled sequences are obtained, possible functions of genes can be assigned through downstream annotation process which is achieved via database similarity searches.

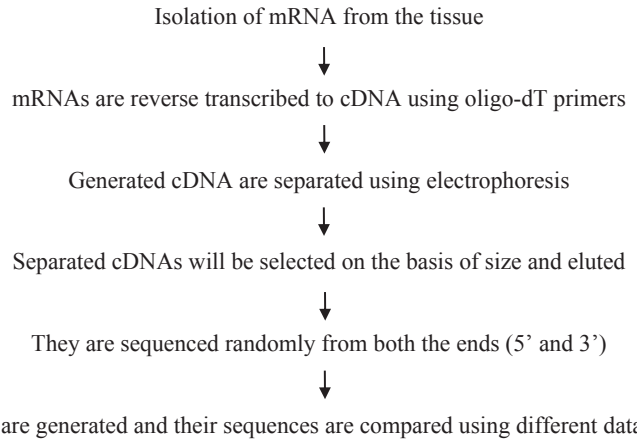

Fig. 1 Steps for generation of ESTs from cDNA library of an isolated mRNA

Processing of cDNA Sequence

Before comparison of sequences with databases, the raw sequences must be processed to remove the contaminant sequences, e.g., sequence of vector, and to achieve this, various steps are followed.

Preprocessing

The generated cDNAs certainly contain many contaminants which have to be processed before final sequencing. To achieve this, different databases are used, which many a times are specific for the type of contaminants one wants to remove. Ribosomal RNA (rRNA), vector, and adaptor sequences are considered to be the major contaminants in ESTs analysis. The vector sequences are trimmed to reduce the overall noise in EST data which in turn improves the efficacy of subsequent analyses. This is generally done using BLAST as it identifies the contaminants before ESTs are clustered (Altschul et al. 1990). VecScreen (www.ncbi.nlm.nih.gov/VecScreen/VecScreen.html), UniVec (www.ncbi.nlm.nih.gov/VecScreen/UniVec.html), and EMVec (www.ebi.ac.uk/blastall/vectors.html) are also used for removal of vector, linker, and adapter sequences from cDNAs (Nagaraj et al. 2006). Other contaminants which are repetitive elements of the genomes like LINEs (long interspersed elements), SINEs (short interspersed elements), etc. are removed using either RepeatMasker (www.repeatmasker.org/; Phred 2006) or MaskerAid (blast.wustl.edu/maskeraid; Bedell et al. 2000). Any low-complexity regions in EST data can also be removed using DUST (ftp.ncbi.nih.gov/blast/; Wan et al. 2003). The isolated mRNA contains poly(A) tail which should be trimmed to 6–10 adenines to achieve high-quality ESTs and is done using Trimest (http://emboss.sourceforge.

net/apps/#Apps) and Trimseq (emboss.sourceforge.net/apps/#App). TIGR plant repeat database (www.tigr.org/tdb/e2k1/plant.repeats/) also helps in identification and removal of plant repeats (Nagaraj et al. 2006).

Clustering and Assembly of EST

The main purpose of clustering is to collect the overlapping sequences of ESTs into a unique cluster which will help in reducing the redundancy and error rates by generating contiguous consensus sequences in each cluster. The EST cluster is generated in such a manner that single gene is grouped in single index class which provides information for particular gene (Burke et al. 1999). There are mainly two ways for clustering according to Ptitsyn and Hide (2005): stringent method and loose method. The former is a conservative technique and requires stringent criteria to join the sequences, with a requirement of high levels for sequence identity. Hence the grouping of ESTs in this method results in relatively accurate clusters but generates shorter sequence consensuses with low coverage of expressed genes, whereas loose clustering generates less accurate but longer-sequence consensuses and also groups two sequences if they share overlaps above a given threshold. Phrap (www.phrap.org/; Ewing and Green 1998) and CAP3 (genome.cs.mtu.edu/cap/cap3.html; Huang and Madan 1999) are among the most extensively used programs for sequence clustering and assembly.

Database Similarity Searches

Finally the processed EST sequences are searched for sequence similarity and to identify the putative functions genes. The sequences can be aligned to the genome sequence of either the same (*cis* alignment) or other related species (*trans* alignment, if a reference genome sequence is not available). Different BLAST programs like BLASTN (to search ESTs against nucleotide sequence database) and BLASTX (to search against protein databases from NCBI) serve as a universal tool for database similarity searches (Altschul et al. 1997). This is discussed further in database for EST analysis section.

Databases for EST Analysis

Plant genes express differently from time to time in various tissues; hence, an EST dataset will contain only the information of expressed genes. Placing ESTs into clusters is a difficult step (Parkinson and Blaxter 2004), and to solve this problem, different databases for clustering analysis are useful in identifying genes of secondary metabolite biosynthesis; for this, many databases are publically available.

One of the major databases for sequence similarity searches is NCBI (National Center for Biotechnology Information; www.ncbi.nlm.nih.gov/) (Altschul et al. 1997) and dbEST (database for Expressed Sequence Tag; www.ncbi.nlm.nih.gov/dbEST/), which is a division of GenBank at NCBI that contains sequence data and other information on EST for a number of organisms including plants. Other databases like DDBJ (DNA Data Bank of Japan; www.ddbj.nig.ac.jp/index-e.html) and EMBL-EBI (European Molecular Biology Laboratory- European Bioinformatics Institute; www.ebi.ac.uk/) contain EST datasets from all available species like plants, animals, and microorganisms. Database such as TIGR (The Institute for Genome Research) Gene Indices (www.tigr.org/tdb/tgi/) helps in searching the individual sequences on the basis of homology between species (Lee et al. 2005), whereas PlantGDB (Plant Genome Database; http://www.plantgdb.org/) contains all the available plant ESTs selected from the above databases (Duvick et al. 2008). Till 2011 PlantGDB has provided EST datasets for 22,93,800 sequences which includes 848 plants distributed in 157 families with 428 genera (Ogata and Suzuki 2011). Database from the Department of Plant Gene Research, Kazusa DNA Research Institute (www.kazusa.or.jp/en/plant/database.html), provides information on EST of *Arabidopsis thaliana* and *Lotus japonicas* and many other databases specific to the plant ESTs for sequence similarity searches (Yonekura-Sakakibara and Saito 2009).

KEGG (Kyoto Encyclopedia of Genes and Genomes) database (www.genome.jp/kegg/plant/) became operational in 1995 and is a link between genomic information with functional information (Kanehisa and Goto 2000). It consists of three databases: PATHWAY, GENES, and LIGAND. PATHWAY is for representation of functional information represented graphically for different cellular processes. GENES database has collection of gene catalogs for all the sequenced/partially sequenced genomes. LIGAND is for the collection of chemical compounds and enzyme molecules and their reactions (Kanehisa and Goto 2000). EGENES (www.genome.jp/kegg-bin/create_kegg_menu?category=plants_egenes) is a new database of the KEGG which links the genomic information with functional information (Kanehisa et al. 2006). The genomic information in EGENES is a collection of EST contigs constructed from assembly of ESTs. There are many plant species whose genome is not sequenced, and for them there is a new expansion in KEGG which uses ESTs for reconstructing metabolic pathways (Masoudi-Nejad et al. 2007). Different steps of processing EST sequences using KEGG have been described previously (Masoudi-Nejad et al. 2004, 2006; Moriya et al. 2005). Table 2 lists some of the commonly used databases for EST analysis.

MetaCyc (www.metacyc.org) and AraCyc (http://arabidopsis.org/tools/aracyc/) are databases which can be used to computationally predict the metabolic pathway complement of the genome (Zhang et al. 2005). There are about 60 plant-specific pathways in MetaCyc, whereas AraCyc is a species-specific database which contains enzymes and pathways present in *Arabidopsis thaliana*. Other user-friendly plant databases are MapMan (Usadel et al. 2005) (gabi.rzpd.de/projects/MapMan/),

Table 2 EST databases

Name	URL
dbEST at NCBI	http://www.ncbi.nlm.nih.gov/dbEST/index.html
DDBJ	http://ddbj.nig.ac.jp/index-e.html
EMBL-EBI	http://ebi.ac.uk/embl.html
Kazusa EST database	http://www.kazusa.or.jp/en/plant/database.html
Plant GDB	http://www.plantgdb.org/
TIGR plant gene indices	http://www.tigr.org/tdb/tgi/plant.html
UniGene at NCBI	http://www.ncbi.nlm.nih.gov/UniGene/
University Minnesota	http://ccgb.umm.edu/
KGENES	http://www.genome.jp/kegg-bin/creare_kegg_
ESTree db	http://www.itb.cnr.it/estree
TbestDB	http://www.tbestdb.bcm.umontreal.ca
Pscroph database	http://www.pscroph.ucdavis.edu
Mendel-GFDb and Mendel-ESTS	http://www.mendel.ac.uk/
US Mirror	http://genome.cornell.edu/
Sputnik	http://www.mips.gsf.de/proj/sputni

Kazusa Plant Pathway Viewer (KaPPA-View) (Tokimatsu et al. 2005), and Atomic Reconstruction of Metabolism (ARM) (Arita 2004) (www.metabolome.jp/software/plonejavaapplet.2006-06-29.9364604635/). Some databases focus on a specific secondary metabolite pathway, e.g., the Flavonoid Viewer (www.metabolome.jp/software/FlavonoidViewer/FlavonoidViewer). TrichOME (www.planttrichome.org/) is database of EST sequence analysis for species containing trichomes (Dai et al. 2010).

Completion of genome sequencing in model plants paves a novel way for the elucidation of genetic principles behind the huge diversity of plant secondary metabolism. Based on this, strategies such as transcriptomics have been developed and successfully employed for gene identification. EST sequences are generated by a single pass and hence may have a higher error rate than sequences that are verified by multiple sequencing runs. Although there is no real substitute of whole genome sequencing, EST analysis will certainly avoid the problems related to large genome size and retrotransposon repetitiveness (Tang et al. 2003). Generation of EST sequences provides the low-cost identification of a target gene and provides information about gene expression and metabolism in the plant tissues. Deriving information from EST datasets requires bioinformatics tools and software that allow comparisons of large sequence datasets. EST databases accelerate the research that helps to understand the expressed genes which are regulators of the secondary metabolite biosynthesis. In the last decade, this technique has been useful in revealing the genes of metabolic pathways and their interconnections. The list of plants in which genes for different secondary metabolite pathways have been identified using this technique are summarizes in Table 3.

Table 3 List of plant species in which the genes of secondary metabolite pathways have been identified using ESTs

Plant	Secondary metabolite pathway	References
Actaea racemosa	Terpenoid/sterol, phenylpropanoid, and alkaloid biosynthesis	Spiering et al. (2011)
Adonis aestivalis	Carotenoid biosynthesis	Li et al. (2008)
Arabidopsis thaliana	Phenylpropanoid biosynthesis	Costa et al. (2003)
	Fatty acid biosynthesis	White et al. (2000)
Artemisia annua	Isoprenoid, flavonoid monoterpene, and sesquiterpene biosynthesis	Bertea et al. (2006)
	Sesquiterpene biosynthesis	Teoh et al. (2006)
Astragalus sinicus	Terpenoids and polyketides biosynthesis	Zhang et al. (2015)
Beta vulgaris	Phenylpropanoid, stilbenoid, diarylheptanoid, gingerol, flavonoid, terpenoid, α-linolenic acid, fatty acid, carotenoid, flavon, flavonol, ubiquinone, terpenoid-quinone, diterpenoid, benzoxazinoid, linoleic, isoquinoline alkaloid, tropane alkaloid, piperidine alkaloid, pyridine alkaloid, sesquiterpenoid, indole alkaloid, caffeine, monoterpenoid, anthocyanin, lipoic acid, and betalain biosynthesis	Fugate et al. (2014)
Bixa orellana	Fatty acid, flavonoid, phenylpropanoids, alkaloids, terpenoids, and steroids biosynthesis	Soares et al. (2011)
Bougainvillea spectabilis 'Speciosas'	Sesquiterpenoid, sesquiterpene, terpene, terpenoid, triterpenoid, phenylpropanoid,, flavonoid, anthocyanins, and betalain biosynthesis	Xu et al. (2015)
Camellia sinensis	Phenylpropanoid and flavonoid biosynthesis	Park et al. (2004)
	Flavonoids and alkaloids biosynthesis	Chen et al. (2005)
	Flavonoid biosynthesis	Phukon et al. (2012)
	Caffeine and catechin biosynthesis	Taniguchi et al. (2012)
	Phenylpropanoid biosynthesis	Wang et al. (2012); Yang et al. (2012)
Cannabis sativa	Cannabinoids, flavonoids, and anthocyanin biosynthesis	Duraisamy et al. (2015)
Catharanthus roseus	Alkaloid, flavonoid, and monoterpenoid indole alkaloid biosynthesis	Murataa et al. (2006)
	Terpenoid indole alkaloid biosynthesis	Shukla et al. (2006)
	Triterpene biosynthesis	Murata et al. (2008)
	Terpenoid indole alkaloid biosynthesis	Mishra et al. (2011)
Centella asiatica	Centelloside biosynthesis	Kim et al. (2014)
Chlorophytum borivilianum	Saponin, flavonoid and alkaloid biosynthesis	Kalra et al. (2013)
Cistus creticus subsp. *creticus*	Flavonoid and terpenoid biosynthesis	Falara et al. (2008)
Citrus sinensis	Phenylpropanoid and flavonoid biosynthesis	Lucheta et al. (2007)

(continued)

Table 3 (continued)

Plant	Secondary metabolite pathway	References
Cryptomeria japonica	Agatharesinol and other norlignan, betailains, flavonoid, anthocyanin, isoquinoline alkaloid, and phenylpropanoid biosynthesis	Yoshida et al. (2007)
Dendrocalamus latiflorus	Flavonoid and flavonol biosynthesis	Gao et al. (2011)
Epimedium sagittatum	Flavonoid biosynthesis	Zeng et al. (2010)
Fritillaria cirrhosa	Steroidal alkaloid biosynthesis	Sun et al. (2011)
Ginkgo biloba	Phenylpropanoid, terpenoid, and alkaloid biosynthesis	Wang et al. (2010)
Glycine max	Isoflavonoid biosynthesis	Livingstone et al. (2009)
Glycyrrhiza uralensis	Glycyrrhizin biosynthesis	Li et al. (2010)
Humulus lupulus	Terpenoid, terpenophenolic, bitter acid, xanthohumol, and flavonoid biosynthesis	Nagel et al. (2008)
	Terpene and prenylflavonoid biosynthesis	Wang et al. (2008)
Huperzia serrata	Alkaloids, terpenoids, flavone/flavonoids, and anthocyanin biosynthesis	Luo et al. (2010a, b)
Jatropha curcas	Fatty acid and triacylglycerol biosynthesis	Costa et al. (2010)
	Fatty acid and flavonol biosynthesis	Natarajan et al. (2010)
	Fatty acid and steroids biosynthesis	Chen et al. (2011a, b)
Lavandula angustifolia	Isoprenoid biosynthesis	Lane et al. (2010)
Lotus japonicas	Flavonoid biosynthesis	Endo et al. (2002)
Lupinus albus	Phenylpropanoid and isoflavone biosynthesis	Tian et al. (2009)
Lycopersicon hirsutum	Methyl ketones biosynthesis	Fridman et al. (2005)
Malus domestica	Terpenoid, ester, flavonoid, and anthocyanin biosynthesis	Newcomb et al. (2006)
Medicago sativa	Steroids and tropinone biosynthesis	Hays and Skinner (2001)
Medicago truncatula	Steroid, phenylpropanoid, flavonoid, and fatty acid biosynthesis	Covitz et al. (1998)
	Triterpene saponin biosynthesis	Suzuki et al. (2002)
	Phenylpropanoid, lignin, flavonoid, isoflavonoid, and terpenoid biosynthesis	Aziz et al. (2005)
Melaleuca alternifolia	Fatty acid, terpene, and phenylpropanoid biosynthesis	Shelton et al. (2002)

(continued)

Table 3 (continued)

Plant	Secondary metabolite pathway	References
Mentha × *piperita*	Monoterpene, sesquiterpene, isoprenoid, and flavonoid biosynthesis	Lange et al. (2000)
	Terpenoid, isoflavonoid, monoterpenoid, menthol, limonene, shikimate, terpene, phenylpropanoid, tetrahydrobenzylisoquinoline, monoterpenoid, monoterpenoid indole alkaloids, and isoquinoline alkaloid biosynthesis	Roy et al. (2011)
Mesembryanthemum crystallinum	Phenylpropanoid, terpenoids, amines, and tetrapyrroles biosynthesis	Kore-eda et al. (2004)
Ocimum basilicum	Phenylpropene and terpene biosynthesis	Gang et al. (2001)
Ocimum basilicum cv Sweet Dani	Monoterpene biosynthesis	Iijima et al. (2004)
Panax ginseng	Ginsenoside biosynthesis	Jung et al. (2003); Kim et al. (2006); Chen et al. (2011a, b); Devi et al. (2012); Li et al. (2013)
	Fatty acid, benzophenanthridine alkaloid, phenylpropanoids, ally alcohols, alkaloids, flavonoids, isoflavonoids, and ginsenoside biosynthesis	Choi et al. (2005)
	Fatty acid, isoprenoid, and ginsenoside biosynthesis	Sathiyamoorthy et al. (2010)
	Isoprenoid and ginsenoside biosynthesis	Sathiyamoorthy et al. (2011)
Pandanus fascicularis	Terpenoid, shikimate, phenylpropanoid, isoprenoid, flavonoids, and alkaloids biosynthesis	Vinod et al. (2010)
Panicum virgatum	Lignin biosynthesis	Srivastava et al. (2010)
Papaver somniferum	Benzylisoquinoline alkaloid and phenylpropanoid biosynthesis	Zulak et al. (2007)
	Benzylisoquinoline alkaloids biosynthesis	Pienkny et al. (2009); Desgagné-Penix et al. (2010)
	Benzylisoquinoline alkaloids biosynthesis	Priya et al. (2012)
Polygonum minus	Flavonoid biosynthesis	Roslan et al. (2012)
Pueraria lobata	Isoflavone biosynthesis	He et al. (2011)
Poplar hybrid H11–11 (*Populus trichocarpa* × *P. deltoides*),	Phenylpropanoid biosynthesis	Christopher et al. (2004)
Ricinus communis	Ricinoleic acid biosynthesis	van de Loo et al. (1995)
	Ricinoleate biosynthesis	Lu et al. (2007)
Rosa chinensis cv. Old Blush	Terpenoid, isoprenoid, carotenoid, sesquiterpene, and anthocyanin biosynthesis	Channeliere et al. (2002)

(continued)

Table 3 (continued)

Plant	Secondary metabolite pathway	References
Rosa hybrid	Sesquiterpene and anthocyanin biosynthesis	Guterman et al. (2002)
Salvia fruticosa	Terpenoid, phenylpropanoid, flavonoid, and alkaloid biosynthesis	Chatzopoulou et al. (2010)
Salvia miltiorrhiza	Terpenoid, diterpenoid, coumarine, lignin, flavonoid, alkaloid, fatty acid, and phenylpropanoid biosynthesis	Yan et al. (2010)
Sesamum indicum	Sesame lignans (sesamin and sesamolin) biosynthesis	Suh et al. (2003)
Solanum habrochaites	Terpenoid and flavonoid biosynthesis	Besser et al. (2009)
Solanum lycopersicum 'M82'	Rutin, chlorogenic acid, and sesquiterpene biosynthesis	Schilmiller et al. (2010)
Sorghum bicolor	Sorgoleone and phenylpropanoid biosynthesis	Baerson et al. (2008)
Stevia rebaudiana	Steviol glycoside biosynthesis	Brandle et al. (2002); Richman et al. (2005)
Sugarcane	Isoprenoid, phenylpropanoid, flavonoid, and indole alkaloid biosynthesis	França et al. (2001)
Ocimum basilicum	Phenylpropanoid, phenylpropenes, and terpenoid biosynthesis	Gang et al. (2001)
Ocimum basilicum (Cv. CIM-Saumya)	Terpenoid, phenylpropanoid/flavonoid, alkaloid, tocopherol, and carotenoid biosynthesis	Misra et al. (2013)
Taiwania cryptomerioides	Flavonoid biosynthesis	Chen et al. (2004)
	Ginsenosides, monoterpenoid indole alkaloids, phenylpropanoid, and terpenoid-quinone biosynthesis	Lee et al. (2006)
Tamarix androssowii	Fatty acid and isoprenoid biosynthesis	Wang et al. (2006)
Theobroma cacao	Phenylpropanoid and fatty acid biosynthesis	Naganeeswaran et al. (2012)
Vitis pseudoreticulata	Phenylpropanoid biosynthesis	Xu et al. (2009)
Vitis vinifera	Flavonoid, isoflavonoid, lignin and terpenoids biosynthesis	Terrier et al. (2001)
	Phenylpropanoid pathway, lignin, flavonol, anthocyanin, proanthocyanidin, and isoflavonoid biosynthesis	da Silva et al. (2005)
Withania somnifera	Carotenoid, terpenoid, and flavonoid biosynthesis	Peng et al. (2007)
	Withanolide biosynthesis	Senthil et al. (2010)

Conclusion

In the era of functional genomics, transcriptomics – a global study of transcription, along with genomics – has undoubtedly contributed to plant science research. Among the different techniques which are used for identification of genes, large-scale EST analysis attracts due to cost-effectiveness and efficiency. ESTs have also

been used for discovery of the genes that are differentially expressed in various tissues, cell types, or developmental stages of the same or different plant genotypes. The method is relatively rapid for identification of novel genes and is commonly used in development of molecular markers. Availability of large amounts of data in the form of ESTs will provide an opportunity for the discovery of novel genes. Apart from gene identification, ESTs have other applications in plant science like marker development and microarray analysis. However there is no real substitute of whole genome sequencing, but due to the large genome size of plants, EST analysis certainly avoids the problems associated with it and overcomes the retrotransposon repetitiveness.

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Terpenoids: An Activator of "Fuel-Sensing Enzyme AMPK" with Special Emphasis on Antidiabetic Activity

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Introduction

The increased prevalence of type 2 diabetes, with the attendant increase in morbidity and mortality, poses a substantial therapeutic challenge. *Type 2 diabetes* is a complex polygenic disease with a strong genetic component, as indicated by the high prevalence in certain ethnic groups and by studies of identical twins. Nevertheless, the rapid increase in the prevalence of obesity-associated disease conditions, including type 2 diabetes, in worldwide populations suggests the contribution of environmental factors. A widely accepted explanation lays on the frequent consumption of processed foods with a high-calorie content and the reduction in physical exercise due to sedentary lifestyle in modern urban environment. Disruption of energy balance has led to an increased prevalence of these conditions. Type 2 diabetes is characterized by altered lipid and glucose metabolism (fasting or postprandial hyperglycemia, dyslipidemia) as a consequence of combined insulin resistance in the skeletal muscle, liver, and adipose tissue and relative defects of insulin secretion by β-cells that may arise due to an imbalance between energy intake and expenditure. Insulin resistance occurs when a normal dose of hormone is unable to elicit its metabolic responses. Insulin is the primary anabolic hormone that stimulates uptake and storage of fuel substrates while inhibiting substrate production in peripheral tissues. It lowers blood glucose levels by facilitating glucose uptake, mainly into skeletal muscle and fat tissue, and by inhibiting endogenous glucose

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production in the liver. Peripheral insulin resistance is associated with lipid partitioning in specific compartments, i.e., muscle and liver, more than with obesity per se. Usually, after an asymptomatic period of insulin resistance, hyperglycemia appears when pancreatic β-cells fail to secrete sufficient amounts of insulin to meet the metabolic demand. In the natural history of type 2 diabetes, pancreatic β-cells initially compensate for insulin resistance by hypersecretion of insulin, but with time, progressive *β*-cell failure leads to insulin deficiency and hyperglycemia ensues. Progression in diabetes leads to the development of chronic complications such as blindness, kidney failure, neuropathy, cardiovascular diseases, and amputations. Thus, novel ways to prevent and treat type 2 diabetes are urgently needed (Squirrell 1999).

AMP-activated protein kinase (AMPK) is the downstream component of a protein kinase cascade that acts as an intracellular energy sensor. Over the last few years, accumulating evidence has demonstrated that AMPK is a major regulator of cellular and whole-body energy homeostasis that coordinates metabolic pathways in order to balance nutrient supply with energy demand. However, it is activated by an increase in the cellular AMP/ATP ratio induced by metabolic stress, hormone, and nutrient signals. Once activated AMPK phosphorylates a number of downstream substrates, the overall effect of which is to switch on catabolic pathways that generate ATP and switch off ATPconsuming anabolic pathways by acute regulation of the activity of key enzymes in metabolism and chronic regulation of the expression of key transcription factors. This pivotal role of AMPK places it in an ideal position of therapeutic drug target in the treatment of diabetes, obesity, and other metabolic disorders. Therefore, the intense search for novel AMPK activators by rational drug design, screening of vast chemical libraries, and testing of various plant extracts has produced numerous promising compounds (Marín-Aguilar et al. 2017).

Among the latest developments is the activation of AMPK by naturally occurring dietary constituents and plant products—termed phytochemicals. Owing to their efficacy and safety, phytochemicals are considered as an alternative to the conventional therapy for diabetes. The rising popularity of using phytochemicals for diabetes therapy is supported by a substantial progress in identifying the molecular pathways involved, including AMPK.

Several herbal plants improve medical conditions. Such plants contain many bioactive phytochemicals. **Terpenoids** (also called “isoprenoids”) constitute one of the largest families of natural products accounting for more than 40,000 individual compounds of both primary and secondary metabolisms. In particular, terpenoids are contained in many herbal plants, and several terpenoids have been shown to be available for pharmaceutical applications, for example, artemisinin and Taxol as malaria and cancer medicines, respectively. Various terpenoids are contained in many plants for not only herbal use but also dietary use. Terpenoids contained in herbal or dietary plants, which can activate AMPK, can “fuel sensor” that control energy homeostasis; daily eating of these terpenoids might be useful for the management for obesity-induced metabolic disorders, such as type 2 diabetes, hyperlipidemia, insulin resistance, and cardiovascular diseases. Since there is a renewed interest on AMPK activators from natural products, therefore, in this review we

have discussed **terpenoids – an activator of "fuel-sensing enzyme AMPK" with special emphasis on antidiabetic activity**.

Plants as a Source of New Medicines

Putting aside the excitement surrounding the human genome, in the present era, we see the emergence of a new class of prescription medicine containing complex mixtures of plant extracts playing an important role in the search for "new medicines and effective therapies" (Clark 1996). Prior to the advances in synthetic chemistry and the discovery of antimicrobials in the late nineteenth and early twentieth centuries, plants provided the major source of medicines. Evidence of their use as long as 50,000 years ago comes from the Middle Eastern grave site of a Neanderthal man containing plant specimens. The hugely diverse plant kingdom, consisting of some 250,000–300,000 species, continues to evolve and adapt to a multiplicity of environmental conditions and to protect from pathogens and predators.

Phytochemicals (from the Greek word phyto, meaning plant) are biologically active, naturally occurring chemical compounds found in plants, which provide health benefits for humans further than those attributed to macronutrients and micronutrients (Phillipson 2001). They protect plants from disease and damage and contribute to the plant's color, aroma, and flavor. In general, the plant chemicals that protect plant cells from environmental hazards such as pollution, stress, drought, UV exposure, and pathogenic attack are called as **phytochemicals**. Recently, it is clearly known that they have roles in the protection of human health, when their dietary intake is significant. More than 4000 phytochemicals have been cataloged and are classified by protective function, physical characteristics, and chemical characteristics, and about 150 phytochemicals have been studied in detail. A wide-ranging dietary phytochemicals are found in fruits, vegetables, legumes, whole grains, nuts, seeds, fungi, herbs, and spices. Broccoli, cabbage, carrots, onions, garlic, whole wheat bread, tomatoes, grapes, cherries, strawberries, raspberries, beans, legumes, and soy foods are common sources. Phytochemicals accumulate in different parts of the plants, such as in the roots, stems, leaves, flowers, fruits, or seeds. Many phytochemicals, particularly the pigment molecules, are often concentrated in the outer layers of the various plant tissues. Levels vary from plant to plant depending upon the variety, processing, cooking, and growing conditions (Koehn and Carter 2005).

Phytochemicals are also available in supplementary forms, but evidence is lacking that they provide the same health benefits as dietary phytochemicals. These compounds are known as **secondary plant metabolites** and have biological properties such as antioxidant activity, antimicrobial effect, modulation of detoxification enzymes, stimulation of the immune system, decrease of platelet aggregation, and modulation of hormone metabolism and antidiabetic and anticancer property. There are more than thousand known and many unknown phytochemicals. It is well-known that plants produce these chemicals to protect themselves. Phytochemicals

are not essential nutrients and are not required by the human body for sustaining life but have important properties to prevent or to fight some common diseases. Because of this property, many studies have been undertaken to reveal the health benefits of phytochemicals. The list of phytochemical compounds present in medicinal herbs in relation to disease management and human health are as follows:

Phenolic Compounds: Phenolic compounds are phytochemicals that have one or more aromatic rings with at least one hydroxyl group. Plant phenolics include:

(a) **Flavonoids**: Flavonoids are low-molecular-weight polyphenolic antioxidants.
(b) **Phenolic acids**: Phenolic acids are aromatic secondary plant metabolites widely spread in plants. Phenolic acids that occur naturally can be divided into two main categories: cinnamic acid derivatives like ferulic acid and caffeic acid and benzoic acid derivatives.
(c) **Stilbenes**: Stilbenes are a family of secondary metabolites derived from phenylpropanoid pathway that consist a trans-ethene double bond substituted with a phenyl on both carbon atoms of the double bond.
(d) **Lignans**: Lignans are plant polyphenolic compounds derived from phenylalanine through dimerization of substituted cinnamic acid alcohols.
(e) **Tannins**: Tannins are polyphenols that are obtained from various parts of different plants belonging to multiple species. It is found in abundance in the tree bark, wood, fruit, fruit pod, leaves, and roots and also in plant gall. Tannins can be classified into two broad groups—hydrolysable tannins and condensed tannins.
(f) **Alkaloids**: Alkaloids are phytochemicals that contain nitrogen and are derived from various amino acids.
(g) **Saponins**: Saponins are plant compounds that occur either as steroid alkaloids, glycosides of triterpenoids, or steroids.
(h) **Cardiac Glycosides**: Cardiac glycosides are plant secondary metabolites that have a glycoside unit and act on the contractile action of the cardiac muscle.
(i) **Sterols**: Phytosterols are subgroup of steroids that have structures and functions similar to cholesterol.
(j) **Terpenoids**: Terpenoids are compounds synthesized from five-carbon isoprene units mainly isopentenyl pyrophosphate and its isomerdimethylallyl pyrophosphate by terpene synthases (Table 1) (Fig. 1).

In this review, we provide an overview of the role of "**Terpenoids – An activator of " *fuel sensing enzyme AMPK*" with special emphasis on antidiabetic activity.**

Terpenoids

The terpenoids are a class of natural products which have been derived from five-carbon isoprene units. Most of the terpenoids have multi-cyclic structures that differ from one another by their functional groups and basic carbon skeletons. These types

Table 1 Biological activities of phytochemicals

Classification	Main groups of compounds	Biological function
NSA (non-starch polysaccharides)	Cellulose, hemicellulose, gums, mucilages, pectins, lignins	Water holding capacity, delay in nutrient absorption, binding toxins, and bile acids
Antibacterial and antifungal	Terpenoids, alkaloids, phenolics	Inhibitors of microorganisms, reduce the risk of fungal infection
Antioxidants	Polyphenolic compounds, flavonoids, carotenoids, tocopherols, ascorbic acid	Oxygen free radical quenching, inhibition of lipid peroxidation
Anticancer	Carotenoids, polyphenols, curcumin, flavonoids	Inhibitors of tumor, inhibited development of lung cancer, antimetastatic activity
Detoxifying agents	Reductive acids, tocopherols, phenols, indoles, aromatic isothiocyanates, coumarins, flavones, carotenoids, retinoids, cyanates, phytosterols	Inhibitors of procarcinogen activation, inducers of drug binding of carcinogens, inhibitors of tumorogenesis
Others	Alkaloids, terpenoids, volatile flavor compounds, biogenic amines	Neuropharmacological agents, antioxidants, cancer chemoprevention

of natural lipids can be found in every class of living things and therefore considered as the largest group of natural products. Many of the terpenoids are commercially interesting because of their use as flavors and fragrances in foods and cosmetics, for example, menthol and sclareol, or because they are important for the quality of agricultural products, such as the flavor of fruits and the fragrance of flowers like linalool. Terpenes are widespread in nature, mainly in plants as constituents of essential oils. Their building block is the hydrocarbon isoprene, $CH2 = C(CH3)–CH=CH2$. Terpene hydrocarbons therefore have molecular formula $(C5H8)n$, and they are classified according to the number of isoprene units:

(a) **Hemiterpenoids**: Consist of a single isoprene unit. The only hemiterpene is the isoprene itself, but oxygen-containing derivatives of isoprene such as isovaleric acid and prenol are classified as hemiterpenoids.
(b) **Monoterpenoids**: Biochemical modifications of monoterpenes such as oxidation or rearrangement produce the related monoterpenoids. Monoterpenoids have two isoprene units. Monoterpenes may be of two types, i.e., linear (acyclic), or contain rings, e.g., geranyl pyrophosphate, eucalyptol, limonene, citral, camphor, and pinene.
(c) **Sesquiterpenes**: Sesquiterpenes have *three isoprene* units, e.g., artemisinin, bisabolol and farnesol, oil of flowers, or cyclic compounds, such as eudesmol, found in eucalyptus oil.
(d) **Diterpenes**: They are composed of four isoprene units. They derive from geranylgeranyl pyrophosphate. There are some examples of diterpenes such as cembrene, kahweol, taxadiene, and cafestol. Retinol, retinal, and phytol

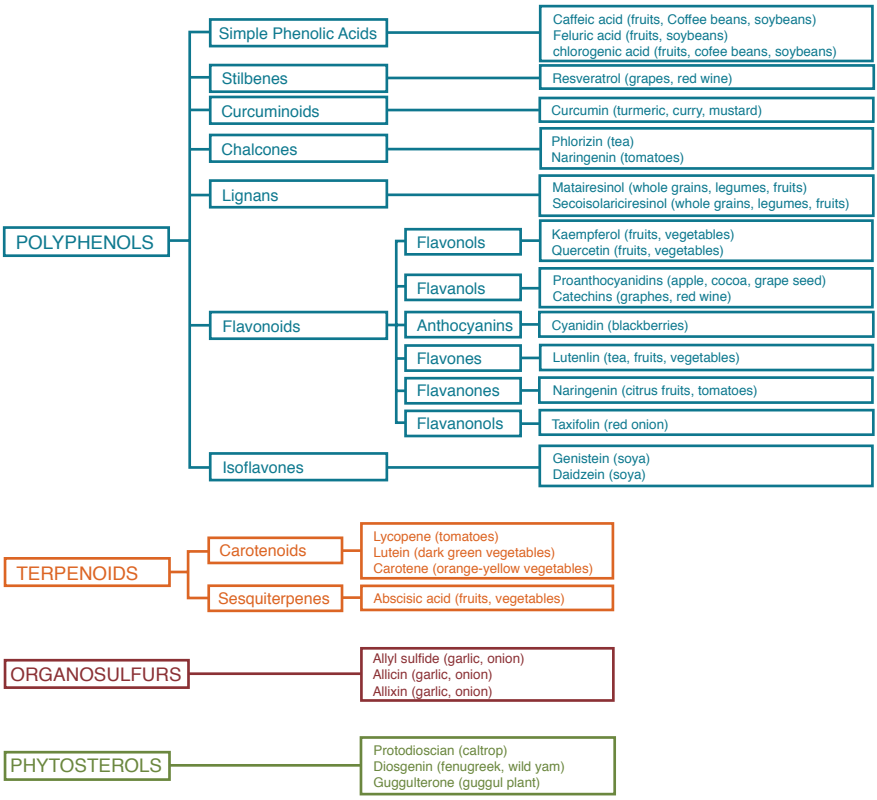

Fig. 1 Classification of dietary phytochemical (Gonzalez-Castejon and Rodriguez-Casado 2011)

are the biologically important compounds while using diterpenes as the base.

(e) **Triterpenes**: It consists of *six* isoprene units, e.g., lanosterol and squalene found in wheat germ and olives (Yadava et al. 2014).

Diversity of Terpenoids in Nature

Nature relies on an intricate network of biosynthetic pathways to produce a lot of small organic molecules required to support life. Terpenoids (also called "isoprenoids") constitute one of the largest families of natural products accounting for more than 40,000 individual compounds of both primary and secondary metabolisms. Most of them are of plant origin, and hundreds of new structures are reported every year. All organisms naturally produce some terpenoids as part of primary metabolism, but many produce terpenoids via secondary metabolism. Isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP) are the universal

five-carbon precursors of all terpenoids. After isotope-labeling studies by Rohmer et al., it has been shown that there is an alternate pathway to terpenoids that do not originate from acetyl-CoA. The complete pathway has been finally elucidated in 2002. This alternative MVA-independent pathway has been named the methylerythritol phosphate (MEP) pathway, which has been identified in both bacteria and plants. Plants use both pathways although they are compartmentalized: MVA to the cytoplasm and possibly to mitochondria to provide sterols, the side chain of ubiquinone, and sesquiterpenes (C15) and MEP to plastids providing plastidial terpenoids, for example, isoprene (C5), monoterpenes (C10), diterpenes (C20, including gibberellins and the phytyl tail of tocopherols and chlorophylls), and carotenoids (C40) (Goto et al. 2010).

Pharmaceutical Application of Terpenoids

Plants have an enormous capacity to synthesize huge amounts of diverse terpenoids, particularly via the combination of the terpenoid biosynthetic route and other secondary metabolic pathways. In addition to universal physiological, metabolic, and structural functions, many specific terpenoids function in various situations, including communication and defense. Members of the isoprenoid group also include industrially useful polymers (e.g., rubber and chicle) and agrochemicals (e.g., pyrethrins and azadirachtin). It is known that several herbal plants improve medical conditions. Such plants contain many bioactive phytochemicals. In particular, terpenoids are contained in many herbal plants, and several terpenoids have been shown to be available for pharmaceutical applications, for example, artemisinin and Taxol as malaria and cancer medicines, respectively. Various terpenoids are contained in many plants for not only herbal medicine use but also dietary use. Terpenoids also improve the skin tone, increase the concentration of antioxidants in wounds, and restore inflamed tissues by increasing blood supply. Terpenoids also improve lung function. Terpenoids have shown to reduce diastolic blood pressure and lower the sugar level in blood in hypertensive and diabetic patients, respectively, in the management of peripheral neuropathy, a complication associated with diabetes mellitus (Saxena et al. 2013).

Diabetes Mellitus

Diabetes mellitus is a disorder caused by the total (or relative) absence of insulin, which manifests clinically as an elevated blood glucose. Diabetes mellitus either classified as insulin-dependent diabetes mellitus (IDDM) or non-insulin-dependent diabetes mellitus (NIDDM). In 1998, a new classification system based upon the etiological factors at work in diabetes was proposed by the

WHO and has now become the accepted system for classifying diabetes mellitus:

1. **Type 1 diabetes**: immune-mediated and idiopathic forms of β-cell dysfunction, which lead to absolute insulin deficiency. This is an autoimmune-mediated disease process which gives rise to absolute deficiency of insulin and therefore total dependency upon insulin for survival.
2. **Type 2 diabetes**: disease of adult onset, which may originate from insulin resistance and relative insulin deficiency or from a secretory defect. This is a disease which appears to have a very strong genetic predisposition and is caused by a combination of inadequate insulin secretion and an insensitivity of the body tissues to insulin so leaving patients with this condition relatively deficient in insulin.
3. **Type 3 diabetes**: this covers a wide range of specific types of diabetes including various genetic defects in insulin action and diseases of the exocrine pancreas.
4. **Type 4 diabetes**: is gestational diabetes.

Morbidity: Diabetes places a huge burden of illness on sufferers and society. People with diabetes in the age group 45–64 years are 23 times more likely to be registered blind than the nondiabetic population of the same age. Diabetic retinopathy is the lead cause of blindness in this age group. Diabetes often affects the kidneys, and up to 40% of people who develop type 1 diabetes before the age of 30 years can expect to develop diabetes-related nephropathy. A significant number of these will progress to renal failure requiring long-term renal dialysis treatment. Thirty percent of people with diabetes develop diabetic neuropathy leading to a range of problems including from foot ulceration, sexual difficulties, cardiac arrhythmias, and sudden death.

Mortality: It is thought that 20,000 people per year die prematurely because of diabetes-associated disease. Most of these deaths are from the macrovascular complications of diabetes such as myocardial infarcts and cerebrovascular accidents. The number of people dying prematurely in the diabetic population is double that of the nondiabetic population.

Clinical Features and Etiology

Type 1 diabetes typically presents in the teens with a short history of weight loss, incredible thirst, and polyuria (passing lots of urine). Such patients are often thin, there is very often no family history of diabetes, and although the cause of the illness is not known, it is thought to be triggered by a viral infection.

Type 2 diabetes typically presents later in life. Such patients are often overweight at diagnosis, and there is often a strong family history of the disease. It is not

known why the disease develops, but it may be related to overeating. In contrast to type 1 diabetes, patients with type 2 diabetes are often asymptomatic when it is diagnosed, and the diagnosis is often made while a doctor is investigating some other complaint.

Type 2 Diabetes

Type 2 diabetes is characterized by the abnormal metabolism of glucose and fat, due to improper resistance to the actions of insulin in the skeletal muscle, liver, and fat. In the natural history of type 2 diabetes, pancreatic β-cells initially compensate for insulin resistance by secreting excess insulin. However with time, progressive β-cell failure leads to insulin deficiency and overt hyperglycemia. Progression in diabetes leads to the development of chronic complications such as retinopathy, neuropathy, nephropathy, etc.

At present, oral therapy for type 2 diabetes relies on several approaches targeted to reduce hyperglycemia, namely, sulfonylureas, which increase insulin release from pancreatic islets; α-glucosidase inhibitors, which inhibit gut glucose absorption; metformin, which acts to reduce hepatic glucose output through inhibition of gluconeogenesis; and peroxisome proliferator-activated receptor-activator thiazolidinediones (TZDs), which promote insulin sensitization. These therapies have either limited efficacy or significant mechanism-based side effects like hypoglycemia, flatulence, body weight gain, or enhancement of gastrointestinal problems.

Evidence accumulated over the past few years indicates that the AMP-activated protein kinase (AMPK) may be a good target for the pharmacologic treatment of type 2 diabetes.

AMP-Activated Protein Kinase (AMPK)

The AMPK belongs to the family of energy-sensing enzymes that are activated by cellular stresses resulting in ATP depletion, thus acting like a "fuel gauge." Upon activation, AMPK functions to restore cellular ATP by both inhibiting ATP consumption processes and accelerating ATP generation processes. The cascade is activated by stresses such as prolonged exercise, electrical stimulation in skeletal muscle, ischemia in heart muscle, heat shock, as well as inhibition of tricarboxylic acid cycle or oxidative phosphorylation. AMPK is activated by an increase in AMP/ATP and creatine/phosphocreatine (pCr) ratios, resulting in allosteric modification, and/or through mechanism involving phosphorylation of the subunit by upstream kinases (AMPKKs).

Structure of AMPK

AMPK is a highly evolutionarily conserved serine/threonine kinase and found in all eukaryotic species. It exists as a heterotrimeric complex consisting of a catalytic (α1 or α2) subunit and two regulating (β1 or β2 and γ1, γ2, or γ3) subunits, all of which are encoded by separate genes; therefore, at least 12 combinations are possible. At the molecular level, the catalytic α-subunits contain a classical serine/threonine kinase domain at the N-terminus while a regulatory domain at the C-terminus, which is involved in complex formation with β and γ subunits. The β subunits contain C-terminal region that interacts with α and γ subunits and serves as the scaffold of the heterotrimeric complex. In addition, the central part of β subunits contains a specific sequence "N-isoamylase domain" required for AMPK complexes binding to glycogen. The γ subunits contain N-terminal four tandem repeats of cystathionine-β-synthase sequences (CBS) to form two Bateman domains, which selectively bind adenosine-containing molecules, such as AMP or ATP. AMPK α consists of 548 amino acids (aa), and it contains catalytic domain (1–312aa), an autoinhibitory domain (312–392 aa), and a subunit-binding domain (392–548 aa). The catalytic domain has a site of phosphorylation at Thr172 which is required to be phosphorylated for its activation by AMPKK (Fig. 2).

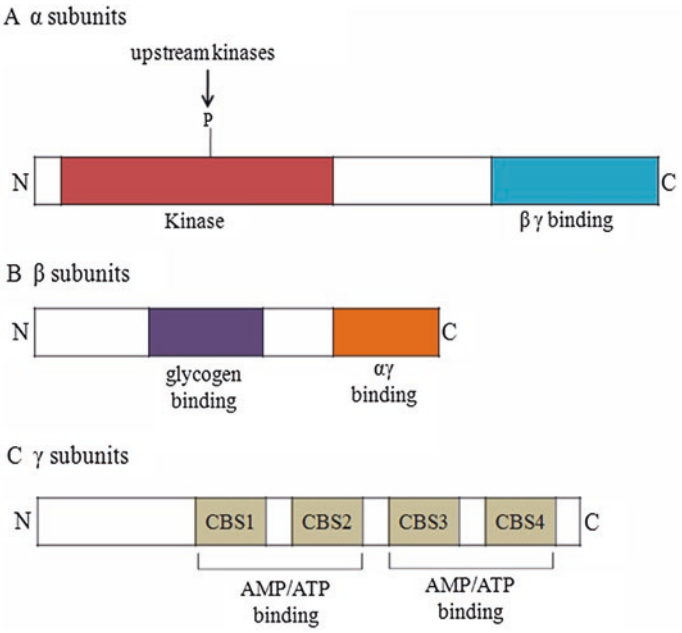

Fig. 2 Typical domain structure of α, β, and γ subunits of AMPK. (Mohammad Nasir Uddin et al. 2013)

Role of AMPK in Skeletal Muscle

Skeletal muscle is the main site for glucose disposal in the body, and there are two ways to stimulate glucose uptake in skeletal muscle: insulin dependent and insulin independent. Insulin resistance is one of the early defects detected in the muscle of diabetic patients, and insulin resistance is caused mainly due to defect in insulin-signaling pathways. Decrease in insulin-stimulated tyrosine phosphorylation of the insulin receptor and insulin receptor-1 substrate (IRS-1) and in IRS-1-associated phosphatidylinositol-3 kinase (PI-3 kinase) activity leading to the problem in translocation of glucose transporter (GLUT4) from microvesicle to membrane.

Currently no pharmacological approach is being pursued to correct these defects in insulin-signaling pathway. Therefore, targeting insulin-independent pathway to restore glucose disposal can be explored as an alternative approach. There are sufficient data available to support the hypothesis that exercise enhances muscle glucose disposal in diabetic patients through an insulin-independent mechanism. **Mimicking exercise-like effect through drug could be an attractive approach to improve blood glucose level**. Muscle contraction leads to increase in AMP/ATP and Cr/pCr levels leading to robust increase in AMPK activity which is well correlated with contraction-mediated glucose uptake in muscle. It's suggested that AMPK plays an important role in contraction-mediated glucose disposal.

Role of AMPK in Liver

In the liver, activated AMPK inactivates ACC at transcriptional and posttranslational level and also inhibits HMG-CoA reductase, the rate-limiting enzyme in cholesterol synthesis by phosphorylation. Like skeletal muscle, in the liver also, activated AMPK decreases malonyl CoA synthesis resulting in increased β-oxidation through enhanced CPT-1 activity. Type 2 diabetic patients are often associated with hypertriglyceridemia and high cholesterol, the potential risk factors for cardiovascular problems. Activated AMPK could reduce this risk by controlling elevated level of free fatty acid, TG, and cholesterol. The elevation of fasting plasma glucose is associated with type 2 diabetes, and it is regulated by gluconeogenesis, a process which makes glucose from noncarbohydrate source in the liver. Gluconeogenic enzymes like phosphoenolpyruvate carboxy kinase (PEPCK) and glucose-6-phosphate dehydrogenase are the major players. Thus, AMPK, by inhibiting hepatic glucose output and increasing muscle glucose uptake, could control elevated blood glucose level in the body.

Role of AMPK in Adipocytokine Signaling

Adiponectin and leptin, the two adipocytokines produced and secreted from adipose tissues, play an important role in the pathogenesis of type 2 diabetes. Adiponectin increases free fatty oxidation through ACC, an AMPK target gene, and also enhances insulin sensitivity both in the skeletal muscle and liver. There is an inverse relationship with the level of blood plasma adiponectin and insulin resistance. These results indicate that adiponectin behaves like an ideal AMPK activator. Leptin has both central and peripheral mechanisms through which it controls energy expenditure. Being secreted from adipose tissue, leptin binds to its receptor in the brain and exhibits its effect through JAK-STAT (Janus kinases-signal transducers and activators of transcription) pathway. The activation of AMPK by leptin causes inhibition of ACC, which in turn stimulates skeletal muscle fatty acid oxidation indicating that its insulin sensitization action might be through reduction in free fatty acid level, the potential precursor of insulin resistance (Misra and Chakrabarti 2007).

AMPK as a Pharmacological Target: Present and Promise

AMPK Activity Is Critical to Cell Physiology in Different Tissues and Organs

AMPK functions as a ser/thr kinase which provides an evolutionary conserved cellular energy sensor. This kinase is a focal point for metabolic control in all eukaryotes, where it regulates many aspects of physiology. It is well-established that AMPK and its yeast ortholog Snf1 control a large number of diverse processes; they include the response to nutrient limitation or other environmental changes, transcription, transport across the nuclear envelope, cell growth, cell cycle progression, mitosis, cell polarity, development, and auto- and mitophagy. As a result of these contributions, AMPK is vital to the function of several organs and tissues in metazoans. Owing to its pivotal role in the control of glucose homeostasis and carbohydrate, lipid, and protein metabolism, AMPK is a key player in many human diseases and disorders. In particular, the low activation state of AMPK could contribute to the increase in type 2 diabetes and obesity. Moreover, as essential regulator of glucose homeostasis and lipid metabolism, AMPK has become an important therapeutic target in type 2 diabetes. AMPK is a global target as it regulates different diversified signals in metabolic pathways.

On the basis of the merits associated with this target, an ideal AMPK activator is expected to increase muscle glucose transport and muscle insulin sensitivity, enhance fat oxidation in muscle and liver, inhibit hepatic gluconeogenesis, decrease cholesterol and triglyceride synthesis in liver, and devoid problems associated with present antidiabetic drugs (gastrointestinal problem, body weight increase, etc.). Three different kinds of AMPK activators have been reported so far. First, PPARg

activators, rosiglitazone and pioglitazone, activate AMPK without direct binding but by increasing the cellular AMP/ATP ratio. Second, AICAR, an analog of natural activator AMP, activates AMPK through direct binding followed by allosteric modification. Lastly, metformin, an AMPK activator, does not affect AMP/ATP ratios or bind to AMPK but acts through an unknown mechanism. PPARg agonist rosiglitazone, the leading antidiabetic drug, although an activator of AMPK, is associated with PPARg-related side effects, like weight gain and edema.

Therefore, it is a great challenge for the pharmaceutical companies to get a safe but efficacious AMPK activator. We also need to remember that there are certain difficulties associated with AMPK, which makes it a difficult pharmacological target:

1. AMPK is a heterotrimeric protein and so far no crystal structure is available.
2. Each subunit contains two or more isoforms.
3. The AMP-binding site is not well defined.

Despite these limitations, several pharmaceutical companies are working on this target and have reported several AMPK activators in preclinical studies (Fig. 3).

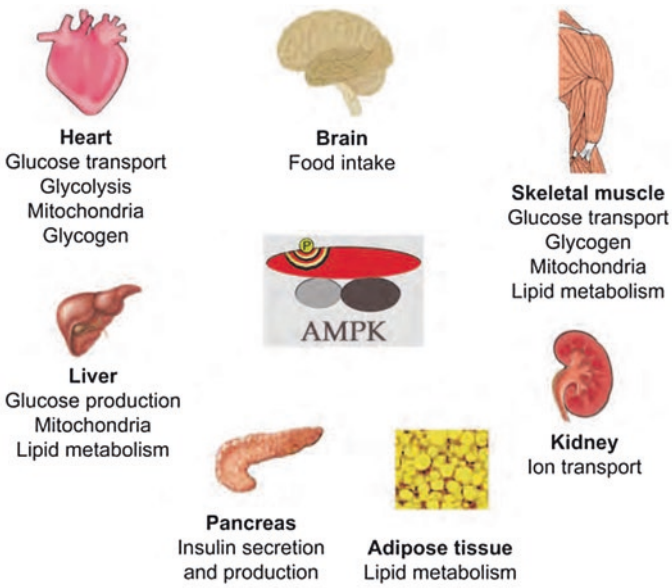

Fig. 3 The role of AMPK in different organs and tissues (Mohammad Nasir Uddin et al. 2013)

Organization and Activation of AMPK

AMPK senses a drop in cellular energy as it is induced by a reduction in glucose availability or other metabolic stresses. The overall consequence of AMPK activation is a change in metabolism; thus, when the AMP/ATP ratio increases, AMPK becomes activated in order to rescue the energy balance. As a result of AMPK activation, the cellular metabolism switches from anabolic to catabolic processes. This metabolic shift is accomplished by the AMPK-dependent phosphorylation of multiple targets which are located in different cellular organelles and compartments. The heterotrimeric enzyme AMPK contains one catalytic subunit that is encoded by two genes (1 and 2). The regulatory and subunits are encoded by two and three genes, respectively. The two subunits (1, 2) can be myristoylated and phosphorylated, and these modifications may impact the activation and intracellular localization of AMPK. The subunits (1, 2, 3) bind AMP and ATP in a mutually exclusive fashion; this AMP binding is important to the activation of the enzyme.

Activators of AMPK

AMP-activated protein kinase can be activated by various types of metabolic stress that lead to ATP depletion, such as conditions of low nutrient supply or prolonged exercise, or via an increase in intracellular Ca^{2+} concentration. The upstream kinases, LKB1 and calcium/calmodulin-dependent protein kinase kinase-β (CaMKKβ), activate AMPK by phosphorylating Thr172 in the activation loop of the catalytic α-subunit. The finding that CaMKKβ can also activate AMPK, independently of LKB1, broadened the potential for AMPK to be used for therapy in cancers that have mutant LKB1 and thus low AMPK activation. The more well-known activators of AMPK include several pharmacological agents that stimulate the LKB1 pathway. Metformin, the most widely prescribed type 2 diabetes drug for more than 30 years, has been shown to activate AMPK in an LKB1-dependent manner. Metformin mimics an energetic stress because it inhibits the mitochondrial complex I in hepatocytes and cancer cells which leads to the decrease in intracellular ATP and an increase in glycolysis and lactate production. Consistent with this, diabetic patients treated with metformin had a lower incidence of cancer compared to those on other medications. Phenformin, a biguanide more potent than metformin, and A-769662, a direct AMPK activator developed by Abbott, also delayed tumorigenesis in a mouse cancer model. 5-Amino-1-β-Dffff-ribofuranosyl-imidazole-4-carboxamide, or AICAR, is an analog of AMP and widely used to activate AMPK in experiments. Interestingly, AMPK is also activated by ionizing radiation (IR) in lung, prostate, and breast cancer cells, independent of LKB1. Among the most recent developments is the activation of AMPK by naturally occurring dietary constituents and plant products (Kim and He 2013).

Activation of AMPK by Phytochemicals

It was not until about 5 years ago that AMPK began to be recognized as a target for various phytochemicals. In addition to pharmaceutical agents, numerous naturally occurring compounds and phytochemicals have been shown to activate AMPK. Among them are:

- **Polyphenols**: a structural class of natural or synthetic products characterized by the presence of multiples of phenol structure units. Despite the structural variance, numerous polyphenols are capable of activating AMPK, and they exert beneficial effects on type 2 diabetes and metabolic syndrome. These include resveratrol from red grapes; quercetin from many plant units including fruits, vegetables, and grains; genistein found in a number of plants such as soybeans; epigallocatechin gallate from green tea; berberine from *Coptis chinensis*; and curcumim from *Curcuma longa*. Mechanisms of activation of AMPK by these compounds appear to require the elevation of AMP levels because many of these compounds are known to inhibit mitochondrial ATP production. Resveratrol, quercetin, epigallocatechin-3-gallate, and curcumin target and inhibit the mitochondrial F_1F_0-ATPase/ATP synthase, whereas berberine is associated with the inhibition of respiratory chain complex I. The molecular mechanism of AMPK activation by resveratrol, berberine, and quercetin has further been supported by the observation that these compounds fail to activate AMPK in cells expressing AMP-insensitive (R531G) AMPKγ2 subunit.
- **Ginsenoside**: *Panax ginseng* has been long known to have favorable effects in type 2 diabetes and metabolic syndrome. Ginsenosides, a class of tetracyclic triterpene glycosides, are the major pharmacological ingredients in ginseng. To date, more than 80 structurally different ginsenosides have been isolated from the plant genus *Panax*, and a number of ginsenosides, including Rb1, Rb2, Rc, Re, Rg1, Rg2, and Rg3, have been reported to activate AMPK, resulting in an increased glucose uptake, decreased hepatic triglyceride and cholesterol levels, and inhibition of lipogenesis and hepatic glucose production. The mechanisms for AMPK activation by ginsenosides are largely unknown; however, presumably these compounds are likely to activate AMPK via AMP-dependent mechanisms because the ginsenoside, Rb1, has been reported to increase the intracellular AMP/ATP ratio.
- **α-Lipoic acid**: α-Lipoic acid (ALA), a naturally occurring dithiol compound derived from octanoic acid, has a critical role in mitochondrial bioenergetics reactions by acting as a cofactor for pyruvate dehydrogenase and α-ketoglutarate dehydrogenase. Owing to its powerful antioxidant property, ALA has gained substantial attention for use in managing diabetic complications. Recent studies have also demonstrated that ALA exerts beneficial effects on metabolic syndrome, lipotoxic cardiomyopathy, and endothelial dysfunction through the activation of AMPK in various tissues. Although the underlying mechanisms for AMPK regulation by ALA are poorly understood, Shen et al. have reported that ALA increases the intracellular calcium level in C2C12 myotubes, suggesting

that CaMKK, but not LKB1, is responsible for AMPK activation. In the hypothalamus, where AMPK is implicated in the regulation of appetite, ALA suppresses AMPK activity, leading to reduced food intake. Further examination is required to understand the molecular mechanism of the regulation of AMPK by ALA.

- **Resveratrol**: Resveratrol (trans-3,5,4′-trihydroxystilbene) is a dietary polyphenol compound, found in a wide variety of plant species including grapes, red wines, berries, and peanuts. Recently, resveratrol has been reported as a potent AMPK activator and useful in the treatment of diabetes and metabolic syndrome. Murase et al. reported resveratrol as an exercise substitutive agent, which, through AMPK activation, induces energy metabolism and is effective for preventing or ameliorating obesity, diabetes, hyperglycemia, insulin resistance, hypercholesterolemia, hepatic hypertrophy, or fatty liver. In this invention, the AMPK activation potential of resveratrol was evaluated on the basis of phosphorylation of AMPK α and β.
- **Nootkatone**: Nootkatone, [4, 4a, 5, 6, 7, 8-hexahydro-6-isopropenyl-4, 4a-dimethyl-2(3H) naphthalenone], present in grapefruit peels, has received much attention as flavor because of its characteristic flavor and taste of grapefruit. Although its physiological activity has hardly been reported, recently, Murase et al. found nootkatone as potent AMPK activator. Murase et al. (2010) claimed that nootkatone has strong AMPK-activating action in muscle cells which promotes glucose and lipid metabolism.
- **Cucurbitane triterpenoid**: Cucurbitane-type triterpenoids are isolated from *Momordica charantia* L (Cucurbitaceae family), and it had been claimed that these compounds may act as glucose uptake stimulator, agonist for translocation of GLUT4 to the cell membrane, and AMPK activator.
- **Obovatol**: Obovatol, a phenolic constituent from *Magnolia obovata*, is well known for its antidepressive and antianxiety effect. Obovatol has been claimed as potent AMPK-activating agents, and it might be used in the treatment of diabetes and metabolic syndrome.
- **Glabridin**: Glabridin is a dietary isoflavan compound, one of the major active flavonoids in licorice (*Glycyrrhiza glabra*). It has been reported as a novel AMPK activator that would exert therapeutic effects in obesity-related metabolic disorders. Moreover, Park et al. (2006) disclosed glabridin as a strong AMPK activator and, therefore, potential drug target for diabetes and metabolic syndrome.
- **Damulin A and B**: Huh et al. (2011) reported two novel dammarane-type glycosides, named damulin A and B from *Gynostemma pentaphyllum* as strong AMPK activator. In vitro study revealed that both compounds, damulin A and B, strongly activate AMPK. Moreover, upon AMPK activation both compounds increased β-oxidation with increasing GLUT4 translocation to the plasma membrane. Taken together, the results clearly indicated that damulin A and B possess high antidiabetic and anti-obesity effect.
- **Other AMPK modulators**: Although intracellular energy levels are a major determinant of AMPK activity, AMPK is highly sensitive to the cellular level of reactive oxygen species (ROS). In many cases, oxidative stress results in intra-

cellular ATP depletion. However, recent studies have revealed that ROS can stimulate AMPK activity even without a decrease in cellular ATP. Oxidative modification of the AMPKα subunit appears to be a major mechanism by which AMPK is activated under conditions of oxidative stress. Therefore, any modulators capable of inducing intracellular ROS generation can activate AMPK without an associated decrease in ATP levels. Such a modulator is cryptotanshinone from *Salvia miltiorrhiza Bunge*, which exerts antidiabetic and anticancer effects through ROS-dependent AMPK activation. DNA-damaging agents, such as cisplatin or metals, including arsenite, vanadate, and cobalt, activate AMPK through ROS generation.

Conclusion

Diabetes has been considered as a global health burden by the World Health Organization (WHO), and it represents one of the leading causes of mortality and morbidity worldwide. Although several synthetic drugs have been developed as antidiabetic agents, their utility has been hampered due to their side effects and poor efficacy. In this scenario, research on natural products has gained importance due to their safety profile in toxicity studies. Terpenoids belong to an important class of natural products, and several terpenoids have been reported as antidiabetic agents. Its shown that various molecular mechanisms involved in the diabetes and progression of diabetic complications by inhibiting glucose absorption, glucose uptake, insulin secretion, enzymes involved in glucose metabolism, prevent the development of insulin resistance, strong antioxidant activity and inhibit the formation of advanced glycation end products, inhibition or expression of several genes which are responsible for diabetes and progression of diabetic complications.

Even several classes of novel natural products are established for the treatment, the utility is less because of untoward effects of drug therapy. So research had done so for on natural products to achieve better treatment than existed therapy with synthetic drugs. As a part of that, terpenoids exhibit promising role in the prevention and treatment of diabetes and diabetic complications like retinopathy, nephropathy, neuropathy, embryopathy, and other vascular dysfunctions.

As a sensor of cellular energy levels that acts on a diverse array of biological pathways, it is not surprising that AMPK plays a key role in the regulation of cell growth and metabolism. Thus, significant progress has been made in the discovery of novel and promising activators of AMPK and in resolving the mechanism of action of agents that activate AMPK in cells. However, considering the complexity of AMPK biology and the biochemical features associated with AMPK complexes, it is highly challenging to discover complex and/or tissue-selective AMPK activators. Nevertheless, the number of recent advances in the field has begun to make AMPK a feasible drug target. Consequently, several natural compounds, reported in the last few years, showed promise as AMPK activator which might have exciting potential for use in the treatment of metabolic diseases.

Terpenoids are capable of stimulating AMPK activity, favoring GLUT4 translocation, weight loss, and metabolic control. Here we propose triterpenes as natural multi-target agents, which represent promising therapeutic agents by acting on various molecular mechanisms leading not only to diabetes but also diabetic complications. Combinatorial therapy using antidiabetic triterpenoids may also be advantageous. Overall, research on the development of triterpenoids in diabetic therapy is in good progress. Safety profile of triterpenoids in toxicity studies represents an advantage for their clinical development. In this context, discovery of antidiabetic new triterpenoids from natural sources and detailed investigations including clinical evaluation on existing antidiabetic triterpenoids are needed for their development as effective and safe drugs for diabetic therapy.

Conflict of Interest The authors do not have any conflict of interest to declare.

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Active Compounds, Health Effects, and Extraction of Unconventional Plant Seed Oils

Hasene Keskin Çavdar

Abbreviations

AEE	Aqueous enzymatic extraction
ALA	α-Linolenic acid
CLnA	Conjugated linolenic acid
DHA	Docosahexaenoic
DPA	Docosapentaenoic acid
EPA	Eicosapentaenoic
GAE	Gallic acid equivalent
GAME	Gas-assisted mechanical extraction
GLA	γ-Linolenic acid
HDL	High-density lipoproteins
LA	Linoleic acid
LDL	Low-density lipoproteins
MAE	Microwave-assisted extraction
PEF	Pulsed electric field extraction
PUFAs	Polyunsaturated fatty acids
SC-CO_2	Supercritical carbon dioxide
SDA	Stearidonic acid
SFE	Supercritical fluid extraction
UAE	Ultrasound-assisted extraction
α-ESA	α-Eleostearic acid

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Introduction

In recent years, several researches have been carried out to discover the active compounds of the oils extracted from the seeds of exploited or underexploited plant species and their effects on human health with increasing health awareness among consumers. Many of these plant species contain significant amount of oil and are considered as a source of dietary or specialty oils with their valuable functional components. Recent researches have shown that those plant seed oils may serve as specialty oils for health promotion and disease prevention due to their special fatty acid composition and other beneficial bioactive components (Yu et al. 2006). The mentioned special plant species with high fixed oil content are defined in the transitional region between the medicinal plants and industrial raw materials.

The rapidly growing functional food and nutraceutical market are targeting bioactive lipid components, such as ω-3 fatty acids, phytosterols, tocopherols, and tocotrienols, for their health benefits (Temelli 2009). The important role of these compounds in the prevention and treatment of some chronic diseases and improvement of immunity system has been demonstrated in many studies. The seeds of black cumin, sesame, flax, nettle, pomegranate, grape, pumpkin, and mustard are the most common specialty oil sources that are used in alternative and folk medicine to prevent some chronic diseases and also improve immune function.

It is critical to consume the high-quality specialty oil preserving its valuable components. These oils are extracted traditionally by cold pressing to preserve the bioactive compounds, while novel extraction methods have been started to be used such as supercritical fluid, ultrasound, microwave-assisted extraction, or enzymatic aqueous extraction. Cold pressing is used substantially in the specialty oil extraction. However, cold pressing is restricted in terms of oil recovery and the high levels of residual oil left in the meal. Moreover, conventional solvent extraction depends on the use of organic solvents, which should be removed with the aid of subsequent evaporation. Heat-sensitive bioactive compounds can be damaged because of the heat applied during solvent evaporation. Additionally, government regulations on the use of organic solvents are getting stricter, and the safety of residual organic solvents in the final product is being questioned (Temelli et al. 2008). The present chapter provides an overview of the specialty oils derived from plant seeds, their bioactive compounds, health benefits, and therapeutic applications and, besides, novel extraction methods for these oils.

Specialty Oils

Some plant-based oils are classified as specialty oils owing to their high content of bioactive constitutes with established health benefits. Specialty oils consist of triacylglycerols mainly with a fatty acid composition rich in unsaturated fatty acids and minor constituents such as tocols, carotenoids, sterols, and squalene (Temelli et al.

2008; Turkay and Gurbuz 2013). Nowadays, these specialty high-value plant seed oils are gaining attention owing to their health benefits which are linked to their high content of special polyunsaturated fatty acids (PUFAs) and other bioactive compounds (Van Hoed et al. 2009). These components give the oil important dietary properties. Nut oils (hazelnut, peanut, pecan, pistachio, and walnut), seed oils (apricot, borage, cherry, evening primrose, flax, grape, hiprose, pumpkin, sea buckthorn, sesame, etc.), cereal oils (oat, rice bran, and wheat germ), and oils of fruits and vegetables (buriti fruit, carrot, cloudberry, olive husk, and tomato) are classified under specialty oils (Temelli 2009). The gourmet and health-promoting specialty oils are distinguished from the major commodity oils by the following characteristics: (1) gentle processing (gentle extraction or cold pressing and gentle or no refining), (2) unique flavor and/or aroma, (3) unique health-promoting properties, (4) lower production amounts, and (5) higher prices (Moreau and Kamal-Eldin 2009).

Although the demand for specialty oils is expanding at a rapid pace, they are available in modest quantities and still regarded as a niche market compared to the commodity oils (Temelli et al. 2008). If the oils are to be used as dietary supplements, as health foods, as gourmet oils, or in the cosmetic industry, it is important that the seeds are to be handled, transported, and stored under conditions that will maintain quality. It may also be necessary to consider growing the crops in such a way as to minimize the level of pesticides (Gunstone 2006). Some trade organizations and government agencies have started to issue guidelines for quality and specifications for some functional specialty oils. However, some issues still remain on what methodologies should be used to validate the health and functional properties of nutraceutical specialty oils and the establishment of regulatory protocols to insure that products meet the quality and efficacy specifications (Hernandez 2016).

Health Effects of Specialty Oils

Traditionally plant seed-derived oil has been primarily used as a medium of cooking and a lubricant and is also utilized in pharmaceuticals and other industries. However, lately, the focus has been on identifying functional ingredients in oilseeds that can find therapeutic applications (Naik and Lele 2012). Numerous physiological and biochemical processes in the human body may produce oxygen-centered free radicals and other reactive oxygen species as by-products due to exposure to oxygen, smoke, exercise, carcinogens, toxins, UV light, or sunlight. Overproduction of such free radicals can cause reversible or irreversible oxidative damage to biomolecules such as lipids, proteins, and DNA. These damages may cause cancer, heart diseases, and arthritis and could accelerate aging of organisms (Cai et al. 2004; Siger et al. 2008; Van Hoed 2010).

The consumption of new and improved products such as cold-pressed specialty oils may improve human health and may prevent certain diseases mentioned (Siger et al. 2008). Most of these beneficial effects are due to antioxidant activity of spe-

cialty oils, especially when the presence of phenolic compounds and tocopherols is involved in the stability of oils. Unsaturated fatty acids, squalene, and carotenoids also affect the pro- and/or antioxidative processes in the oils (Tuberoso et al. 2007). Epidemiological studies have demonstrated that many of these antioxidant compounds possess anti-inflammatory, anti-atherosclerotic, antitumor, antimutagenic, anticarcinogenic, antibacterial, or antiviral activities to a greater or lesser extent (Cai et al. 2004).

Nutraceutical Applications of Specialty Oils

The list of the high-value seed oils rich in bioactives is almost endless. Some of the specialty oils are listed below including their main characteristic compositions, functional properties, and nutraceutical applications. The specialty oils mentioned were selected considering their prevalences documented in the literature and in the lists of specialty oil market. Moreover, some specialty oils which are getting attention in recent years were also given.

Almond (*Oleum amygdalae*) Oil

Almond oil includes significant amounts of the essential fatty acids. Almond oil is rich in β-zoosterol, squalene, and α-tocopherol, all of which are important constituents of healthy-looking skin (Ahmad 2010). It is used as a cosmeceutical and commonly used in skin care products. It is also marketed as an antiaging and anti-wrinkle skin product and a promoter of healthy hair and scalp (Hernandez 2016). Historically, almond oil had been used in ancient Chinese, Ayurvedic, and Greco–Persian schools of medicine to treat dry skin conditions such as psoriasis and eczema. Cardiovascular benefits have also been identified with almond oil increasing the levels of "good cholesterol" and high-density lipoproteins (HDL), while it reduces low-density lipoproteins (LDL) (Ahmad 2010).

Amaranth (*Amaranthus cruentus*) Seed Oil

Amaranth seed oil contains relatively high levels of squalene (Berganza et al. 2003). The oil comprises significant proportions of linoleic and oleic acid (León-Camacho et al. 2001). It has been sold as a nutritional supplement for improvement of the immune system. It is widely sold to improve skin health (Hernandez 2016). Regular consumption of amaranth oil reduces blood pressure and cholesterol levels improving antioxidant status and some immune parameters since amaranth seed oil may be a benefit for those with hypertension and cardiovascular disease (Martirosyan et al. 2007).

Apricot (*Prunus armeniaca*) Seed Kernel Oil

The main fatty acids are oleic (58.3–73.4%) and linoleic (18.8–31.7%). It also contains neutral lipids, glycolipids, phospholipids, and phytosterols (Turan et al. 2007). Apricot seed oil is used in cosmetics, particularly as a skin-conditioning agent, and is also available as a specialty oil for food use (Gunstone 2006).

Bitter Gourd (*Momordica charantia* L.) Seed Oil

The bitter gourd seed oil is unique since it comprises high amounts of conjugated fatty acid α-eleostearic acid (50–60%, α-ESA), a positional and geometric isomer of α-linolenic acid (C18:3 ω-3 or ALA) (Yoshime et al. 2016). This oil has been used in traditional folk medicine for the treatment of many diseases, such as diabetes and atherosclerosis. α-ESA suppresses the growth of DLD-1 human colon cancer cells by inducing apoptosis via lipid peroxidation. α-ESA, which is converted to conjugated linoleic acid in vivo, had a stronger suppressive effect than the conjugated linoleic acid on tumor cell growth (Nerurkar and Ray 2010; Tsuzuki et al. 2004). Grossmann et al. (Grossmann et al. 2009) have shown that α-ESA blocks breast cancer cell proliferation and induces apoptosis.

Black Cumin (*Nigella sativa*) Seed Oil

Black cumin seed oil is rich in linoleic and oleic acids as well as bioactive phytosterols and tocopherols (Ramadan et al. 2012). Black cumin seed and its oil have been traditionally used to promote health and prevent diseases. Their immunopotentiating, immunomodulating, and interferon-like activities have been documented in the literature (Lutterodt et al. 2010). Black cumin seed oil can be taken in the capsule form, and it can be used externally for curing skin diseases such as psoriasis and eczema (Ramadan 2007). Black cumin crude seed oil is mainly desirable for skin eruptions, paralysis, hemiplegia, back pain, rheumatism, and related inflammatory diseases (Lutterodt et al. 2010). The seed oil has been reported to possess antitumor, antioxidant, antibacterial, anti-inflammatory, hypoglycemic, central nervous system depressant, antioxidant, and immunostimulatory activities. These activities have been attributed to the fixed oil, volatile oil, or their components (Amin et al. 2010).

Black Currant (*Ribes nigrum*) Seed Oil

Black currant seed oil is of interest and of value because it contains γ-linolenic acid (C18:3 ω-6 or GLA) and stearidonic acid (C18:4 ω-3 or SDA) which are important metabolites of linoleic and linolenic acids, respectively. Black currant seed oil is also a rich source of tocopherols (1700 mg/kg). It is used in cosmetics and also as

dietary supplements (Gunstone 2006). It has a moderate immune-enhancing effect (Wu et al. 1999). Black currant seed oil reduces LDL cholesterol level and is helpful in premenstrual syndrome (Fa-lin et al. 2010; Philp 2003).

Borage (*Borago officinalis* L.) Seed Oil

Borage seed oil has one of the highest amounts of γ-linolenic acid (30–40%) of seed oils (Asadi-Samani et al. 2014). It is used for the treatment of various diseases such as multiple sclerosis, diabetes, heart diseases, rheumatoid arthritis, seborrheic dermatitis, neurodermatitis, and eczema (Hernandez 2016; Asadi-Samani et al. 2014).

Cactus Pear (*Opuntia ficus-indica* L.) Seed Oil

Cactus pear seed oil has high content of polyunsaturated fatty acids and vitamins. It is characterized by a high degree of unsaturation wherein linoleic acid (C18:2 ω-6 or LA) is the major fatty acid (56.1–77%) (Ramadan and Mörsel 2003). Nowadays, this oil is getting more attention. It is traditionally used as a cosmeceutical and commonly used in skin care products. Moreover, Ennouri et al. (Ennouri et al. 2006) observed a decrease in plasma total cholesterol and in cholesterol with no change in HDL-cholesterol concentrations after the addition of seed oil (25 g/kg) to the diet in rats (Ennouri et al. 2006).

Coriander (*Coriandrum sativum*) Seed Oil

Coriander seed oil is unique because of its high amount of petroselinic acid (C18:1 ω-12). The location of unsaturation in this fatty acid is at the 6,7-position, which is rare among octadecanoic acid and can hence produce unique derivatives that cannot be achieved with other seed oils (Placek 1963; Sahib et al. 2013). It is handled in cosmetics, body-care products, and perfumes (Opdyke 1973). It reduces triglyceride levels and LDL and very-low-density lipoprotein (VLDL) cholesterol and improves HDL cholesterol. Coriander oil has also mild anti-inflammatory effect with good skin tolerance (Sahib et al. 2013; Reuter et al. 2008).

Evening Primrose (*Oenothera biennis* L.) Seed Oil

The primrose seed oil is rich in γ-linolenic acid and γ-tocopherol. Evening primrose seed oil was found to contain lipophilic triterpenoidal esters such as 3-O-*trans*-caffeoyl derivatives of betulinic and oleanolic acid (Boskou 2017). Evening primrose oil is used as a supplement for skin disorders such as eczema, psoriasis, and acne. It is also used for rheumatoid arthritis and weak bones (osteoporosis) (Hernandez 2016).

Fenugreek (*Trigonella foenum-graecum* L.) Seed Oil

It consists of mainly unsaturated acids, namely, linoleic, linolenic, and oleic acids (Ciftci et al. 2011). The oil is used in folk medicine owing to its evaluated hypolipidemic, hypoglycemic, antidiabetic, reno-protective, and antioxidant effects (Hamden et al. 2010). Al-Oqail et al. (Al-Oqail et al. 2013) demonstrated the decrease in the cell viability of the cancerous cells exposed to seed oil of fenugreek. Abdel-Daim et al. (Abdel-Daim et al. 2014) evaluated the role of fenugreek oil as a protective agent against deltamethrin-induced toxicity in rats. The authors showed that fenugreek oil maintained the hematological parameters, namely, red blood cell ranges, platelet counts, hemoglobin, and hematocrit values. Fenugreek oil restored the biochemical changes such as cholesterol, triglycerides, urea, uric acid, creatinine, and enzyme alanine aminotransferase to normal levels. Additionally, fenugreek oil also prevented the lipid peroxidation and oxidative stress in a dose-dependent manner (Venkata et al. 2017). The oil is used in flavoring many canned foods and syrups and as an ingredient in some perfumes (Gu et al. 2017).

Flax (*Linum usitatissimum* L.) Seed Oil

Flaxseed oil (or linseed oil) contains a high level of essential fatty acids, quality proteins, soluble and insoluble fibers, flavonoids, and phenolic acids. This oil is desirable since more than 70% of flaxseed oil consists of the polyunsaturated fatty acids and most of the fatty acids constitute essential ω-3 and essential ω-6 fatty acids (Sovilj 2010). It is the most abundant plant source of ω-3 fatty acid, specifically α-linolenic acid. Supplementation of ALA from flaxseed oil significantly reduced inflammatory markers, including C-reactive protein, serum amyloid A, IL-6, and soluble VCAM-1, in dyslipidemic patients (Rallidis et al. 2003; Rallidis et al. 2004). Flaxseed oil can reduce colon cancer (Dwivedi et al. 2005; Williams et al. 2007) and decrease the growth and metastasis of human estrogen receptor-negative breast cancer in nude mice fed diets containing flaxseed products (Wang et al. 2005). More detailed benefits of the flaxseed oil were reviewed by Oomah and Verghese et al. (Oomah 2001; Verghese et al. 2011).

Grape-Seed Oil

Grape-seed oil is rich in unsaturated fatty acids, especially linoleic acid (Martinello et al. 2007), tocopherols, antioxidant-effective tocotrienols (Matthäus 2008), and high levels of hydrophilic constituents, such as phenolic compounds, and lipophilic constituents, such as vitamin E, unsaturated fatty acids, and phytosterols. Grape-seed oil has beneficial properties for health that are mainly detected by in vitro studies, such as anti-inflammatory, cardioprotective, antimicrobial, and anticancer properties, and may interact with cellular and molecular pathways (Garavaglia et al. 2016). Moreover, this seed oil is a good anticholesteremic, dietetic oil, which

reduces LDL cholesterol and raises HDL cholesterol, providing the anticholesterol effect and protecting against heart problems (Beveridge et al. 2005).

Hemp (*Cannabis sativa* L.) Seed Oil

Hemp seed oil is 80% polyunsaturated fatty acids and is an exceptionally rich source of the two essential fatty acids linoleic acid (18,2, ω-6) and α-linolenic acid (18,3, ω-3) (Callaway 2004). It possesses a well-balanced proportion of linoleic acid and α-linolenic acid in the ratio 3:1. This balance is unique among the specialty oils and has been claimed optimal for human nutrition (Simopoulos 2002). The oil, because of this feature and the presence of γ-linolenic acid, is ideal as an ingredient for light body oils and lipid-enriched creams, known for their high penetration into the skin (Oomah et al. 2002a). Hemp seed oil has health-promoting effects over a wide range of acute and chronic conditions, e.g., from the rapid healing of simple cuts and burns to influenza, various skin problems, other allergic symptoms, and inflammatory diseases. These effects may be linked to the unique fatty acid profile of hemp seed oil and its direct impact on the subsequent metabolism of dietary essential fatty acids to eicosanoids, which include prostaglandins and other important metabolites (Callaway 2004; Leizer et al. 2000). Hemp seed oil has also demonstrated positive health effects on lipid metabolism, cardiovascular health, immunomodulatory effects, and dermatological diseases (Montserrat-De La Paz et al. 2014).

Milk Thistle (*Silybum marianum* L.) Seed Oil

This oil includes essential phospholipids and a relatively high content of vitamin E (Fathi-Achachlouei and Azadmard-Damirchi 2009). Milk thistle oil is a rich source of antioxidants and flavonolignans (silymarin). The oil can be used in the treatment of many diseases including viral hepatitis and cirrhosis (Harrabi et al. 2016). This oil is popular because of its benefit on the liver. It supports the regeneration of the liver and generates a natural barrier against such poisons as alcohol, medicine, pesticides, or heavy metals. It provides the production of bile and prevents the creation of gallstones.

Niger (*Guizotia abyssinica* Cass) Seed Oil

The seed oil is rich in linoleic acid and α-tocopherol, and thus, it is a good source of vitamin E. It is used for both edible and industrial purposes (Gunstone 2006). The high level of vitamin K1 may be the most unique health-promoting characteristic of niger seed oil. Niger seed oil appears to be nutritionally valuable, as the high content of linoleic acid is known to prevent cardiovascular diseases and to be the precursor of structural components of plasma membranes and of some metabolic regulatory compounds (Ramadan et al. 2012).

Pomegranate (*Punica granatum* L.) Seed Oil

Pomegranate seed oil has a high level of ω-5 punicic acid, gallic acid, and phytosterols (Carvalho Filho 2014). Pomegranate seed oil has been well reported for its potential health benefits, such as antioxidant properties, lipoperoxidation, and activity of antioxidant enzymes, immune function, lipid metabolism, estrogene content, skin photoaging inhibition effect, and protective effect against nephrotoxicity (Goula et al. 2018). The polyphenols and phytosterols of pomegranate seed oil are considered to be useful for wound healing, tempering inflammatory responses, preventing wrinkle formation, reducing redness, and alleviating itchy skin (Khoddami et al. 2014). It reduces hepatic triacylglycerol accumulation and acts as a chemopreventive agent against hormone-related human cancers (prostate, breast) (Caligiani et al. 2010).

Pumpkin (*Cucurbita pepo* L.) Seed Oil

This oil has a high level of linoleic and oleic acids (Boskou 2017). Pumpkin seed oil is used in foods like salad dressing to add flavor and as supplement that is sold for suggested health benefits including prevention of cardiovascular disease and prevention of benign prostatic hyperplasia (Hernandez 2016). The most critical health benefit attributed to pumpkin seed oil is preventing the growth and reducing the size of the prostate. Pumpkin seed oil can retard the progression of hypertension and mitigate hypercholesterolemia and arthritis. Reduced bladder and urethral pressure and improved bladder compliance have been linked to pumpkin seed lipid components. Pumpkin seed oil has been found to alleviate diabetes by promoting hypoglycemic activity (Stevenson et al. 2007).

Sesame (*Sesamum indicum* L.) Seed Oil

Sesame oil is a good source of polyunsaturated fatty acids. Vitamin E and phenols, mainly γ-tocopherol and the lignans sesamin and sesamolin, display an abundance of biological activities (Boskou 2017). Sesame oil is used in the West for specialized cooking for its characteristic flavor. It also commonly used in Asian countries in supplements and in therapeutic and cosmetic applications. It suppresses oxidative stress in vivo, lowering of cholesterol level in blood, and protection of the liver from oxidative damage (Hernandez 2016). Phytochemical compounds in sesame seed such as sesamin, sesamol, and anthrasesamone F have been proven to have in vitro/in vivo antioxidant activity. Moreover, sesamin and sesamolin showed anti-inflammatory, antihypertensive, and anticarcinogenic effects in several studies (Zhou et al. 2016). The oil has wide medical and pharmaceutical application. The oil has been used for healing wounds for thousands of years. It is naturally antibacterial for common skin pathogens and fungi. Sesame oil neutralizes oxygen radicals when it is used before and after radiation treatments as UV protector. Oil-soluble

toxins on the skin are attracted to sesame oil molecules that can then be washed away with hot water and a mild soap. Internally, the oil molecules attract oil-soluble toxins and carry them into the blood stream and then out of the body as waste (Anilakumar et al. 2010).

White Mahlab (*Prunus mahaleb*) Seed Oill

White mahlab seed oil has a high content of unsaturated fatty acids, high quantity of tocopherols with γ-tocopherol as the major tocopherol isomer. α-Eleostearic acid, which is a conjugated fatty acid, is rarely found in vegetable oils and has beneficial effects on human health. It is used in various industries including the food and pharmaceutical industries (Sbihi et al. 2014). Mahlab has been used as a tonic for sensory organs and the heart in folk medicine, in the treatment of asthma, and in relief of pains arising from the liver, kidney, and gastrointestinal troubles. The plant is robust and insensitive to diseases and is used as a stock in the grafting of cherry and marasca. Also in Arabia, the seeds are used as sedative and vasodilator as well as for scenting and preservation purposes (Alma et al. 2012).

Bioactive Compounds and Their Health Effects

Although a precise consensus definition of bioactives has not yet been established, the term generally describes food components considered to possibly provide health benefits beyond that of previously accepted understanding of fundamental nutrition (Hernandez 2016). Bioactive compounds in plants can be defined as secondary plant metabolites eliciting pharmacological or toxicological effects in human and animals (Azmir et al. 2013). Edible unrefined oils are good sources of valuable natural bioactive substances such as polyunsaturated fatty acids, tocopherols and tocotrienols, free and esterified sterols, various phenols, lignans, squalene, triterpene alcohols, carotenoids, and chlorophylls which are very important for human health (Boskou 2017; Muneeshwari et al. 2017). Bioactive components, especially antioxidative constituents, play an imperative role in the nutritional and health impact of edible oils (Muneeshwari et al. 2017). The potential health benefits of bioactives can encompass reduced heart disease, mitigated cancer risk, improved plasma cholesterol levels, reduced inflammation, enhanced brain function, improved gastrointestinal health, loss of retained body fat, and many others (Puri et al. 2012).

Polyunsaturated Fatty Acids

High-value seed oils are taken to be those that contain one or more polyunsaturated fatty acids with desirable bioactivity (Catchpole et al. 2009). Polyunsaturated fatty acids are fatty acids that contain two or more double bonds in the carbon chain.

Most PUFAs are essential fatty acids and have to be provided to the body through an adequate diet. They are usually classified as ω-3 and ω-6, depending on the position of the first double bond from the methyl end of the carbon chain. α-Linolenic, eicosapentaenoic (20,5ω-3 or EPA), docosapentaenoic (22,6 ω-3 or DPA), and docosahexaenoic (22,6 ω-3 or DHA) acids are examples of ω-3 PUFAs, whereas linoleic acid and γ-linolenic acid are examples of ω-6 PUFAs (Temelli et al. 2008). The main sources of EPA, DPA, and DHA are fish oils. Since the objective of this chapter is plant-derived specialty oils, only PUFAs such as LA, ALA, and GLA will be discussed (Temelli et al. 2008). The most commonly extracted specialty oils are those that are rich in γ-linolenic acid and/or α-linolenic acid (Catchpole et al. 2009).

Linoleic Acid

Linoleic acid, an essential fatty acid of ω-6 series, has a physiological role in maintaining the water permeability barrier of the skin as a constituent of acylglycosyl ceramides. Besides its structural role of polyunsaturated fatty acids in cell membranes, linoleic acid gives rise to arachidonic acid, which is the major precursor of a series of bioactive metabolites called *eicosanoids*, which regulate a large number of physiological processes. Linoleic acid deficiency, which can be cured/prevented by an intake in as low as 1% of the dietary energy, results in poor growth and development of infants, a scaly dermatitis, and an impaired immune response (Sanders 2016). Linoleic acid reduces LDL cholesterol with minimal effects on HDL cholesterol (Mensink and Katan 1992). However, in recent years, there are concerns about the effects of elevated dietary linoleic acid on human health related to its role in inflammation and its possible activity as a promoter of cancer in animals. There seems to be no clear path to understanding if it has a role in the promotion of cancer, although it is intriguing to consider the use of very low linoleic acid diets to hinder tumor growth by deprivation of cell membrane structural requirements. Studies of the effects of a wide range of linoleic acid consumption may help determine dietary recommendations that are optimal for human health (Jandacek 2017). Linoleic acid comprises more than 50% of the total fatty acids found in black cumin, evening primrose, poppy, grape, and hemp seed oils (Table 1).

α-Linolenic Acid

α-Linolenic acid belongs to the ω-3 family and is referred to as the essential precursor of the longer chain ω-3 PUFA (commonly known as ω-3 fatty acids) because it is the metabolic precursor from which longer chain ω-3 fatty acids are synthesized (Barceló-Coblijn and Murphy 2009). ALA can be metabolized into eicosapentaenoic acid and docosahexaenoic acid (Shahidi and Senanayake 2006). Long-chain *ω*-3 fatty acids, including *α*-linolenic, eicosapentaenoic, and docosahexaenoic acids, have been shown to exert beneficial effects in the prevention of cancer, heart disease, hypertension, and autoimmune disorders (Yu et al. 2006). ALA could be

Table 1 Fatty acid composition of some selected specialty seed oils

Oilseed source	C14:0	C15:0	C16:0	C16:1	C17:0	C17:1	C18:0	C18:1 ($n-12$)	C18:1 ($n-9$)	C18:1 ($n-7$)	C18:2 ($n-6$)	C18:3 ($n-3$)
Apricot kernel	–	–	4.92	0.63	0.05	0.12	1.21	–	70.83	–	21.96	0.08
Bitter gourd	–	–	1.50	–	–	–	32.4	–	1.5	–	2.6	–
Black cumin	1.51	–	12.5	–	–	–	3.16	–	24.1	–	55.3	0.99
Black currant	0.1	–	6.1	0.2	–	–	1.72	–	11.62	–	45.52	15.62
Cactus pear	0.6	–	27.3	1.7	–	–	2.6	–	14.6	–	45.3	7.3
Coriander	–	–	5.54	–	–	–	1.36	67.0	7.86	–	15.9	–
Evening primrose	–	–	6.0	0.1	–	–	1.6	–	8.3	0.6	73.8	0.1
Fenugreek	0.1	0.1	11.2	0.1	0.4	0.2	3.9	–	15.6	–	47.3	18.5
Flax	–	–	6.86	0.14	–	–	4.59	–	15.07	0.87		58.31
Grape	–	–	4.5	–	–	–	2.1	–	17.6	0.8	64.5	0.6
Hemp	–	–	5.62	0.31	–	–	–	–	11.90	–	55.05	16.70
Milk thistle	–	–	8.4	–	–	–	4.6	–	27.7	–	51.7	0.2
Mustard	–	–	4.15	0.11	–	–	1.4	–	26.28	–	10.68	8.16
Neetle	–	–	25.4	0.1	–	–	2.3	–	4.8	–	22.7	6.6
Niger	4.4	–	12.0	–	–	–	3.0	–	13.5	–	65.4	0.1
Pomegranate	–	–	4.40	–	–	–	2.29	–	7.12	–	6.63	–
Poppy	–	–	9.79	0.13	–	–	1.93	–	11.94	1.09	74.47	0.60
Pumpkin	–	–	11.5	0.1	–	–	5.6	–	37.7	–	44.0	0.2
Sesame	–	–	9.4	0.1	–	–	5.6	–	41.8	–	41.3	0.7

C18:3 ($n-6$)	C18:3 ($n-9$)	C18:3 ($n-5$)	C18:4 ($n-3$)	C20:0	C20:1	C20:2 ($n-6$)	C20:5 ($n-3$)	C22:0	C22:1 ($n-9$)	C22:6 ($n-3$)	C24:0	References
–	–	–	–	0.10	0.10	–	–	–	–	–	–	Turan et al. (2007)
–	61.5	–	–	–	–	–	–	–	–	–	–	Nyam et al. (2009)
–	–	–	–	–	–	2.44	–	–	–	–	–	Ramadan (2007)
14.04	–	–	3.2	0.2	1.2	0.3	–	0.1	–	–	<0.1	Bakowska-Barczak et al. (2009)
0.3	–	–	–	–	–	–	–	–	–	–	–	Dubois et al. (2007)
1.0	–	–	–	–	–	–	–	–	0.77	0.57	–	Ramadan et al. (2003)
9.5	–	–	–	0.3	0.3	0.1	–	0.1	0.1	–	–	Kapoor and Huang (2006)
1.3	–	–	–	–	0.2	0.1	–	0.7	0.1	–	0.3	Ciftci et al. (2011)
–	–	–	–	–	–	–	–	–	–	–	–	Bozan and Temelli (2008)
0.4	–	–	–	0.2	–	–	–	–	–	–	–	Dubois et al. (2007)
3.4	–	–	–	2.50	1.44	–	–	0.40	–	–	–	Montserrat-De La Paz et al. (2014)
–	–	–	–	2.9	0.9	–	–	2.3	–	–	0.7	Fathi-Achachlouei and Azadmard-Damirchi (2009)
–	–	–	–	0.53	9.68	–	–	0.53	38.76	–	–	Ryan et al. (2007)
–	–	–	–	–	2.1	–	–	–	1.2	–	–	Guil-Guerrero et al. (2003)
–	–	–	–	0.2	–	–	0.9	0.3	–	–	0.1	Dubois et al. (2007)
–	–	78.23	–	0.40	0.70	–	–	–	–	–	–	Khoddami et al. (2014)
–	–	–	–	–	–	–	–	–	–	–	–	Bozan and Temelli (2008)
–	–	–	–	0.4	0.1	–	–	0.1	–	–	–	Haiyan et al. (2007)
–	–	–	–	0.6	0.2	–	–	0.1	–	–	–	Haiyan et al. (2007)

beneficial by simply acting as the precursor of EPA and DHA. The studies on the health benefits of DHA alone or together with EPA preceded the interest in the potential beneficial effects of ALA (Barceló-Coblijn and Murphy 2009). Many of the chronic conditions, cardiovascular disease, diabetes, cancer, obesity, autoimmune diseases, rheumatoid arthritis, asthma, and depression are associated with increased production of thromboxane A_2, leukotriene B_4, IL-1β, IL-6, tumor necrosis factor, and C-reactive protein. All these factors increase by increases in ω-6 fatty acid intake and decrease by increases in ω-3 fatty acid intake, either ALA or EPA and DHA (Simopoulos 2002). More detailed positive health effects of ALA on cardiovascular diseases, plasma lipid level, and its anti-arrhythmic, anti-inflammatory, and neuroprotective properties were reviewed by Barceló-Coblijn and Murphy (Barceló-Coblijn and Murphy 2009). Flaxseed and perilla seed oils are the richest sources of the ω-3 fatty acid and α-linolenic acid, typically in the range of 55–60%. A third important source of ALA is hemp seed oil, which contains about 20% ALA in addition to 0.5–2% stearidonic acid and 1–4% γ-linolenic acid. Other sources of ALA include the less exploited seed oils of chia, kiwi, and lingonberry (Hernandez and Kamal-Eldin 2013a).

γ-Linolenic Acid

The most common polyunsaturated fatty acids occurring in seed oils are linoleic acid and α-linolenic acid, but in a few species, the α-linolenic acid is accompanied or replaced by GLA that is now recognized as an interesting fatty acid with beneficial health properties (Gunstone 2006). The isomeric ω-6 fatty acid, GLA (*cis* 6, *cis* 9, *cis* 12-octadecatrienoic acid), is produced in the body as an intermediate in the metabolism of linoleic acid by the action of enzyme delta-6-desaturase. However, this reaction is very slow and is further restricted during nutritional deficiencies of vitamins and minerals and also during inflammatory conditions like arthritis and psoriasis (Kapoor and Huang 2006). Because of the limitations, supplementation with preformed GLA is becoming important. γ-Linolenic acid and GLA-rich oils have been studied for several beneficial biological effects in areas such as platelet aggregation, blood lipids, obesity, atopic eczema, and immune functions (Hernandez 2016). GLA induces inhibition of platelet aggregation and thrombosis and inhibition of smooth muscle proliferation and reduction in both systolic and diastolic blood pressure (Hernandez and Kamal-Eldin 2013a). γ-Linolenic acid may promote health via the modulation of prostaglandin balance (PGE1 versus PGE2) (Moreau and Kamal-Eldin 2009). GLA has been shown to exert tumoricidal activity against a variety of cancers including cancers of the breast, pancreas, colon, brain, etc. (Kapoor and Huang 2006). Kapoor and Huang (Kapoor and Huang 2006) reported that this fatty acid can play an important function in the modulation of the inflammatory processes linked to several pathologies such as cancer, diabetes, heart disease, arthritis, and Alzheimer's disease. GLA is found naturally in limited plant species. The significant commercial sources of GLA include the seed oils of borage (18–26 g/100 g GLA), black currant (15–20 g/100 g GLA), and evening primrose (7–10 g/100 g GLA). It is also found in hemp seed oil at 1–6% (Huang and Huang 2006).

Conjugated Linolenic Acid (CLnA)

Conjugated linolenic acid is a mixture of positional and geometric isomers of octadecatrienoic acid (α-linolenic acid, *cis* 9, *cis* 12, *cis* 15–18:3 ω-3) found in plant seeds. Conjugated linolenic acid has been widely studied for its bioactive properties. Indeed, CLnA isomers have been attributed to exhibit several health benefits including anticarcinogenic, lipid metabolism regulation, anti-inflammatory, antiobese, and antioxidant activities (Yuan et al. 2014). CLnA is present in high amounts in some seed oils. Takagi and Itabashi (Takagi and Itabashi 1981) reported the occurrence of calendic acid (8t,10t,12c-18:3; 62.2%) in pot marigold seed oil, punicic acid (c9c,11t,13c-18:3; 83.0%) in pomegranate seed oil, α-eleostearic acid (9c,11t,13t-18:3) in tung (67.7%), and bitter gourd (56.2%) seed oils, as well as catalpic acid (9t,11t,13c-18:3; 42.3%) in catalpa seed oil. Among plant seeds containing CLnA, bitter gourd and pomegranate are edible plants, and catalpa is used in Chinese medicine. Bitter gourd in particular is an important cultivated food crop in Asia (Shahidi 2006).

The metabolic products of the ω-6 fatty acids promote inflammation, blood clotting, and tumor growth, while the ω-3 fatty acids are generally viewed as anti-inflammatory. Therefore, it is important to maintain a balance of ω-3 and ω-6 fatty acids in the diet as these two substances work together to promote health (O'brien 2009). An unbalanced ω-6/ω-3 ratio in favor of ω-6 PUFAs is highly prothrombotic and pro-inflammatory, which contributes to the prevalence of atherosclerosis, obesity, diabetes, cancer, heart disease, arthritis, and depression (O'brien 2009; Simopoulos 2016). The optimal dose or ratio of ω-6/ω-3 varies from 1/1 to 4/1 depending on the disease under consideration (O'brien 2009). Table 1 presents fatty acid compositions of different specialty oils. The fatty acid composition of plant seed oils varies depending on plant origin, genetic factors, ripening grade of fruit, and specific climatic conditions (Velasco et al. 2005). Among the oils presented, mustard seed had a different fatty acid profile, insofar as the fat was primarily monounsaturated due to its exceptionally high content of erucic acid (C22:1). There is some suggestion that erucic acid-rich mustard may bear a cardiotoxic or prooxidant substrate (Ryan et al. 2007).

Phenolic Compounds

Phenolic compounds are organic molecules with a hydroxylated benzene ring. They are secondary metabolites in plants, synthesized both in normal and in stress conditions. Although seeds are very rich in phenolic compounds, a relatively small amount remains in the seed oil upon pressing/extraction. Thus, it is not expected that levels of phenolic compounds will reach levels in seed oils as high as in, for example, extra virgin olive oil, which is known to be very rich in phenolics (Van Hoed 2010). Oilseeds generally contain phenolic compounds of various chemical natures, including flavonoids, lignans, phenolic acids, tannins, and tocopherols (Wanasundara et al. 1997).

Phenols present in the seed oil have the potential for applications in the promotion of health and prevention of oxidative damages caused by radicals (Siger et al. 2008; Dimitrios 2006). Researchers and food manufacturers have become more interested in polyphenols due to their potent antioxidant properties, their abundance in the diet, and their credible effects in the prevention of various oxidative stress-associated diseases. The preventive effects of phenolic compounds in terms of cardiovascular and neurodegenerative diseases and cancer are deduced from epidemiologic data as well as in vitro and in vivo and result in respective nutritional recommendations (Dai and Mumper 2010). Phenolic compounds exhibit a wide range of health-promoting effects, such as antiallergenic, anti-artherogenic, anti-inflammatory, antimicrobial, antioxidant, antithrombotic, cardioprotective, and vasodilatory effects. The beneficial effects derived from phenolic compounds have been attributed to their antioxidant activity. Most of their activities are directly linked with their reducing capabilities, but research indicates as well their capacity to modulate enzymes and to bind to proteins (Van Hoed 2010).

The main compounds reported for most seed oils are phenolic acids, such as protocatechuic acid (typical for pumpkin seed oil), p-(or 4-) hydroxybenzoic acid, vanillic acid (widely present), salicylic acid, quercetin, cinnamic acid, p-coumaric acid, ferulic acid, sinapic acid, elenolic acid, 3,4-dihydroxybenzoic acid, ellagic acid, and gallic acid. Further, vanillin and 3,4-dihydroxybenzaldehyde are reported in some seed oils. In grape-seed oil, oligomeric phenolic compounds have been reported, such as catechin, epicatechin, epicatechin-3-O-gallate, and oligomeric procyanidins, as well as traces of resveratrol (Van Hoed 2010; da Silva and Jorge 2017). The individual phenolics of pumpkin seed oil were defined as tyrosol, vanillic acid, vanillin, luteolin, and sinapic acid by Andjelkovic et al. (Andjelkovic et al. 2010). The antioxidant activity and the various healthful properties of sesame seed and sesame seed oil are attributed to the presence of lignans such as sesamin, sesamolin, sesaminol, sesangolin, 2-episalatin, and others (Dimitrios 2006). Further, more detailed studies are needed to elucidate the complex phenolic profiles in these oils, and standardized methods are needed to assess uniformly their levels in the oils (Van Hoed 2010). For specialty oils, the highest total phenolic contents in terms of gallic acid equivalent (GAE) were reported for caraway seed oil (3530 mg GAE/kg) (Yu et al. 2005), followed by black cumin seed oil (3500 mg GAE/kg) (Ramadan et al. 2012) and milk thistle seed oil (3070 mg GAE/kg) (Parry et al. 2006). A slightly higher phenolic content was recorded for carrot seed oil (1980 mg GAE/kg) and cranberry seed oil (1610 mg GAE/kg) (Yu et al. 2005). Moderate amounts of total phenolic content was found in hemp seed oil (440 mg GAE/kg) (Yu et al. 2005) and grape-seed oil (59–360 mg GAE/kg). Pomegranate seed oil (93.42 mg GAE/kg) (Amri et al. 2017), kenaf seed oil (32 mg GAE/kg) (Chew et al. 2017), pumpkin seed oil (24.71–50.93 mg GAE/kg) (Andjelkovic et al. 2010), quince seed oil (64.03 mg GAE/kg), flaxseed oil (17.67 mg GAE/kg), sesame seed oil (28.06 mg GAE/kg), and poppy seed oil (Górnaś et al. 2014) have lower total phenolic contents.

Tocol

Tocopherols and tocotrienols (collectively known as tocols) are monophenolic compounds and summarized under the term vitamin E and are a group of fat-soluble antioxidants with a chromanol ring and a hydrophobic side chain (phytyl in the case of tocopherols, isoprenyl in the case of tocotrienols) (Schwartz et al. 2008; Shahidi and Ambigaipalan 2015). Tocopherols and tocotrienols have similar basic chemical structures. The difference between tocopherol and tocotrienols is that the tocopherols have a saturated side chain, while the tocotrienols have an unsaturated side chain containing three double bonds. The tocopherol and tocotrienol homologues are named α, β, γ, and δ depending on the position and number of the methyl substitutions on the aromatic side of the chromanol ring (Seppanen et al. 2010).

Tocols have also attracted the attention of nutritionists and clinicians because of their possible role in the prevention of cancer and atherosclerosis due to their antioxidant potential (Bonvehi et al. 2000). These molecules serve as antioxidants by preventing propagation of free radical reactions. Indeed, these molecules can scavenge free radicals in the body, thereby preventing them from damaging cell membranes and genetic material and changing the character of fats and proteins. One example is the protection that vitamin E grants to polyunsaturated fatty acids, which are especially vulnerable to destructive oxidation. It is predicted that vitamin E requirements increase if the intake of polyunsaturates is high; a ratio of 0.4 mg α-tocopherol/g polyunsaturates is recommended (Foster et al. 2009; Ju et al. 2009). Cohort studies show that tocopherols, as effective antioxidants, can protect against carcinogenesis. Some studies found a significant inverse association between dietary intake of vitamin E and the risk of lung cancer (Ju et al. 2009). *γ*-Tocopherol, for instance, has been reported to be more potent than *α*-tocopherol in decreasing platelet aggregation and LDL oxidation and delaying intra-arterial thrombus formation. Likewise, tocotrienols have been shown to inhibit cholesterol biosynthesis and are discussed in the context of reducing the risk of breast cancer. Hence, concurrent administration of various tocopherols and tocotrienols may result in increased antioxidant, antitumor, and hypocholesterolemic potential (Schwartz et al. 2008).

Tocopherols and tocotrienols must be obtained from the diet because humans cannot synthesize them (Foster et al. 2009). The tocopherols are mainly present in oilseeds, oils, meats, and green parts of higher plants, while the tocotrienols are mostly found in the germ and bran fraction of certain seeds and cereals. The most abundant natural antioxidants in vegetable oils are the α- and γ-tocopherols (Seppanen et al. 2010). Table 2 reports the tocopherol content of some specialty oils. Pomegranate seed oil is an excellent source of β-tocopherol with a content of 2794 mg/kg oil (Caligiani et al. 2010), while white mahlab seed oil is a superior source of γ-tocopherol with a content of 1925 mg/kg (Sbihi et al. 2014). Borage seed oil has high level of δ-tocopherol (1432 mg/kg) (Czaplicki et al. 2011). Rosehip seed oil is one of the specialty oils containing lowest tocopherol content (Zlatanov 1999).

Table 2 Tocopherols (mg kg^{-1}) in some specialty seed oils

Specialty oil	α-Tocopherol	β-Tocopherol	γ-Tocopherol	δ-Tocopherol	References
Amaranth	234	191	102	90.9	Czaplicki et al. (2011)
Apricot kernel	19.51	0.38	475.11	12.64	Turan et al. (2007)
Bitter gourd	442.2	196.6	539.3	172.1	Nyam et al. (2009)
Black cumin	260	30	24	50	Ramadan et al. (2012)
Black currant	36.9	0.2	55.4	6.9	Zlatanov (1999)
Borage	nd	nd	171	1432	Czaplicki et al. (2011)
Cactus pear	56	12	33	5	Ramadan and Mörsel (2003)
Camelina	69.5	nd	599	5.15	Czaplicki et al. (2011)
Coriander	86	672	162	347	Ramadan et al. (2003)
Evening primrose	222	nd	433	7.72	Czaplicki et al. (2011)
Fenugreek	824	12	18	1	Ciftci et al. (2011)
Fig	46	–	3918	76.50	Cihat Icyer et al. (2017)
Grape-seed	325.39	nd	31.73	1.63	Ramadan et al. (2003)
Hemp	34	6	733	25	Oomah et al. (2002b)
Kenaf	200.1	790	638.6	nd	Nyam et al. (2009)
Linseed	64.4	nd	495	3.46	Czaplicki et al. (2011)
Milk thistle	378.6	20.9	18.3	28.1	Fathi-Achachlouei and Azadmard-Damirchi (2009)
Mustard	178.78	nd	400.15	13.09	Shrestha et al. (2013)
Niger	861	331	570	185	Ramadan et al. (2003)
Pomegranate	423	2794	27	207	Caligiani et al. (2010)
Poppy	91.7	nd	201	nd	Czaplicki et al. (2011)
Pumpkin	151.9	nd	613.2	41.4	Nyam et al. (2009)
Rosehip	19	nd	71	1.8	Zlatanov (1999)
Roselle	36.8	31.9	706.5	46.0	Nyam et al. (2009)
Sesame	nd	nd	837	nd	Czaplicki et al. (2011)
White mahlep	258.4	nd	1925	432.2	Sbihi et al. (2014)

nd not detected

Sterol and Stanols

With the high demand for "natural" products by consumers, more companies are searching for new sources of natural antioxidants. One potential source of natural antioxidants is a class of substances called sterols (Segall and Artz 2006). Plant sterols and plant stanols, known commonly as phytosterols, are plant-derived compounds that are structurally related to cholesterol. Plants produce more than 40 different types of phytosterols (or plant sterols) (Kerrihard and Pegg 2015). Most plant sterols have a double bond in the C-5 position in the nucleus, while others are totally saturated and are called stanols (Yuan et al. 2017). Plant oils are major dietary sources of phytosterols, whereas phytostanols occur mainly in cereals, such as wheat and rye (Weber and Mukherjee 2006).

Phytosterols exist in all foods of plant origin and are known to have several bioactive properties with possible benefits for human health (Cherif 2012). Many beneficial effects have been shown for the sitosterol, β-sitosterol, campesterol, and stigmasterol which are the most common and have an average 20% of sterols in our diets. Phytosterols, in general, are of interest, due to their antioxidant activity and impact on health (Hamden et al. 2017). Phytosterols are also considered to have anti-inflammatory, antibacterial, anti-atherosclerotic, antioxidative, anti-ulcerative, and antitumor properties in humans (Cherif 2012). In recent years, phytosterols also have been reported to play a role in reducing the incidence of several common cancers, possibly by slowing the cell cycle progression, the induction of apoptosis, and the inhibition of tumor metastasis (Hernandez 2016). The mechanism of action in cancer reduction is unclear, but current evidence suggests that phytosterols may reduce oxidative stress by promoting the activity of antioxidative enzymes (Kerrihard and Pegg 2015; Vivancos and Moreno 2005). Moreover phytosterols are known to reduce plasma's low-density lipoprotein cholesterol, although the precise mechanism of action is not fully understood. The main effect of phospholipid is thought to be due to a decrease in intestinal cholesterol absorption due to the displacement of cholesterol in mixed micelles (Hernandez 2016; Plat and Mensink 2005).

It is believed the typical modern Western diet does not include high enough concentrations of phytosterols for optimal health. Although phytosterol-enriched products have been introduced into the European and the US markets in recent years, consumption rates of phytosterols are still well below optimum levels (Hamden et al. 2017). However, it is worth noting that the consumption of the recommended effective dose of 2 g/day is highly infeasible when consuming phytosterols in their endogenous form. For example, one would need to consume approximately 80 g of rice bran oil to achieve the desired quantities of phytosterols, and other specialty oils would require even higher rates of consumption (Kerrihard and Pegg 2015).

The best dietary sources of phytosterol are unrefined plant oils, seeds, nuts, and legumes (Awad and Fink 2000). Table 3 presents the contents of the most common sterols found in specialty seed oils. Black currant seed oil has the distinctly highest

Table 3 β-Sitosterol, campesterol, stigmasterol, and total phytosterol content (mg/100 g) of selected specialty seed oils

Specialty oil	β-Sitosterol	Campesterol	Stigmasterol	Total	References
Almond	207.1	5.5	5.17	Nr	Maguire et al. (2004)
Amaranth	559.5	198	18.5	1249	Szterk et al. (2010)
Apricot kernel	273.67	12.19	2.22	335.7	Turan et al. (2007)
Bitter gourd	265	60.2	57.8	886.2	Yoshime et al. (2016)
Black cumin	1190	230	320	376	Ramadan et al. (2012)
Black currant	3984–5053	403.3–511.2	23.7–31.0	5787–6894	Bakowska-Barczak et al. (2009)
Borage	56.6	43.8	nd	196	Czaplicki et al. (2011)
Cactus pear	675	166	30	933	Ramadan and Mörsel (2003)
Camelina	221	82	traces	349	Czaplicki et al. (2011)
Coriander	146.4	50.8	154.8	518.6	Ramadan and Mörsel (2002c)
Evening primrose	749	55.3	traces	857	Czaplicki et al. (2011)
Fenugreek	598–668	123–317	30–104	1420–1883	Ciftci et al. (2011)
Flax	162.5	97.5	2.4	513.2	Szterk et al. (2010)
Grape	6.66–6.74	0.01–0.93	1.02–1.08	Nr	Garavaglia et al. (2016)
Hemp	320	71	11	Nr	Mo et al. (2013)
Kenaf	589	72.6	40.9	703	Chew et al. (2017)
Milk thistle	61.79–68.9	6.8–8.44	10.07–13.27	1790–2010	Fathi-Achachlouei and Azadmard-Damirchi (2009)
Niger	203	71.3	66	422.2	Ramadan et al. (2003)
Pomegranate	604.9	57.9	21	Nr	Caligiani et al. (2010)
Primrose	749	55.3	traces	857	Czaplicki et al. (2011)
Pumpkin	nd	nd	nd	179	Czaplicki et al. (2011)
Pumpkin	81.38	nd	9.85	154.1	da Silva and Jorge (2017)
Quince	260	32	20	356	Nogala-Kalucka et al. (2010)
Sea buckthorn	158	33	8	511	Nogala-Kalucka et al. (2010)
Sesame	241	62.6	15	516	Czaplicki et al. (2011)
Viper's bugloss	137	263	18	744	Nogala-Kalucka et al. (2010)

Nr not reported; *nd* not detected

total phytosterol content in the range of 5787–6894 mg/100 g, followed by milk thistle seed oil (1790–2010 mg/100 g), fenugreek seed oil (1420–1883 mg/100 g), and amaranth seed oil (1249 mg/100 g) (Table 3). Pumpkin and borage seed oils have the lowest phytosterol contents of 154 and 196 mg/100 g, respectively. Czaplicki et al. (Czaplicki et al. 2011) reported that pumpkin seed oil does not contain the major sterols, while it contains Δ7-avenasterol (32.6 mg/100 g oil), α-spinasterol + β-sitosterol (96 mg/100 g oil), Δ5,24-stigmastadiene (42.8 mg/100 g oil), and Δ7,22,25-stigmastatrienol (7.6 mg/100 g oil). On the other hand, (da Silva and Jorge 2017) reported that total sterol content of pumpkin seed oil is 154.1 with a composition of β-sitosterol (81.38 mg/100 g), stigmasterol (9.85 mg/100 g), and stigmastanol (61.64 mg/100 g).

Squalene

Squalene is a highly unsaturated aliphatic hydrocarbon ($C_{30}H_{50}$) with important biological properties (Nyam et al. 2009). Squalene is synthesized by all plants and animals, including humans, but in widely varying quantities. It is a precursor of steroid hormones, vitamin D, cholesterol in animals, and phytosterols in plants. It can reduce the level of reactive oxygen species in vitro and act against oxidative DNA damage in human mammary epithelial cells (Yuan et al. 2017).

Squalene has been shown to act as a potent scavenger of singlet oxygen, thereby inhibiting oxidative damage induced by UV radiation and presenting possible protection against the formation of cancerous tumors in the colon, breast, and prostate (Kerrihard and Pegg 2015). A number of animal studies showed that dietary squalene has distinct anticarcinogenic effects. It was shown that squalene presents inhibitory action in carcinogenesis models of skin, colon, and lung cancer (Temelli et al. 2008). Another possible benefit of squalene related to cancer is that it has been shown to reduce side effects associated with chemotherapy. Squalene is also used as an ingredient in delivery systems for vaccines. Squalene, in conjunction with surfactants, is capable of enhancing the immune response to antigens. The enhanced response promotes an increased effectiveness of the vaccine (Kerrihard and Pegg 2015).

Most vegetable oils contain very small amounts of squalene with olive oil, one of the highest, containing between 0.41 and 0.54% (599 mg/100 g) (Gunstone 2006; Tuberoso et al. 2007). *Amaranthus* oil is unusual among vegetable oils in that it has a relatively high level (6–8%, 2260–5940 mg/100 g) of squalene, and this concentration can be raised tenfold by short-path high-vacuum distillation (Gunstone 2006) (Table 4). Kiwi, cranberry, and pumpkin seed oils are also good sources of squalene, 826.2, 671.5, and 352.9 mg/100 g, respectively. Squalene was not detected in flaxseed, black cumin, poppy, and sesame seed oils (Table 4).

Table 4 Squalene (mg/100 g) in some specialty plant seed oils

Specialty oil	Squalene	References
Amaranth	2260–5940	Berganza et al. (2003)
Kiwi	826.2	Van Hoed et al. (2009)
Cranberry	671.5	Van Hoed et al. (2009)
Pumpkin	352.9	Tuberoso et al. (2007)
Blueberry	178.1	Van Hoed et al. (2009)
Pomegranate	84.6	Caligiani et al. (2010)
Kiwi	82.6	Van Hoed et al. (2009)
Apricot kernel	12.6–43.9	Rudzińska et al. (2017)
Blackberry	17.0	Van Hoed et al. (2009)
Roselle	14.51	Nyam et al. (2009)
Bitter gourd	12.95	Nyam et al. (2009)
Almond	9.5	Maguire et al. (2004)
Hemp	8.05	Montserrat-De La Paz et al. (2014)
Cactus pear	5.5	R'bia et al. (2017)
Kenaf	3.69	Nyam et al. (2009)
Flax	1–4.3	Tańska et al. (2016)
Milk thistle	0.95	Dabbour et al. (2014)
Quince	0.067	Górnaś et al. (2013)
Evening primrose	Traces	Czaplicki et al. (2011)
Black cumin	nd	Rudzińska et al. (2016)
Poppy	nd	Czaplicki et al. (2011)
Sesame	nd	Czaplicki et al. (2011)

nd not detected

Carotenoids and Vitamin A

Carotenoids are widely distributed in nature and synthesized by photosynthetic organisms. The primary chemical structure of carotenoids is a symmetrical C-40 polyisoprenoid with an extensive conjugated double-bond system (Wanasundara et al. 1997). Carotenoids are highly colored minor components of many oils. The main bioactivity of the common carotenoids is as provitamin A and as high-strength antioxidants (Catchpole et al. 2009). Of all carotenoids, β-carotene has the highest provitamin A activity, approximately twice that of α- and γ-carotene (Temelli et al. 2008). Vitamin A is essential for vision and immunity (Hernandez and Kamal-Eldin 2013a). Besides the provitamin A function performed by carotene, the carotenoids present antioxidant capacity, as they protect the cell against lipid oxidation, thus preventing the risk of degenerative diseases, such as cancer, heart diseases, and cell degeneration (da Silva and Jorge 2017). Recently, it has been demonstrated that intake of carotenoid-rich foods can decrease the risk of free radical-mediated cancers, tumors, and cardiovascular disease (Wanasundara et al. 1997). The antioxidant potentials of carotenoids have been reported for the prevention of free radical-initiated diseases, including atherosclerosis, cataracts, age-related muscular degeneration, and multiple sclerosis (Lee and Min 2006). Dietary vegetable oils not only

Table 5 β-Carotene content (mg/kg) of selected specialty seed oils

Specialty oil	β-Carotene	References
Coriander	892	Ramadan et al. (2003)
Niger	702	Ramadan et al. (2003)
Black cumin	593	Ramadan et al. (2003)
Cactus pear	525	Ramadan and Mörsel (2003)
Hemp	19.9	Oomah et al. (2002b)
Grape	11.94	da Silva and Jorge (2017)
Japanese quince	10.77	Górnaś et al. (2014)
Pumpkin	6.84	Górnaś et al. (2014)
Pomegranate	3.17	Amri et al. (2017)
Flax	1.87	Górnaś et al. (2014)
Blueberry	1.32	Parry et al. (2005)
Poppy	1.04	Górnaś et al. (2014)
Almond	0.49	Górnaś et al. (2014)
Sesame	0.37	Górnaś et al. (2014)
Red raspberry	0.082	Parry et al. (2005)
Milk thistle	nd	Parry et al. (2006)

nd not detected

provide variable levels of carotenoids to the human body, but they also aid carotenoid absorption (Hernandez and Kamal-Eldin 2013a).

Among fats and oils, vitamin A is available in considerable amounts in butter and in very high amounts in fish liver oils, especially cod liver oil (Ramadan and Mörsel 2003). Total carotenoid-rich oils are mainly buriti fruit, carrot, rosehip, tomato, and wheat germ oils in terms of specialty oils (Temelli et al. 2008). Coriander seed oil is a good source of β-carotene (892 mg kg) among the specialty oils. It is followed by niger seed oil (702 mg/kg), black cumin seed oil (593 mg/kg), and cactus pear seed oil (525 mg/kg) as tabulated in Table 5. However, the β-carotene content can be influenced by the stage of fruit ripeness, the extraction process, and the storage conditions. Thus, oils extracted from older fruits may contain more carotene pigments and oils from younger fruits more chlorophyll pigments (Ramadan and Mörsel 2003).

Vitamin K

Vitamin K is present in green leafy vegetables, and a form of the vitamin is synthesized by the resident bacteria in the large intestine and then absorbed from the cecum. Some vegetable oils are also good sources of this important vitamin, although the content of the different oils varies quite considerably (Foster et al. 2009). The most important form of the vitamin K complex is phylloquinone (vitamin K_1), which is provided by plants. The other forms are the menaquinones (vitamin K_2), which are different by the length and the unsaturation degree of the side chain (Leray 2015).

The significance of dietary vitamin K has recently increased. Vitamin K is a fat-soluble vitamin that functions as a coenzyme and is involved in the synthesis of a number of proteins participating in blood clotting and bone metabolism. Vitamin K also plays a role as a cofactor for posttranslational carboxylation of specific glutamate residues to γ-carboxyglutamate residues in several blood coagulation factors and coagulation inhibitors in the liver, as well as a variety of extrahepatic proteins such as the bone protein osteocalcin. Vitamin K reduces the risk of heart disease, kills cancer cells, enhances skin health, and may have antioxidant properties (Damon et al. 2005; Otles and Cagindi 2007; Ramadan 2012; Shearer 1992).

The phylloquinone requirement of the adult human is extremely low. The vitamin K_1 level is very low in most foods. Among edible oils, the best sources of phylloquinone were rapeseed oil (*ca* 1.5 μg/g) and soybean oil (*ca* 1.30 μg/g) (Piironen et al. 1997; Ramadan 2011). Ramadan and Mörsel (Ramadan and Mörsel 2002a) reported that niger, black cumin, and coriander seed oils were characterized by high levels of phylloquinone, especially niger seed oil which contains more than 0.2% of total oil vitamin K_1. Vitamin K_1 content of niger and black cumin seed oil is 263 and 116.2 mg/100 g (Ramadan and Mörsel 2002a). Coriander seed oil has 42.8 mg vitamin K_1 in 100 g (Ramadan and Mörsel 2002a), while cactus pear seed oil has 4.7 mg vitamin K_1 in 100 g (Ramadan and Mörsel 2003). Pumpkin seed oil, a specialty of Styria in Austria, contains a high phylloquinone concentration of 113 μg/100 g (Jakob and Elmadfa 1996). For mustard seed oil, among the carotenoids, lutein was predominant (78.15 μg/g oil) in the mustard seed oil, with only a trace amount of β-carotene (Vaidya and Choe 2011).

Sphingolipids

Sphingolipids and their phosphorylated derivatives are ubiquitous bioactive components of cells. They are structural elements in the lipid bilayer and contribute to the dynamic nature of the membrane (Michaelson et al. 2016). Plant sphingolipids do not only serve as structural components of cellular membranes but are also involved in different physiological functions (Tellier et al. 2014). The hydrolyzed products of sphingolipids are used by cells to regulate growth, differentiation, and apoptosis. There is evidence that sphingolipids inhibit colon carcinogenesis in experimental animals at a human diet-equivalent concentration. They may reduce colon cancer risk in humans and inhibit skin cancer development (Ratha et al. 2006; Vesper et al. 1999). In addition to colon cancer inhibition activity, sphingolipids were found to have other beneficial effects. In short- and long-term animal studies (feeding experiments with rats), sphingolipids were found to reduce plasma cholesterol, a risk factor for atherosclerosis. Also, the sphingolipids in foods may protect humans against bacterial toxins and viruses (Fang et al. 2006). It has been suggested that dysregulation of sphingolipid metabolism can lead to diseases such as neurodegenerative diseases, cardiovascular diseases, chronic inflammation, or cancer (Gangoiti et al. 2010). However, the molecular determinants of sphingolipid functions are still

poorly understood. Because of their structural complexity, powerful analytical tools are required to identify a large number of individual sphingolipid molecules with a high degree of structural accuracy (Tellier et al. 2014). Sphingolipids form a significant proportion of the lipids present in higher plants, with some studies suggesting that they constitute up to 10% of plant lipids. Novel plant lipid structures are still being discovered, and over 200 have been identified in various different species to date (Michaelson et al. 2016).

Phospholipids

Phospholipids are the primary component of cell membranes and play an important role in all cell functions (Foster et al. 2009). Phospholipid content of seed oils may be as high as 10% of their total lipids, depending on the type of seed examined. Several studies have indicated that phospholipids chelate trace metals, thus acting as a secondary antioxidant, and subsequently increase oxidative stability of the oil. However, it has been documented that phospholipids can act as synergists with tocopherols and flavonoids. It has also been suggested that phospholipids may participate in antioxidant activity by releasing protons and bringing about rapid decomposition of hydroperoxides without the formation of free radicals (Wanasundara et al. 1997). As a class of natural antioxidants, phospholipids represent the most controversial group of antioxidants in that in many in vivo assays, they have been shown to be pro-oxidant. By contrast, many lipid model studies show that phospholipids, alone and in conjunction with other antioxidants, have the ability to stabilize lipids. Phospholipids have cholesterol-reducing and liver-protecting effects as well as brain-improving functions (Ramadan and Mörsel 2002b). Black currant and rosehip contain 1.3 and 1.4 g phospholipid per kg of seed oils, respectively (Zlatanov 1999).

Plant Seed Oil Extraction Methods

The extraction and refining processes of commodity oils are designed to produce a final product that is mostly neutral triglycerides, and thus, most other potential bioactive compounds are removed during the refining steps (Hernandez 2016).

New specialized uses of edible oils and other lipids in food supplements, pharmaceutical products, and cosmetic applications require more sophisticated methods of processing and chemical modification (Hernandez and Kamal-Eldin 2013b). The production of specialty oil from oilseeds is an important business, and food industry is always searching the best extraction technology to improve the oil output of the seeds as well as ways to control the composition of the oil itself. Specialty oils are generally produced using conventional methods of mechanical pressing and/or solvent extraction similar to commodity oils (Temelli et al. 2008). The major chal-

lenges of solvent extraction are longer extraction time, requirement of costly and high-purity solvent, evaporation of the huge amount of solvent, low extraction selectivity, thermal decomposition of thermolabile compounds, and undesirable solvent residue in the oil. The cold-pressing procedure involves neither heat nor chemical treatments; however, a major drawback of cold pressing is the high level of residual oil left in the meal. To overcome these limitations of conventional extraction methods, new, clean, and promising extraction techniques are introduced. These techniques are referred to as nonconventional extraction techniques and becoming alternatives to replace conventional extraction (Temelli 2009; Azmir et al. 2013). These novel extraction techniques include ultrasound-assisted extraction (UAE), microwave-assisted extraction (MAE), supercritical fluid extraction (SFE), aqueous enzymatic extraction (AEE), pulsed electric field extraction (PEF), and gas-assisted mechanical extraction (GAME). Some of these techniques are considered as green techniques. Those methods are designed in such a manner that potential beneficial minor components in the oil are preserved, and in the case of gourmet oils, the flavor and aroma of the oil remain in the final product (Hernandez 2016).

Conventional Extraction Methods

Chemical Extraction

Soxhlet is a standard technique and is still considered as one of the reference methods to compare success of newly developed extraction alternatives (Azmir et al. 2013; Wang and Weller 2006). Wide industrial applications, better reproducibility and efficiency, and less extract manipulation are the advantages of Soxhlet extraction over the other novel extraction methods (Wang and Weller 2006). However, the main disadvantage of conventional Soxhlet extraction for specialty oils rich in bioactive compounds is the degradation of fat-soluble bioactive compounds and undesirable solvent residue left in the oil (Temelli et al. 2008). Thermal decomposition and degradation of fat-soluble bioactive components cannot be ignored since the extraction is usually performed at the boiling point of the solvent for a long time and solvent is removed from the final product at harsh processing conditions (Wang and Weller 2006). Those decomposition and degradations cause damages and modifications in the bioactivity of the final product.

The chemical extraction of edible oil typically involves organic solvents such as hexane. When the oil has been obtained after solvent is removed, a trace percentage of the solvent may still be present in the final oil. Hexane use is under greater scrutiny due to increasing government restrictions, consumer concerns regarding the safety on the use of organic solvents in food processing, and demands are for "natural" or organic products, processed without the use of organic solvents (Temelli 2009; Hernandez and Kamal-Eldin 2013a; Wang and Weller 2006).

Cold Pressing

Over the last few years, increased interest in cold-pressed plant oils has been observed as these oils have better nutritive properties than those after refining. Cold pressing is the oldest, simple, and ecological method and does not require much energy (Siger et al. 2008). Even though cold pressing at temperatures below 60 °C is used extensively in the specialty oil market, cold pressing is limited in terms of oil recovery and the high levels of residual oil left in the meal (Temelli 2009; Temelli et al. 2008). Oil extraction efficiency with hot pressing is higher than cold pressing, but due to the heat generated during the compressing, the quality of the resulting oil is lower and the oil extracted by cold pressing preserves its natural properties and is free of chemical materials; therefore, the demand for oils obtained by cold-pressing approach is getting increased (Bakhshabadi et al. 2017).

Cold pressing has the advantages of pure, fresh products processed in an environmentally friendly manner. They are *trans*-fat free and retain nutrients and natural flavors. Also, the conventional refining processes used on commodity oils normally cause the removal of these valuable bioactives; as a result, the majority of specialty oils from seeds, nuts, and fruits are recovered by cold pressing (Hernandez 2016).

Novel Extraction Techniques

Supercritical Fluid Extraction

Supercritical carbon dioxide (SC-CO_2) extraction is a promising technology getting popular as a cost-effective and environmentally friendly method and meets the consumer demand for "natural" products (Mitra et al. 2009). SC-CO_2 has attracted interest as an alternative "green" solvent for the extraction of oils to organic solvents used in fat and oil processing (Wang and Weller 2006; Sahena et al. 2009). SFE uses no or only minimal organic solvent in extraction (Wang and Weller 2006). Research carried out has shown that SC-CO_2 is effective in recovering specialty oils rich in bioactive compounds from nuts, seeds, cereals, fruits, and vegetables. The use of SC-CO_2 to extract high-value nutraceutical lipids and other bioactive compounds is now being used in several commercial applications (Temelli et al. 2008).

Advantages of extraction with SC-CO_2 include (1) low processing temperatures; (2) minimal thermal degradation of the minor components of interest; (3) ease of separation of extraction solvent, resulting in no solvent residue left in the product; and (4) the fact that processing in the CO_2 environment minimizes undesirable oxidation reactions, which is especially beneficial for the sensitive bioactive components of specialty oils such as sterols, tocols, carotenoids, and polyunsaturated fatty acids (Temelli et al. 2008). Low temperature and absence of oxygen and light avoid thermal degradation and decomposition of possible labile compounds during extraction (Brusotti et al. 2014). Moreover, SC-CO_2 is physiologically harmless at the very low levels at which it is present in foods (because it is easily removed by sim-

ple expansion to common environmental pressures) (Sahena et al. 2009). SFE can achieve higher yield and quality of plant seed oil than conventional extraction. Bernardo-Gil et al. (Bernardo-Gil et al. 2002) reported that the contents of free fatty acids, sterols, triacylglycerols, and tocopherols in the hazelnut oil extracted by SFE were comparable with those obtained with *n*-hexane extraction. However, the SFE-extracted oil was more protected against oxidation of the unstable polyunsaturated fatty acids than the *n*-hexane-extracted oil (Wang and Weller 2006). Mohammed et al. (Mohammed et al. 2016) demonstrated that the black seed oil extracted using SC-CO_2 has higher biological activity compared with those of oil obtained by cold pressing. The physicochemical properties of black seed oil for supercritical CO_2 extraction showed that better quality oil could be obtained using SC-CO_2 (Mohammed et al. 2016).

Gas-Assisted Mechanical Extraction of Oilseeds

The application of SC-CO_2 for oil extraction may be limited due to the high CO_2 consumption and the long extraction duration. Recently, it was proposed to combine supercritical fluid extraction with mechanical expression. This technology called gas-assisted mechanical expression has been successfully applied to recover oil from oilseed crops, showing higher yields, shorter extraction time, and less consumed CO_2 (Mhemdi et al. 2016). In that process, CO_2 is dissolved in the oil contained in the seed cells. Then expression of the oil–CO_2 mixture is done in a hydraulic press or in a screw press. The resultant press cake has a lower residual oil content than that produced by conventional pressing at the same conditions, because part of the oil has been displaced by CO_2. The oil obtained via this process is unfractionated, of high quality, and free of harmful solvents. This technique is desirable since it combines the high yields of (supercritical) solvent extraction and the high oil quality of hydraulic pressing and supercritical extraction but does not require the large quantities of solvent used in supercritical solvent extraction (Willems and ABd 2012).

Aqueous Enzymatic Extraction

Enzyme-based aqueous oil extraction from plant seed is a potential alternative to conventional solvent-based extraction methods. This method offers the possibility of greener chemistry as pressure mounts on the food industry and even pharmaceutical companies to identify cleaner routes for the extraction of new compounds (Puri et al. 2012). This process is environmentally friendly, safer, and healthier, and simultaneous oil and protein extraction can be done without compromising the quality. As a consequence, aqueous enzymatic extraction is a promising green technique both for oilseed processing and also to extract the desired compound (Kumar et al. 2017). The eco-friendly technology for oil extraction uses water as solvent instead of organic chemicals relevant to safety and health concerns (Puri et al. 2012).

However, aqueous extraction processes have the disadvantage of resulting in low oil yields. The low extraction yields can be overcome by using enzymes, such as cellulases, hemicellulases, and pectinases, that hydrolyze the structural polysaccharides forming the cell wall of oilseeds or that hydrolyze the proteins, which form the cell and lipid body membranes and facilitate oil release from the oil bodies. Thus, the soluble components diffuse into water, and the released oil forms a separate liquid phase (Goula et al. 2018). As a result, better release and more efficient extraction of bioactives are achieved (Puri et al. 2012). Aqueous extraction of oilseeds, with or without enzyme, produces oil with similar or better qualities than conventional extraction methods such as direct hexane extraction or prepress solvent extraction (Jung et al. 2012).

Latif and Anwar (Latif and Anwar 2011) found that enzyme-assisted aqueous extraction increased the oil extraction yield from sesame seed as well as the quality of the extracted oil. Oxidative stability, antioxidant activity, and tocopherol profile of sesame seed oil obtained after enzymatic extraction were better than that obtained after hexane extraction (Latif and Anwar 2011; Marathe et al. 2017).

Although aqueous enzyme oil extraction has huge potential, application of this technology is still limited because of the factors such as high cost for enzyme production and downstream processing, long incubation time, and unavoidable added step (de-emulsification) in the process. Nevertheless, due to the wide applications of aqueous enzymatic extraction, commercial enzyme production has been expedited, and as of now the enzyme production has become cheaper. Similarly, the downstream processing costs could be minimized by adapting suitable technologies than the conventional process (Kumar et al. 2017). If the above limitations can be overcome, then enzyme-based extraction could provide an opportunity to not only increase extraction yields but also to enhance product quality by enabling the use of milder processing conditions such as lower extraction temperatures (Puri et al. 2012). In the last decade, it has been proposed that those limitations may overcome with the aid of accelerated enzyme-catalyzed reaction technologies (Goula et al. 2018; Jiao et al. 2014; Long et al. 2011).

Ultrasound-Assisted Extraction

Ultrasound is an emerging technology that has been utilized in food science for processing, preservation, and extraction. Ultrasound is a form of energy generated by sound waves of frequency range that encompasses from 20 KHz (that exceeds the hearing limit of human) to GHz with division between power (Vilkhu et al. 2008). Ultrasound has been recognized for potential application in the extraction of herbals and oils, proteins, and bioactive compounds from plant or animal materials (Ahmadi Kamazani et al. 2014).

Numerous oleaginous seeds have been extracted under ultrasound. In the past years, researchers have shown ultrasound-assisted extraction to result in high yields and high-quality oils, allowing faster extraction with great recoveries. Ultrasound can also reduce the operating temperature allowing the extraction of thermolabile

compounds such as bioactive compounds (Wang and Weller 2006; Gayas and Kaur 2017). Furthermore, ultrasound-assisted extraction can enhance extraction rates with or without using solvents and provide the opportunity to use alternative generally recognized as safe solvents by improving their extraction performance (Gayas and Kaur 2017; Tiwari 2015).

When used as a pretreatment before extraction, alone or in combination with other novel or conventional techniques, ultrasound has enhanced the oil extraction yield for almond, apricot, and rice bran, and scanning electron micrographs showed a destructuring of cell walls due to ultrasonic cavitation (Rostagno and Prado 2013). Ultrasonic pretreatment of the almond and apricot seeds before aqueous oil extraction and aqueous enzymatic oil extraction provided significantly higher yield with reduction in extraction time (Vilkhu et al. 2008). The use of supercritical CO_2 in combination with an ultrasonic probe with a working frequency of 20 kHz introduced inside a high-pressure supercritical fluid extractor-enhanced oil yields by ~30% from almond while reducing the extraction time (Tiwari 2015). A combination of ultrasound and SC-CO_2 significantly increased the extraction rate of amaranth oil from seeds (Bruni et al. 2002) and almond oil (Riera et al. 2004). The scale-up to industrial applications still needs to be explored and optimized and represents a major step with respect to sustainable utilization and exploitation of molecules in real life (Tiwari 2015).

Microwave-Assisted Extraction

In the last decade, microwave-assisted extraction techniques have been widely used for high-added value compound extraction and vegetable oil recovery from plant materials (Koubaa et al. 2016). Microwave technology is a rapid, safe, and low-cost technique for the extraction of essential oils, fats, and oils and does not need samples devoid of water. However, it still uses organic solvents, such as hexane, and therefore cannot be considered as a green technology (Danlami et al. 2014). Besides, Kazamani et al. (Koubaa et al. 2016) reported that microwave pretreatment and microwave-assisted extraction of oilseeds represent a great alternative for conventional extraction methods of oils. Assisting the extractions by microwave leads to enhanced yields and/or nutraceutical value of the seed oil.

The extraction mechanism of microwave-assisted extraction is supposed to involve three sequential steps described by Alupului et al. (Alupului et al. 2012): (1) separation of solutes from active sites of sample matrix under increased temperature and pressure, (2) diffusion of solvent across sample matrix, and (3) release of solutes from sample matrix to solvent. Generally, elevated temperatures result in improved extraction efficiencies. However, for the extraction of thermolabile compounds, high temperatures may cause the degradation of extracts. In this case, the chosen power during MAE has to be set correctly to avoid excess temperatures, leading to possible solute degradation (Wang and Weller 2006). Open-vessel operation at atmospheric pressure condition is better suited to thermolabile species (e.g., organometals) since it uses low temperatures relative to closed-vessel systems (Castro and Priego-Capote 2012). Moreover, in some cases, only selective heating

of the sample matrix is brought about by immersing the sample in a microwave transparent solvent (hexane and chloroform). This approach is useful for thermolabile compounds to prevent their degradation (Danlami et al. 2014).

Compared to the untreated samples, most of the studies conducted on oil-assisted extraction or oilseed pretreatment by microwave showed similar or higher oil quality. Microwave-treated seeds generate oils with an enhanced content of desirable nutraceuticals, such as phytosterols and tocopherols, canolol, and phenolic compounds, which increase the oxidative stability of the oil and extends its shelf life (Koubaa et al. 2016). Alternatively, microwave irradiation can be utilized in accelerated enzyme-catalyzed reactions for natural products and oil extraction (Jiao et al. 2014). This microwave-assisted aqueous enzymatic extraction may be a promising alternative for novel and green extraction of plant seed oil.

Pulsed-Electric Field Extraction

The application of pulsed electric fields and high-voltage electrical discharges (HVED) for oil extraction from oilseeds and its functional ingredients has received increasing interest during the last past decade (Shorstkii et al. 2017). This minimally invasive method allows avoidance of undesirable changes in heat-sensitive biological materials, which are typical for other techniques such as thermal, chemical, and enzymatic ones (Boussetta et al. 2012). The main advantages of PEF pretreatment are related to the possibility of nonthermal extraction even at high electric field strength ($E > 20$–30 kV/cm) due to its short pretreatment time (10^{-5}–10^{-2} s) without any significant temperature increase. With no or little addition of organic solvents or enzymes, extraction assisted by PEF pretreatment is usually conducted in green solvent such as water or ethanol (Yu et al. 2016). HVED may be the other alternative for the enhancement of aqueous extraction from oilseeds which has the disadvantages of lower oil yield, high water consumption, and the addition of de-emulsification operation (Grémy-Gros et al. 2008). PEF results in the rupture of cell membranes when submitted to an external electric field, increasing the electrical conductivity and the permeability of intracellular material. HVED affects both the cell walls and membranes and can cause more extensive damage to the product. This technology is based on the phenomenon of electrical breakdown in water, which induces physical (e.g., shock waves) and chemical (e.g., formation of O_3) processes that affect the cell, enhancing the release of intracellular components. PEF can increase mass transfer during extraction by destroying membrane structure of plant materials for enhancing extraction yield and decreasing extraction time (Azmir et al. 2013).

PEF has been used in applications such as oil extraction from sesame (Sarkis et al. 2015), peanut (Zeng et al. 2010), and oil recovery from other plant products, such as maize, olives, and soybeans (Mercer and Armenta 2011). Guderjan et al. (Guderjan et al. 2007; Guderjan et al. 2005) reported the application of PEF treatment for the improvement of recovery and quality of oils extracted from oil-rich plants (Boussetta et al. 2012). The authors showed that the pretreatment with PEF improved the oil extraction yield significantly. Moreover, this electrical pretreatment had a significant effect on the oil quality, increasing the concentrations of

bioactive compounds such as of tocopherols, polyphenols, total antioxidants, and phytosterols in the obtained oils (Sarkis et al. 2015).

Conclusions and Future Perspectives

This chapter reviews the unconventional plant seed-derived specialty oils, their bioactive compounds, functional properties, nutraceutical/therapeutic applications, and novel extraction techniques. The use of unconventional cold-pressed seed oils introduced to the market quite recently seems to be accelerated in the near future owing to the presence of health-promoting bioactive compounds. These oils have been widely used in traditional, nutritional and medicinal applications. Specialty seed oils are rich in bioactive compounds such as polyunsaturated fatty acids, tocopherols, phytosterols, squalene, carotenoids, and phenolic acids which are linked to positive health benefits of these oils. The potential health benefits of bioactives can encompass reduced heart disease, mitigated cancer risk, improved plasma cholesterol levels, reduced inflammation, enhanced brain function, improved gastrointestinal health, loss of retained body fat, and many others (Puri et al. 2012). Recently several epidemiological studies have demonstrated that unconventional plant seed oils and their bioactive compounds have extensive anti-inflammatory, antiatherosclerotic, antitumor, antimutagenic, anticarcinogenic, antibacterial, or antiviral activities to a greater or lesser extent. However, more scientific research and clinical studies are required to evaluate the health-promoting effects, functional properties, biological activities, and nutritional value of unconventional plant seed oils. By this way, these compounds will have more potential roles in modern diets, pharmaceutical, cosmetics, and food industry.

These oils are traditionally extracted by cold pressing that involves no heat or chemical treatment and thus may preserve more health beneficial components. Novel alternative eco-friendly and safe extraction methods have been started to be used such as supercritical fluid, ultrasound, microwave-assisted extraction, pulsed electrical field, enzymatic aqueous extraction, or their combinations presenting advantages of lower extraction time, improved extraction yields, and higher reproducibility. However, most of these techniques are successfully applied at the laboratory or bench scale. Thus, further investigations are needed to enhance the design and scale-up of these new extraction methods for their industrial applications.

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Nutritional and Bioactive Profiles of Sprouted Seeds of Mangrove Wild Legume *Canavalia cathartica*

Dorothy D. Anita and Kandikere R. Sridhar

Introduction

Continues increase in population resulted in high demand for food leading to outpace the rate of food production from agricultural lands and oceans. Owing to insufficiency in food supply, the hungry population in developing countries has raised by 1% for every 2–2.5% increase in prices by mismatching purchase power and cost of food commodities (Gahukar 2009). In addition to inadequate food supply, population in the developing countries of tropical region are facing protein-energy malnutrition (PEM) due to overdependence on cereal-based diets, scarcity of fertile farmland and degradation of natural resources (FAO 2000). One of the possible avenues to fulfil the demands for protein-energy requirements is to depend on indigenous or underutilized or traditional wild legumes as evidenced by the African wild legumes (Ezeagu et al. 1996; Petzke et al. 1997; Bhat and Karim 2009). Exploring the wild legumes with promising quantity of proteins, fibre, minerals, essential amino acids, essential fatty acids, vitamins and nutraceuticals would strengthen the nutrition, health and food security at least at the regional level. The traditional knowledge of tribals or local people in exploitation of wild legumes as source of food is utmost importance for popularization and invention of future food source.

Canavalia cathartica is one of the common wild legumes in southwest coast of India (Rao and Suresh 2001; Rao and Sherieff 2002; Seena and Sridhar 2006; Seena et al. 2007). The landrace of *C. cathartica* exits in mangroves differs from that of coastal sand dunes of Southwest India (Seena et al. 2007). Nutritional and antinutritional qualities of seeds, ripened beans, repined split beans and tender pods of *C. cathartica* distributed in coastal regions have been studied by various

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investigators (Seena and Sridhar 2006; Bhagya and Sridhar 2007; Bhagya et al. 2006; D'Cunha 2009; Sridhar et al. 2016). Ripened split beans of *C. cathartica* as well as *C. maritima* serve as nutritional source for local dwellers and fishermen community in the Southwest India (Sridhar et al. 2016). In addition, the ripened split beans are endowed with bioactive components and antioxidant potential capable to serve as good nutraceutical source (Shreelalitha et al. 2018).

Germination of seeds has been considered as one of the nutritionally beneficial processes. For instance, sprouting edible legume seeds significantly increases the proteins (Osman 2007; Alonso et al. 2000; Mubarak 2005), vitamin C (Riddoch et al. 1998), protein digestibility (Schelze et al. 1997; Alonso et al. 2000; Shimelis and Rakshit 2007; Ghavidel and Prakash 2007; Sangronis and Machado 2007) and improves the sensory attributes of the food product (Vanderstoep 1981; Deshpande et al. 1984). In addition, sprouted seeds confer other advantages like decreased total/resistant starch (Urbano et al. 1995), decreased nitrogen-free extractives owing to induction of amylolytic enzymes (Osman 2007), decreased/knockoff nonprotein amino acid canavanine (Bell 1960; Rosenthal 1970; Nakatsu et al. 1996) and decreased antinational components (e.g. alkaloids, phytates, trypsin inhibitors and α-galactosidases) (Muzquiz et al. 1998; Trugo et al. 2000). Seed germination shortens the cooking time, cooking enhances the protein digestibility and decreases the antinutritional components (Trugo et al. 2000). In view of inadequate information on the impact of germination of *C. cathartica* seeds, the present chapter focuses on the nutritional and bioactive potential of germinated seeds collected from mangroves of Southwest India.

Seed Samples and Processing

Seeds from the dry pods of *Canavalia cathartica* Thouars from five locations of the Nethravathi mangroves, Mangalore, Southwest India (12°50′ N, 74°49′ E), were harvested during the post-monsoon season (February–April). Healthy seeds were separated in the laboratory and sun-dried for 2 days, and seed characteristics were studied. The hilum portion of the seed was mechanically scarified to break the dormancy and allowed to germinate in five sets on the wet cotton until the radical emerged about 1–1.5 cm (~4–5 days). The seed coat was removed, and each set was divided in to two parts. The first part was sun-dried until the moisture decreases below 10% (3–4 days), while the second part was cooked in a pressure cooker (6.5 L; Deluxe stainless steel; *TTK* Prestige™, Prestige Ltd., India) with freshwater (1.3 v/v) followed by sun-drying. The dried uncooked and cooked germinated seeds were powdered (Wiley mill, mesh # 30), and the flour was stored in airtight containers in refrigerator for analysis.

Assessment of Nutritional and Bioactive Components

Proximate composition like moisture, ash, total lipids, crude fibre (AOAC 1995), crude protein (Humphries 1956), carbohydrates (Müller and Tobin 1980), starch (Sadasivam and Manickam 1992) and calorific value (Ekanayake et al. 1999) were assessed. Minerals such as sodium, potassium, calcium, phosphorus, magnesium, iron, copper, zinc and manganese were assessed according to the AOAC (1995). The amino acids (Brand et al. 1994; Hofmann et al. 1997, 2003), protein fractions (Humphries 1956; Gheyasuddin et al. 1970) and nonprotein nitrogen (Humphries 1956; Sadasivam and Manickam 1992) were assessed by different methods. The in vitro protein digestibility (Akeson and Stahmann 1964), essential amino acid (EAA) score, protein digestibility corrected to amino acid score (PDCAAS) and protein efficiency ratio (PER) (Alsmeyer et al. 1974; FAO-WHO 1991, 1998) were assessed or calculated by standard methodology and formulae.

Bioactive components like total phenolics (Rosset et al. 1982), tannins (Burns 1971), canavanine (D'Cunha and Sridhar 2010), trypsin inhibition activity (Kakade et al. 1974) and hemagglutinin activity against human erythrocytes (Occenã et al. 2007) were assessed by different methods.

Significant difference in nutritional and bioactive components between uncooked and cooked germinated seeds was assessed based on the *t*-test using Statistica version # 8.0 (StatSoft Inc. 2008).

Nutritional Composition

Seed Qualities

The wild legume *C. cathartica* chosen in the present study produces considerable quantity of seeds in mangrove habitats. The whole seeds are large with L-B ratio 1.44, and mass of cotyledons is up to 72.1% that facilitates easy handling (Table 1). Germination of seed followed by thermal treatment provides additional information for optimal use in food products owing to adequate quantity of proteins, carbohydrates, energy and low lipid content.

Proximal Qualities

The moisture content in uncooked and cooked geminated seeds is less than 9% with significant difference ($p < 0.05$) (Table 2). The crude protein significantly decreased on cooking (34.4 vs. 30.2%) ($p < 0.01$), so also the starch content (99.9 vs. 50.9 mg/100 g) ($p < 0.001$). The total lipid (2.7 vs. 3.3 g/100 g; $p < 0.001$) and

Table 1 Physical characteristics of dry seeds of *Canavalia cathartica* of mangroves

Dry weight	
Seed (g/seed)	0.86 ± 0.12 (100%)
Cotyledon (g/seed)	0.62 ± 0.09 (72.1%)
Coat (g/seed)	0.24 ± 0.03 (27.9%)
Dimension	
Length (cm)	1.72 ± 0.11
Width (cm)	1.21 ± 0.16
Thickness (cm)	0.84 ± 0.07
Hilum (cm)	1.10 ± 0.11
L-B ratio[a]	1.44 ± 0.20

$n = 20$; mean ± SD
[a]Length-breadth ratio

Table 2 Proximate composition of germinated seeds of *Canavalia cathartica* of mangroves on dry weight basis

	Uncooked	Cooked
Moisture (g/100 g)	8.01 ± 0.32*	7.34 ± 0.29
Crude protein (g/100 g)	34.37 ± 0.44**	30.21 ± 0.88
Crude lipid (g/100 g)	2.65 ± 0.10	3.32 ± 0.09***
Crude fibre (g/100 g)	2.02 ± 0.25	2.54 ± 0.35
Ash (g/100 g)	3.88 ± 0.51	3.89 ± 0.62
Carbohydrates (g/100 g)	57.08 ± 1.70	60.14 ± 1.62*
Starch (mg/100 g)	99.9 ± 3.44***	50.85 ± 0.90
Calorific value (kJ/100 g)	1627 ± 29	1634 ± 18

$n = 5$; mean ± SD; *t*-test: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

carbohydrates (57.1 vs. 60.1 g/100 g; $p < 0.05$) were significantly increased on cooking. The crude fibre, ash and calorific value of uncooked seeds are not significantly altered on cooking.

The crude protein content of germinated seeds of *C. cathartica* is higher than seeds of many underutilized edible legumes like winged bean (*Neonotonia wightii*) (Viswanathan et al. 2001), velvet bean (*Mucuna monosperma*), green gram (*Phaseolus aureus*), black gram (*Phaseolus mungo*, *P. lunatus* and *P. vulgaris*) (Gupta and Wagle 1978; Baudoin and Maquet 1999; Trugo et al. 2000), pigeon pea (*Cajanus cajan*) (Nwokolo 1987), chickpea (*Cicer arietinum*) (Khan et al. 1979; Jambunathan and Singh 1980), mung bean, cowpea (*Vigna radiata* and *V. unguiculata*) (Khan et al. 1979; Nwokolo and Oji 1985) and thermally treated gila beans (*Entada scandens*) (Vadivel et al. 2008) (30.2 vs. 19.1–23.3%). The crude protein content of germinated seeds is higher than germinated seeds of jack bean (*Canavalia ensiformis*) (34.4 vs. 20%) (Akpapunam and Sefa-Dedeh 1997) and four cowpea cultivars of Nigeria (*Vigna unguiculata*) (34.4 vs. 21.7–26.6%) (Giami et al. 2001).

The total lipids in cooked seeds by Soxhlet extraction is higher than dry seeds and germinated seeds of coastal sand dune landrace (3.3 vs. 1.3–2.7%) (Arun et al. 2003; D'Cunha et al. 2009) but lower than many wild legumes and edible legumes

(e.g. *Atylosia scarabaeoides*, *Canavalia gladiata*, *Lablab purpureus*, *Neonotonia wightii, Sesbania bispinosa* and *Vigna trilobata*) (3.3 vs. 4.6–12.3%) (Arinathan et al. 2003; Pugalenthi et al. 2004).

The crude fibre in germinated seeds increased on cooking (2 vs. 2.5%; $p > 0.05$), which is higher than roasted and cooked seeds of mangrove *C. cathartica* but lower than other *Canavalia* spp. (*C. ensiformis*, *C. gladiata* and *C. maritima*: 8.5–17.43%) (Bressani et al. 1987). The crude fibre content is lower than the four varieties of germinated Nigerian cowpea (2 vs. 2.6–3.6%) (Giami et al. 2001). Although low fibre diets are nutritionally appreciable in improvement of digestibility by trapping less proteins and carbohydrates (Balogun and Fetuga 1986), the high fibre in diet is known for several health benefits (e.g. lowering blood cholesterol and reducing risk of large bowel cancer) (Anderson et al. 1995; Salvin et al. 1997).

The carbohydrates in germinated seeds are significantly increased cooking ($p < 0.05$), which is higher than *C. maritima* of coastal sand dunes (57.1–60.1 vs. 50.5%) (Seena et al. 2005) and edible legumes like peanuts (*Arachis hypogea*) and soybean (*Glycine max*) (57.1–60.1 vs. 20.9–26.1%) (Rao et al. 1999). It is also higher than three germinated Nigerian cowpea varieties (57.1 vs. 53.9–55.4%) (Giami et al. 2001). The high quantity of carbohydrates in seeds is helpful in combating the intestinal cancer and responsible for lowering the glycemic index leading to prevention of type II diabetes (Venn and Mann 2004). The quantity of starch was low in germinated seeds and significantly decreased on cooking. The starch content in seeds is known to provide desired texture to the processed foods.

The calorific value of germinated seeds did not significantly vary on cooking (1627 vs. 1634 kJ/100 g; $p > 0.05$), which is higher than edible legumes (1358–1426 kJ/100 g) as well as wild legumes (e.g. *Mucuna pruriens* and *Entada scandens*: 1516–1541 kJ/100 g) (Vadivel and Pugalenthi 2007; Vadivel et al. 2008).

Mineral Composition

Among the nine minerals assessed, the calcium content is highest in uncooked seeds followed by magnesium, phosphorus, potassium and sodium (Table 3). Except for phosphorus, copper and zinc, rest of the minerals decreased significantly on cooking. The Na-K and Ca-P ratios ranged between 0.26–0.38 and 1.39–1.43, respectively. Unlike non-germinated seeds, germinated cooked seeds did not drain the minerals severely on cooking (Seena et al. 2006). Except for copper, none of the minerals fulfilled the NRC-NAS (1989) stipulated pattern for adults. It is interesting to note that the mineral content of germinated seeds of mangrove *C. cathartica* is lower compared to the coastal sand dune landrace (D'Cunha et al. 2009). As seen in cooked seeds, seed roasting also did not decrease the minerals in *C. cathartica* (Seena et al. 2006). However, the Na-K and Ca-P ratios are desirable because low Na-K ratio (<1) is known to control high blood pressure (Yusuf et al. 2007) and high Ca-P ratio (>1) prevents the loss of calcium in urine as well as restores calcium in bones (Shills and Young 1988).

Table 3 Mineral composition of germinated seeds of *Canavalia cathartica* of mangroves on dry weight basis (mg/100 g)

	Uncooked	Cooked	Dietary allowance[a]
Sodium	50.71 ± 6.31***	29.37 ± 4.27	500
Potassium	133.17 ± 16.59**	110.81 ± 14.42	2000
Calcium	222 ± 27.64*	200.65 ± 26.10	800
Phosphorus	155.07 ± 19.31	144.86 ± 18.84	800
Magnesium	160.52 ± 19.99**	100.65 ± 13.09	280–350
Iron	7.32 ± 1.01*	6.65 ± 0.87	10
Copper	1.52 ± 0.20	1.39 ± 0.18	1.5–3
Zinc	5.35 ± 0.67	5 ± 0.65	15
Manganese	1.37 ± 0.17**	1.11 ± 0.14	2–5
Na-K ratio	0.38	0.26	0.25
Ca-P ratio	1.43	1.39	1.00

$n = 5$, mean ± SD; t-test: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$
[a]NRC-NAS 1989 Pattern for adults

Amino Acid Composition

Among the amino acids, the glutamic acid is highest followed by aspartic acid and leucine in uncooked as well as cooked seeds, while tryptophan is not detectable (Table 4). Cooking significantly increased all amino acids except for glutamic acid. The EAA ratio of cystine + methionine, leucine, tyrosine + phenylalanine and lysine in uncooked seeds and cystine + methionine, tyrosine + phenylalanine and lysine in cooked seeds are below 100. Overall, the amino acid profile of cooked seeds is better than uncooked seeds. Based on the EAA score, threonine, valine, isoleucine, leucine and histidine are comparable or higher than FAO-WHO (1991) recommended pattern for adults. Except for sulphur amino acids, other EAA are comparable to seeds of soybean, rice and germinated soybeans (Livsmedelsverk 1988; Bau et al. 1994; Trugo et al. 2000). However, the amino acid profile of uncooked as well as cooked germinated seeds of *C. cathartica* of coastal sand dunes is higher than that of mangrove landrace (D'Cunha et al. 2009).

Protein Bioavailability

The total proteins decreased significantly in cooked seeds (29.5 vs. 26.2%) ($p < 0.001$) (Table 5). The albumins constituted the major protein fraction in uncooked (62.4%) as well as cooked (69.2%) seeds without significant difference ($p > 0.05$). The globulins and glutelins decreased drastically on cooking (5 vs. 1.2%) ($p < 0.001$). However, in uncooked seeds the prolamins ($p < 0.01$) and glutelins ($p < 0.001$) are significantly high. The total protein content of uncooked and cooked germinated seeds (26.2–29.5%) is comparable with dry seeds of coastal sand dunes

Table 4 Amino acid composition of germinated seeds of *Canavalia cathartica* of mangroves on dry weight basis (g/100 g protein)

	Uncooked	Cooked	Soybean[a]	Rice[b]	FAO-WHO Pattern[c]
Glutamic acid	15.03 ± 0.16***	11.50 ± 0.28			
Aspartic acid	9.02 ± 0.10	9.50 ± 0.23**			
Serine	4.18 ± 0.05	4.74 ± 0.12***			
Threonine	3.54 ± 0.04 (104.1)	3.92 ± 0.10*** (105.3)	3.8	3.2	3.4
Proline	2.92 ± 0.03	3.27 ± 0.08***			
Alanine	3.33 ± 0.04	3.82 ± 0.09***			
Glycine	2.98 ± 0.03	3.42 ± 0.08***			
Valine	3.60 ± 0.04 (102.9)	4.15 ± 0.10*** (118.6)	4.6	6.6	3.5
Cystine	0.70 ± 0.01	0.81 ± 0.07***	1.7	1.2	2.5[d]
Methionine	0.26 ± 0.003 (38.4)[d]	0.34 ± 0.01*** (46)[d]	1.2	2.6	
Isoleucine	3.18 ± 0.04 (113.6)	3.66 ± 0.09*** (130.7)	4.6	4.3	2.8
Leucine	5.93 ± 0.07 (89.9)	6.79 ± 0.17*** (102.9)	7.7	8.2	6.6
Tyrosine	2.92 ± 0.03	3.26 ± 0.08***	1.2–3.4	3.7	6.3[e]
Phenylalanine	3.30 ± 0.04 (98.7)[e]	3.78 ± 0.09*** (88.9)[e]	1.29–4.8	5.1	
Tryptophan	BDL	BDL	0–1.2	1.3	1.1
Lysine	4.33 ± 0.05 (74.7)	4.99 ± 0.12*** (86)	6.1	3.7	5.8
Histidine	2.13 ± 0.02 (112.1)	2.22 ± 0.05* (116.8)	2.5	2.4	1.9
Arginine	3.61 ± 0.04	4.28 ± 0.11***			

$n = 5$, mean ± SD; EAA score in parenthesis; *t*-test: *$p < 0.05$; **$p < 0.01$, ***$p < 0.001$
[a]Bau et al., 1994; [b]Livsmedelsverk 1988; [c]FAO-WHO 1991 pattern for adults; [d]cystine + methionine; [e]Tyrosine + phenylalanine

(*C. cathartica*: 28.3%; *C. maritima*, 29.3%) (Seena et al. 2005, 2006) and exceeded than *Psophocarpus* (15.2%) (Viswanathan et al. 2001), *Canavalia gladiata* (20.8%), *C. ensiformis* (Rajaram and Janardhanan 1992) and *Cassia floribunda* (16.3–17.7%) (Vadivel and Janardhanan 2001). The total protein content is more than the germinated seeds of *C. cathartica* of coastal sand dunes (26.2–29.5 vs. 8.4–19.8% (D'Cunha et al. 2009). Among the protein fractions, albumins are highest followed by globulins, while in dry seeds, it was opposite (Seena et al. 2006). The albumins of germinated seeds did not decrease significantly on cooking (18.4 vs. 18.1%). In addition, the A-G ratio drastically increased in cooked seeds (3.7 vs. 15) depicts lowering or elimination of antinutritional factors. As the albumins are known to be rich in sulphur amino acids and other EAA (Baudoin and Maquet 1999), there seems to be significant raise of several EAA in cooked seeds (see Table 6).

Table 5 Protein fractions and nonprotein nitrogen of germinated seeds of *Canavalia cathartica* of mangroves on dry weight basis (g/100 g)

	Uncooked	Cooked
Total proteins	29.54 ± 0.57** (100)	26.15 ± 0.62 (100)
Albumins	18.42 ± 0.01 (62.36)	18.10 ± 0.20 (69.22)
Globulins	4.99 ± 0.29** (16.89)	1.21 ± 0.07 (4.63)
Prolamins	1.15 ± 0.02 (3.89)	3.40 ± 0.29* (13.08)
Glutelins	4.96 ± 0.27** (16.89)	3.42 ± 0.31 (13.08)
A-G ratio[a]	3.69	14.96
Nonprotein nitrogen	4.61 ± 0.23**	3.62 ± 0.14

$n = 5$, mean ± SD; % in parenthesis; *t*-test, *$p < 0.01$; **$p < 0.001$
[a]Albumin-globulin ratio

Table 6 In vitro protein digestibility (IVPD)

	Fresh	Cooked
IVPD (%)	56.88 ± 4.04	63.89 ± 2.41*
PDCAAS[a]		
Threonine	59.22	73.66
Valine	58.51	75.76
Cystine + methionine	21.84	29.39
Isoleucine	64.64	83.51
Leucine	51.11	65.73
Tryptophan	0	0
Tyrosine + phenylalanine	56.16	71.09
Lysine	42.46	54.97
Histidine	63.77	74.65
PER[b]		
PER_1	1.88	2.56
PER_2	1.92	2.27
PER_3	0.61	1.02

$n = 5$, mean ± SD; *t*-test, *$p < 0.05$ and protein digestibility corrected amino acid score (PDCAAS) and protein efficiency ratio (PER) of germinated seeds of *Canavalia cathartica* of mangroves
[a]Calculated according to FAO-WHO, 1991; [b]calculated according to Alsmeyer et al., 1974

The IVPD is significantly higher in cooked than in uncooked seeds (63.9 vs. 56.9%) ($p < 0.05$) (Table 6). Increase in PDCAAS in cooked seeds is evident in almost all EAA except for tryptophan. The PER is also substantially increased in cooked seeds. The IVPD of cooked germinated seeds of mangrove *C. cathartica* is lower than coastal sand dune landrace (63.9 vs. 71.7%) (D'Cunha et al. 2009), but it is higher than the

Table 7 Antinutritional and nutraceutical components of germinated seeds of *Canavalia cathartica* on dry weight basis

	Fresh	Cooked
Total phenolics (g/100 g)	2.06 ± 0.36* (100)	1.02 ± 0.12 (49.52)
Tannins (g/100 g)	0.001 ± 0.0001* (100)	0.0004 ± 0.0001 (40)
Canavanine (g/100 g)	0.12 ± 0.001** (100)	0.08 ± 0.001 (66.67)
Trypsin inhibition activity	NP	NP
Hemagglutinin activity (Hu/g)		
A^+	100	NA
B^+	120	40
O^+	120	NA

$n = 5$, mean ± SD; t-test: *$p < 0.01$, **$p < 0.001$; % in parenthesis; NP, not present, NA, no agglutination

velvet bean (*Mucuna pruriens*) (63.9 vs. 49.7) (Bhat and Sridhar 2008). Significant increase in IVPD between uncooked and cooked seeds depicts partial elimination or inactivation of antinutritional factors (Poel et al. 1991). The IVPD in defatted soybean flours was also linked with decreased protein-dependent antinutrients (e.g. trypsin and chymotrypsin) by Abu-Tarboush (1998). In our study, the IVPD of cooked germinated seeds increased owing to lack of trypsin inhibitors and significant decrease of other antinutritional factors (e.g. total phenolics, tannins, canavanine and lectins) (Cheryan 1980; Reddy et al. 1985) (see Table 7). Due to increased IVPD, the PDCAAS is also raised in EAA except for tryptophan. Determination of PER is one of the important measures to evaluate the protein quality. Friedman (1996) has classified proteins into three groups based on PER (poor, <1.5; moderate, 1.5–2; high >2). In our study, PER_1 and PER_2 in cooked germinated seeds are high (2.27–2.56), while PER_3 is relatively poor (1.02). However, the average of PER belongs to the moderate category (1.95). It is possible to uplift the PER in cooked germinated seeds of *C. cathartica* through blending suitable flours depending on the type of food product.

Fatty Acids

Extraction of lipids from uncooked and cooked germinated seeds of mangrove *C. cathartica* by hot-extraction (Soxhlet) (AOAC 1995) and cold-extraction (chloroform-methanol-water) (Bligh and Dyer 1959) methods showed difference in fatty acid methyl esters (FAMEs) (Anita et al. 2014). On evaluation of FAMES (Padua-Resurreccion and Banzon 1979; Nareshkumar 2007), lauric, myristic, arachidic and palmitoleic acids exceeded the quantities present in soybean on hot-extraction (Wahnon et al. 1988; Cho 1989). In both methods oleic, linoleic and linolenic acids were higher in germinated seeds than in cooked seeds. The cold-extraction resulted in favourable ratios of TUFA/TSFA, C14:0 + C15:0 + (C16:0/C18:0) and ω-6/ω-3

(Simopoulos 2002). Uncooked and cooked germinated seeds in both methods of extraction resulted in ω-6/ω-3 ratio ranging from 2.2 to 3.1. Those food stuffs possessing a ratio 2.5 are known to reduce the cell proliferation of colorectal cancer, while a ratio between 2 and 3 is known to suppress the inflammation by rheumatoid arthritis.

Bioactive Components

Among the antinutritional or nutraceutical components, total phenolics ($p < 0.01$), tannins ($p < 0.01$) and canavanine ($p < 0.001$) were significantly decreased on cooking, while seeds were devoid of trypsin inhibition activity (Table 7). The hemagglutinin activity was completely eliminated in blood groups A^+ and O^+, while it decreased substantially against blood group B^+. The canavanine content in germinated seeds is very low compared to *C. gladiata* and coastal sand dune *C. cathartica* (0.1 vs. 2.5–5.1%) (Ekanayake et al. 2007; D'Cunha 2009) may be due to impact of germination. Its concentration in uncooked and cooked germinated seeds is comparable to the seeds of *Vicia articulata* and *V. ervilia* (0.08–0.12 vs. 0.05–0.3%), which has been considered nutritionally safe/beneficial (Enneking and Wink 2000). Cooking tender pods, dry seeds and germinated seeds significantly decreased the canavanine content (D'Cunha 2009). Total phenolics and tannins are well-known antioxidants (Hertog et al. 1997; Hagerman et al. 1998) while the canavanine as an anticancer agent (Swaffar et al. 1994). Usually the globulins of seeds consist of lectins, in which decreased of more than fourfold in cooked germinated seeds (5 vs. 1.2%) might be the major cause for drastic reduction of hemagglutinin activity.

Conclusion

In view of protein-energy malnutrition in developing countries, there is an urgent need to identify indigenous wild food resources with desired traits for human nutrition. Recently, three landraces of coastal *Canavalia* gained importance, and their seeds have been evaluated for feasibility for human consumption. Based on the evaluation of seeds (dry, sprouted and ripened) and ripened split beans, the latter devoid of seed coat and testa on cooking showed suitable nutritional qualities (high protein, high energy, low fat, essential fatty acids, essential amino acids and high protein bioavailability). Similar to the ripened split beans, the cooked germinated seeds of mangrove landrace *Canavalia cathartica* endowed with most of the desired qualities suitable as human diet. In addition, germination has eliminated the antinutritional factors or reduced below threshold level. In addition to nutritional advantages, the cooked germinated seeds of *C. cathartica* seem serving as nutraceutical in preventing lifestyle-dependent human ailments. Future studies need to focus on the cultivation of coastal landraces of *Canavalia* under different agroclimatic conditions to fulfil soil binding, soil quality improvement and green manure and to supplement livestock fodder.

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Contribution of Jojoba (*Simmondsia chinensis*) Products in Human Health

Jameel R. Al-Obaidi

Introduction

Jojoba [*Simmondsia chinensis*, (Link) Schneider] pronounced ho-**HO**-ba is an always green, dioecious woody bush native to the desert of North and Central America (Kumar et al. 2012). The plant also has been cultivated for many years worldwide, such as in Argentina, Chile, India, Tunisia, the Palestinian territories and Egypt, due to its high commercial importance. The gender of jojoba plant can only be distinguished from their flowers. Jojoba seedlings can take more than 4 years to produce flowers. As a dioecious crop, the plant produces female and male flowers. Jojoba leaf shape helps to bring wind-borne pollens to the female flower (Fig. 1a). The oil produced from jojoba seeds which make up more than half of the plant seed is commonly known as liquid wax, which has importance in the pharmaceutical, cosmetic and lubricant industries (Cappillino et al. 2003; Al-Soqeer et al. 2012). Native Americans used the crushed dry seed oil for skin treatment and therapeutic purposes (McKeon 2016). The knowledge about the plants and its medicinal values has been transferred through Spanish missionaries during the early eighteenth century. Jojoba oil is exceptional in its molecular structure and high shelf stability under high pressure and heat conditions. Different from other plant-based oil, it has a chain of fatty acids and higher unsaturated alcohol-based esters (Jangra et al. 2014; Fouillen et al. 2013; Fouts et al. 2015; Miklaszewska and Banaś 2016). The wax formula features of jojoba oil make it capable to lasts significantly longer than other crop oils, making it an addition to any skincare product. Jojoba has scientists and agriculturists concern worldwide over the last few decades as a promising industrial crop for semiarid lands (Coates et al. 2006). However, the area used for jojoba plantation is reducing significantly due to a number of restraints (Reddy and Chikara 2010).

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Fig. 1 (**a**) Jojoba leaves. (**b**) Jojoba fruits. (**c**) Jojoba dry seeds

The plant oil production was estimated around 1800 kg/ha (Yang et al. 2014). This plant has been planted to avert desertification issue in some parts of India, Egypt and Saudi Arabia (Ashraful et al. 2014), and for that reason, the plant may have multiple roles: medicinal, economical and environmental.

Jojoba Oil and Its Involvement in Human Health

Jojoba seeds are dark brown coffee bean-like in shape but larger in size (Fig. 1b, c). More than 60% of those seeds are golden liquid and were called "jojoba wax" or "jojoba oil". The oil composes of single esters of long-chain fatty acids and C_{20} and C_{22} alcohols, together with small triglyceride esters. Being almost glycerine-free, jojoba oil is distinctive among the rest of the plant-based oils (Sánchez et al. 2016). A summary of different medicinal, pharmacological and human health-related applications of jojoba plant is listed in Table 1. Traditionally, the Native Americans used extracts from crushed seeds to treat sunburn, wounds, renal colic, hair loss treatment, headache and sores (Ranzato et al. 2011). In comparison with hundreds of thousands of known plant species, jojoba seeds produce very unique ester-based waxy oil similar to the natural human sebaceous gland oil (Fig. 2) (Ayerza and Coates 2004) which make the oil the best candidate for skincare products (Henderson 2015). Previously, a study has revealed the existence of compounds called tocopherols in jojoba oil (Tobares et al. 2003). It has been reported that γ-tocopherol as a main basic component compose more than 79%, followed by α-tocopherol which makes up to 20% of the tocopherol compound; which is available in the form of vitamin E and helps in eliminating free radicals (El-Mallah and El-Shami 2009). It is believed that jojoba's oil molecular structure is more stable in comparison to other plant oils. As can be noticed in Fig. 2, the double bonds in jojoba oil structure are located far from each other and are uneven from the centre. In typical plant oils, the double bonds are close to each other. These close double bands may attract free radicals making these kinds of oil not suitable to be applied on the skin and difficult to manipulate (Busson-Breysse et al. 1994). This is one of the reasons for not recommending plant-based oils for skin health-related product. However, this is not the case for jojoba oil which recently reported to be involved in skin nourishment for its antioxidant, anti-ageing and prevention from free radicals (Bakry et al. 2016). Due

Table 1 Summary of the medicinal and industrial applications of jojoba (*Simmondsia chinensis*)

Plant parts or products	Applications	References
Ethanolic seed extract	Inhibit oxidative stress induced by fumonisins (mycotoxins)	Abdel-Wahhab et al. (2010)
Meal (left over after oil extraction)	Livestock feed	Bouali et al. (2008)
Oil	Bioenergy	Le Dréau et al. (2009)
Simmondsin and its derivatives	Antifungal	Abbassy et al. (2007)
Oil	Anti-inflammatory (e.g. treatment for throat inflammation, wound treatment)	Habashy et al. (2005), Ranzato et al. (2011)
Oil	Relief for headaches	Ranzato et al. (2011)
Oil and seed extracts	Antimicrobial and antifungal	Abu-Salem and Ibrahim (2014), Elnimiri and Nimir (2011), Menghani et al. (2012)
Leaves (flavonoid compounds)	Antioxidant and lipoxygenase inhibitor	Abdel-Mageed et al. (2014)
Leaves and seed coats	Antibacterial and anticancer	Al-Qizwini et al. (2014)
Oil	Lipoxygenase inhibitor	Abdul-Hafeez et al. (2014)
Simmondsin and its derivatives	Antioxidant	Al-Qizwini et al. (2014), Manoharan et al. (2016)
Oil	Pharmaceuticals	Sánchez et al. (2015)
Oil	Skincare treatment/skin health	Henderson (2015), Bakry et al. (2016)
Meal	Anti-rodent	Chaudhary and Tripathi (2015)
Oil	Antifungal/insecticidal properties	Abdel-Mageed et al. (2016)
Crude extracts	Cyclooxygenase inhibitor (anticarcinogenic)	Abdel-Mageed et al. (2016)
Oil	Free radical elimination	El-Mallah and El-Shami (2009)
Oil	Skin anti-ageing	Ainbinder and Touitou (2017), Cvačka and Vrkoslav (2016)
Oil	Replacement of sperm whale oil	Miwa et al. (1979), Wisniak (1987)
Oil	Stroke and diabetes treatment	Manoharan et al. (2016)
Oil	Anti-virus properties	Sánchez et al. (2015)
Oil	Hair health	Haskin and Aguh (2017)

to the structure of jojoba oil, it is proposed that the oil has the ability to prevent the accumulation of molecules in the first layer of the human skin which is usually affected by direct sun exposure (Ainbinder and Touitou 2017; Cvačka and Vrkoslav 2016). Large-scale production of jojoba oil started in the early 1970s last century, as an effort of finding replacement for the high-value sperm whale oil (Miwa et al. 1979; Wisniak 1987). By comparing the structures in Fig. 2, and due to the similarity between the two structures, there is no wonder why jojoba has been used for that purpose (Hill and Hofer 2009). Besides its importance in cosmetic and skincare,

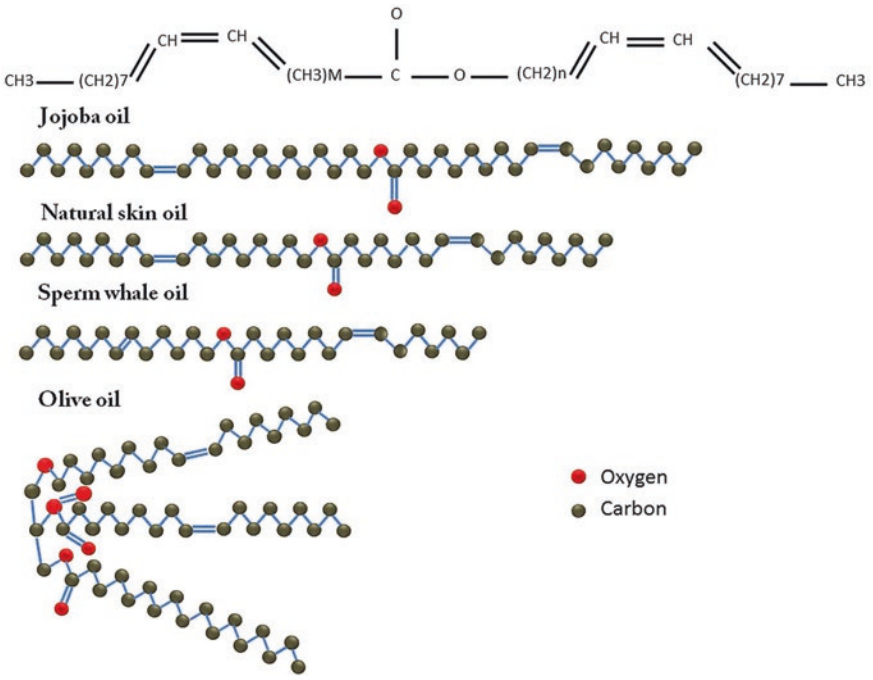

Fig. 2 Jojoba oil structure

jojoba oil also has anti-inflammatory activity (Habashy et al. 2005). The oil exhibits the anti-inflammatory activities against rat and resulted reduction in oedema and prostaglandin E2 content. Besides the oil, alcoholic seed extract showed antioxidant effects and can protect rat liver against FB1-induced hepatotoxicity. This might be due to the presence of jojobenoic acid in the seed extract (Abdel-Wahhab et al. 2016). In a recent report, phytochemical screening of jojoba oil showed antioxidant properties with potential use for inhibition and managing of many sicknesses such as stroke and diabetes (Manoharan et al. 2016). Such reports indicating the possible ability of using jojoba oil as antioxidant for treatment of some chronic diseases will open new prospects in pharmaceuticals and health-related industries; however more in-depth research on the antioxidant properties and more trail on animals or cell lines are needed to confirm these claims. Jojobyl alcohols (11-eicosenol, 13-docosenol and 15-tetracosenol) are produced from jojoba oil using biorefinery process. This compound is produced through two-step crystallizations using waste from fish processing industry as a catalyst (Sánchez et al. 2015). This compound has pharmaceutical and medicinal properties against viruses which make jojoba oil more valuable (Sánchez et al. 2015). Jojoba oil as carrier oil is reported to be involved in hair health (Haskin and Aguh 2017). The oil plays an important role in reducing white hair and promotes hair health by decreasing scalp dryness. In conclusion, jojoba oil has high medicinal value for its anti-inflammatory, antioxidant and potential anticancer properties. The oil is also considered very important in

human skin and health applications with an ability to heal acne by acting as a deep cleanser. As a liquid wax, it can enter deep into the hair follicle and dissolve the sebum deposits and help remove the comedone, clearing out the blockage. Eczema and psoriasis treatment also can be performed using jojoba oil-based products. Treatment of dry hand and cracked feet also can be done by applying jojoba oil which forms a waxy layer that helps the skin to keep moisture in and gives a smooth feel to the skin (Bright 2015).

The Use of Jojoba Leaves and Root Extracts in Health Applications

The most famous product when it comes to jojoba plant is the liquid wax (jojoba oil) and its derivatives. But the importance of this plant is not solely depending on the production of seed oil, but it extends to the other parts of the plant. The extract from the leaves of jojoba together with other plant extracts is reported to be used in the production of cosmetic products to treat sensitive skin stress with anti-inflammatory, cell activator agents and antioxidants due to the ability of those extracts in decreasing active oxygen molecules which promote ageing process (Tanaka et al. 2006). Leaf extract from jojoba contains many flavonoids and lignans (Abdel-Mageed et al. 2014). Alcoholic extract from the jojoba leaves is subjected to antioxidant assay with DPPH, and those results showed that ten flavonoid compounds exhibit antioxidant and lipoxygenase inhibitory effects, while lignans have shown moderate antioxidant effect (Abdel-Mageed et al. 2014). Besides the leaf extract (male and female), extract from seed shelf also exhibits antioxidant activity (using DPPH assay) (Al-Qizwini et al. 2014). The importance of this plant as a good source of phenolics in particular the flavonoid content may increase the interest of research concerning this plant and also help pharmaceutical as well as general medication industries to use this plant as a source of natural starting material. Another report had been published recently showing the importance of leaf extract and its potential pharmaceutical and medicinal properties which had been conducted by Abdul-Mageed and his group (Abdel-Mageed et al. 2016). The group found two compounds with promising cyclooxygenase-2 (COX-2) inhibition activities. It has been reported that COX-2 play an important role in the production of prostaglandins, apoptosis inhibition, spread of tumour cell and carcinogenesis (Wang 2005; Xu et al. 2014; Echizen et al. 2016). For that reason, COX-2 inhibitors such as jojoba leaf extract have shown potent anticancer activity by increasing apoptosis rate, decreasing angiogenesis and reducing invasiveness, which make it a decent goal in cancer treatment (Huss et al. 2002; Cao et al. 2010). Due to the link between health concerns and chemical-based food additive, there are growing demands by consumers for food product prepared with natural preservatives such as plant-based additives (Delves-Broughton 2012). That directed the research to seek of natural antimicrobials suitable for food industry. Alcoholic extract from jojoba root which

contains compound such as alkaloids, saponins and steroids showed promising antimicrobial activity against few food-borne pathogenic bacteria such as *Bacillus cereus*, *Salmonella typhimurium* and *Staphylococcus aureus* as well as two pathogenic fungi *Candida albicans* and *Aspergillus flavus*, and this suggests potential use as natural plant-based additives in food industry against the causative agent of food contamination and food spoilage (Abu-Salem and Ibrahim 2014).

Simmondsin and Its Derivatives: Contribution to Human Health

Simmondsin (2-cyanomethylene)-3 hydroxy 4,5 dimethoxycyclohexyl ß-d-glucoside) and its derivatives are molecules extracted from jojoba plant (Lievens et al. 2003). These molecules exhibit direct and indirect interaction with human health. Earlier reports showed levels of toxicity of simmondsin (Booth et al. 1974). However, many reports following that study indicate the ability of simmondsin to control animal diet and reduce food intake and control body weight (Boozer and Herron 2006; Flo et al. 1998; Tang et al. 2005). The fact that simmondsin can control appetite through oral administration has been studied widely (Lievens et al. 2003, 2009). However, there is no solid concrete conclusion about the side effect of using simmondsin products; in another word there is no answer for the question "is the use of simmondsin products safe for human?" For example, earlier reports have shown the ability to detoxify simmondsin for safer use (Banigan and Verbiscar 1980; d'Oosterlynck 1997); the ability of simmondsin to reduce lipid and prevent cancer after being administered with metabolite detoxification agents has been claimed (Wong et al. 2003). While abnormal physiological changes has been noticed when simmondsin given to Wistar rats suggesting that simmondsin not only facilitates satiation but have multiple positive and negative effects (Lievens et al. 2009). More in-depth comparative studies should be useful by comparing the simmondsin effect before and after detoxification in different doses on different host animals.

Conclusion and Perspective

Jojoba (*Simmondsia chinensis*) has an important value and great potential for commercial cultivation as a profitable crop for medicinal and pharmaceutical applications, not only because it is a source of nonedible lubricant but also a source of many metabolites with remarkable biological activities. As stated in this chapter, these compounds were extracted and isolated from different parts of jojoba. Some studies have been conducted in the past for examining medicinal, pharmacological and cosmetic uses. The importance of the plant not only comes from the unique liquid wax of the plant seed but also from the leaf extracts, roots and seed shelf.

Jojoba meal resulted from the oil extraction process from the seed contains a high protein yield. It can be used as fish male replacement.

However, regarding jojoba medicinal properties, further studies are still essential to examine important popular uses of the jojoba products and to observe jojoba detoxified compound through various in vivo and in vitro experiments. Such studies could encourage people to prioritize this plant, since many popular applications in various cosmetic and healthcare product purposes have been reported, showing a great potential of pharmacological relevance. Additionally, future phytochemical studies of this plant are important to obtain the best knowledge of the chemical composition of different extracts of the plant, in order to distinguish the actual important compounds related to healthcare and medicinal products. In conclusion, this chapter may provide visions for future research direction at both medicinal and pharmacological validations of jojoba oil/extracts and validation as a good source of bioactive compound for natural products for potential application in human health industry. Considering the fact that the plant can be cultivated in arid and semiarid areas, the expansion in plant cultivation will not affect the traditional farming practice.

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Aflatoxins in Plant-Based Foods

Amir Ismail, Muhammad Riaz, Yun Yun Gong, Saeed Akhtar, and Jin Sun

Introduction

Aflatoxins are the secondary metabolites of five different species of *Aspergillus* (*A.*) mold including *A. niger*, *A. flavus*, *A. fumigatus*, *A. nomius*, and *A. parasiticus*. Aflatoxins were discovered in the 1960s due to the death of thousands of turkeys who feed on fungal-contaminated groundnut meal. Until now 17 different types of aflatoxins are reported, but the aflatoxins most toxic to human health are aflatoxin B_1 (AFB_1), aflatoxin G_1 (AFG_1), aflatoxin B_2 (AFB_2), and aflatoxin G_2 (AFG_2), respectively. Aflatoxins are reported in a number of food commodities of both plant and animal origins such as cereals, spices, fruits, animal feed, meat and meat products, and milk and milk products. However, the higher reported values of aflatoxins are from plant-based foods. Aflatoxins are named on the basis of their fluorescence property: AFB_1 gives blue color under ultraviolet (UV) light, and AFG_1 gives green color, while aflatoxin M_1 (AFM_1) is named on the basis of its presence in milk (Ismail et al. 2016).

More than five billion people mostly residing in the developing countries are exposed to aflatoxin through contaminated plant-based food primarily. Aflatoxins now have been regarded as serious concern for public health as both AFB_1 and AFG_1 are classified as group 1 category carcinogen, while aflatoxin M_1 (AFM_1) is categorized as group 2B category carcinogen by the International Agency for Research on

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Cancer (IARC). Aflatoxins are reported as cytotoxic, genotoxic, hepatotoxic, and immunosuppressing agents. On account of their highly toxic impacts on human health, strict regulations are adopted worldwide for aflatoxins.

High levels of aflatoxin contamination are reported mostly in tropical or subtropical areas as compared to temperate zones due to the suitable environmental conditions for the growth of fungus responsible for the production of aflatoxins. However, the trade of food commodities all around the world means that aflatoxin-contaminated foods might be consumed by the people living in every corner of the world. Plant foods are especially rich in aflatoxins as compared to animal-based foods due to the fact that plants are more exposed to environmental contaminants like fungi and are ultimately rich in mycotoxins such as aflatoxins. Worldwide a number of reports are published which confirm the prevalence of aflatoxins above their permissible limits in different plant groups.

In this chapter we will describe the toxic impacts of aflatoxins on human health, regulations adopted for aflatoxins in different countries, different techniques used for the quantification of aflatoxins, and prevalence of aflatoxins in some major plant groups like cereals, fruits, spices, and animal feeds. Furthermore, different strategies to limit the production of aflatoxins will also be discussed.

Aflatoxin Impact on Human Health

Aflatoxicosis or early known as turkey X disease was first discovered in 1960 when hundred thousands of turkey died in England, the investigations of which lead to the discovery of aflatoxins. Perhaps the most severe aflatoxicosis in history occurred in Kenya in 2004 and has led to more than 100 deaths. Most recently in Tanzania, more than ten deaths and many hospitalized cases were reported to be associated with aflatoxin acute exposure. Aflatoxins are reported to functionally damage the liver, the kidney, and the immunity system of humans. The most toxic and most abundant aflatoxin is AFB_1 which can be permeable through the placenta and hence can adversely affect the fetus. AFB_1 is absorbed in the duodenum, while it is converted into its most toxic form, i.e., exo-AFB_1-8,9-epoxide (AFBO), inside the liver by cytochrome P450 enzymes, primarily CYP 3A4 subtype in human. AFBO is highly active so that it binds covalently with DNA, resulting in DNA mutation especially the mutation at codon 249 of tumor inhibition gene P53 which initiates tumor development. International agency for research on cancer has classified AFB_1 as group 1 category carcinogen (IARC); however, in most of the food/feed items, aflatoxins exist together as mixtures, and more recently this mixture of aflatoxins is also being categorized as group 1 category carcinogen. AFM_1 is a less toxic metabolite of AFB_1 produced in the liver of dairy animals consuming AFB_1-contaminated feed and classified as group 2B carcinogen by IARC. It has been estimated that the health implications of aflatoxicosis in humans result in around $143 million loss each year (IARC Publication list 2012; Monson et al. 2015).

The health impacts of aflatoxins are dependent on the amount of toxin ingested and the exposure time; based on these factors, aflatoxin toxicity might be acute or chronic. In mammals, the reported symptoms of aflatoxin acute toxicity are ataxia, loss of appetite, jaundice, lethargy as well as inflammation in the liver, and death in severe cases. On the other hand, the symptoms of chronic exposure to aflatoxins are cirrhosis and hepatocellular carcinoma, immunity system disorders, loss of appetite, reduced milk production, and reduced overall growth. In humans the most prominent health impact of aflatoxin toxicity is hepatocellular carcinoma especially when co-occurring with hepatitis B virus infection. Globally reported cases of hepatocellular carcinoma are 0.55–0.60 million cases/year, and out of these 0.025–0.15 million cases are linked with aflatoxin-contaminated foods (Ismail et al. 2017; Verheecke et al. 2016; Wild and Gong 2010).

Animal species are reported to have varying levels of sensitivity toward aflatoxin toxicity. The most sensitive animals toward both acute and chronic toxicities are duck and turkeys. The dose response may vary considerably with respect to type of food, race, age, and gender. In a number of species, the females are reported more resistant as compared to males, while the sensitivity is much higher in young as compared to adults (WHO 2002).

Aflatoxin Regulations

Owing to their highly toxic nature and global prevalence, the aflatoxins are strictly being monitored, and more than 120 countries are reported to have legislations against aflatoxins in food and feed items. The major purpose of setting maximum allowable limits for aflatoxins in different food commodities is to protect the consumer health. The variations in aflatoxin legislations have a huge impact on world food trade as food commodities on the basis of their aflatoxin level can be accepted by some countries but not by others due to the differences in maximum allowable limits for aflatoxins within countries. The possible factors in setting the legal limits for aflatoxins are socioeconomic condition, climatic characters, agricultural profile, data available regarding the prevalence of aflatoxins, food security status, instrumental techniques, political situation, and business prospects (Bui-Klimke et al. 2014).

Although several countries have established their own maximum permissible limits, the most widely acceptable limits are those recommended by the international standard agencies such as Codex Alimentarius Commission and Food and Agriculture Organization. Aflatoxins' permissible levels vary among different food types. The strictest legislations are adopted for AFM_1 in milk and milk products due to the fact that dairy products are more frequently consumed by the infants and elderly who have least immunity level among all age groups. The European Union, Australian, Sweden, and Iranian limit for aflatoxin M_1 in milk is 0.05 μg/kg, while in the USA, Serbia, India, and Brazil, it is 0.5 μg/kg. European Union maximum allowable limit for AFM_1 in human milk is 0.025 μg/kg. The maximum limit for

AFM_1 in cheese adopted by the European Union and Switzerland is 0.25 μg/kg. The maximum allowable limit for AFM_1 in butter adopted by Switzerland, Iran, and the Netherlands is 0.02 μg/kg (Ismail et al. 2016).

The maximum limits for aflatoxins range between 0 and 50 μg/kg. The European Union limit for total aflatoxins in food items is 4 μg/kg and is applicable in more than 29 countries including Germany, Italy, and France. The maximum limit for aflatoxins in food items as adopted by more than 17 countries including the USA, Mexico, and Brazil is 20 μg/kg. The maximum permissible limit for AFB_1 in more than 29 countries including Germany, the Netherlands, and France is 2 μg/kg and is the most widely acceptable limit. The second most widely adopted permissible limit for AFB_1 is 5 μg/kg and is adopted by more than 21 countries including Italy and Israel (Egmond and Jonker 2003).

Maximum limit of aflatoxins in animal feed samples is generally higher as compared to food samples. The maximum permissible limit for aflatoxins in animal feed samples adopted by most of the countries is 20 μg/kg, and European Union maximum permissible limit for aflatoxins in animal feed samples is 5 μg/kg, while in Korea it is 50 μg/kg (Khayoon et al. 2010).

Analytical Techniques

Foods being a complex of different nutrients and anti-nutritional factors require extraction of aflatoxins with the help of suitable solvents such as methanol, chloroform, etc. prior to their analysis. The most commonly employed methods for the detection and quantification of aflatoxins are the enzyme-linked immunosorbent assay (ELISA) and high-performance liquid chromatography (HPLC) with fluorescence detection or mass spectrophotometric method which has seen more increasing use recently, in particularly when multiple mycotoxin targets are analyzed simultaneously. The oldest technique for the detection of aflatoxins is thin-layer chromatography (TLC), but it is seldom used now. Depending on matrix types and aflatoxin concentration in the matrix, liquid-liquid extraction method or solid-phase extraction using C18 or imuno-affinity columns has been used for sample preparation in order to improve sensitivity. Less commonly employed methods for the detection of aflatoxins are mass spectrophotometric method, spore-based, and immune sensor-based enzyme assays.

A major hurdle in the detection of aflatoxins especially in developing countries is the cost of analysis. Both HPLC and ELISA techniques are costly and therefore are difficult to afford. TLC being a cheaper technology is used in a number of developing countries, but the researchers have to face the difficulties in getting the results published by using this technology. Moreover, the detection of aflatoxins through TLC suits best for qualitative analysis only; for quantitative analysis the technique is not much reliable. A more economical approach is to use TLC as initial sorter of positive samples, and then only the positive samples can be quantified by using HPLC or ELISA.

Prevalence of Aflatoxins in Different Plants

Aflatoxins are reported in a number of plant-based food commodities including cereals, spices, dry fruits, and plant-based animal feed items. The presence of aflatoxins in animal products such as milk, cheese, or butter also finds its roots in plants due to the fact that when dairy animals consume fungal-contaminated fodder, they secrete aflatoxins in their milk. Chances of aflatoxin contamination are more in tropical areas as compared to temperate climates due to environmental suitability for the production of aflatoxins by responsible fungus species. Aflatoxin contamination is more in stored food/fodder as compared to fresh commodities. Food and feed items from developing countries are reported more contaminated as compared to developed countries due to less implementation of rules and regulations, improper storage conditions, economic constraints, and lack of knowledge.

Prevalence of Aflatoxins in Cereals

Cereals are most vulnerable to mold attack, and therefore aflatoxins might be produced in the fields or after harvest, i.e., during storage or transportation. It has been estimated that more than 25% of the world's cereal production is contaminated by mycotoxins especially the aflatoxins. Reports on the prevalence of aflatoxins in different cereals from various countries of the world are presented in Table 1. In 9 studies from different countries, from a total of 3714 samples, 1246 samples (33.54%) were found positive for aflatoxins. Maize, rice, and barley samples from

Table 1 Global prevalence of aflatoxins in different types of cereals

Country	Product	Aflatoxin type	Positive sample/ total sample	Range/mean (μg/kg)	Reference
Zimbabwe	Maize	AFB_1	80/388	0.75–26.60/3.21[a]	Murashiki et al. (2018)
Vietnam	Maize	AFB_1	799/2370	1.0–34.80/13.10	Lee et al. (2017)
Pakistan	Rice	Total AFs	73/208	<0.04–32.20/6.36	Iqbal et al. (2016)
China	Rice	AFB_1	235/370	0.03–20/0.60[a]	Lai et al. (2015)
Turkey	Wheat flour	AFB_1	0/69	–	Kara et al. (2015)
	Maize flour	AFB_1	16/24	0.041–1.12/0.19	
Spain	Cereals	Total AFs	0/67	–	Vidal et al. (2013)
Algeria	Wheat	AFB_1	30/53	<0.05–37.42/…	Riba et al. (2010)
Ethiopia	Barley	AFB_1	13/115	0.00–12.30/8.70[a]	Ayalew et al. (2006)

[a]Mean of positive samples only; *AFs* aflatoxins; *AFB_1* aflatoxin B_1

Vitenam, Pakistan, and Ethiopia, respectively, were found to have maximum aflatoxin levels. Among the cereals maize and barley are the most contaminated crops by the aflatoxins, while relatively less aflatoxin levels are reported in wheat and rice samples. However, on the basis of frequency of consumption, most aflatoxin consumption by humans occurs through rice and wheat, and therefore the total intake of aflatoxins from these two crops would be higher than any other food commodities. Furthermore, a number of factors affect the contamination levels of aflatoxins in cereals such as storage time, processing conditions, season of the year, and geographical location Ismail et al. 2018; Kos et al. 2014).

Prevalence of Aflatoxins in Fruits

Fruits have a protective covering or peel that prevents the inside of the fruit from microbial attacks as well as from mycotoxins. However, the fruit wounds may become the site of microbial attacks and ultimately result in the site for production of aflatoxins. In general the edible portions of fresh fruits such as orange, apple, and mangoes are reported to have less aflatoxins. The chances of aflatoxin contamination are more in dry fruits due to the fact that in drying process, the fruits are sun dried openly in uncontrolled environment providing optimum conditions for the production of aflatoxins. The chances of aflatoxin contamination in dried fruits may be reduced if firm and ripened fruits are dried under controlled conditions. The production of aflatoxins depends on a number of factors. Aflatoxin production increases with an increase in total soluble solids; the unripen fruits contain less aflatoxins; while the over ripened contain the highest contamination levels, less aflatoxin production is reported if fruits are handpicked and solar dried as early as possible to a water activity level (a_w) below 0.80 and are also protected from moisture regain (Jackson and Al-Taher 2008).

The prevalence of aflatoxins in different fruits is reported in Table 2. From a total of 828 fruit samples, 277 samples (33.45%) were found positive for aflatoxins. Alarmingly higher levels of aflatoxins (<0.006–136,000 μg/kg) were reported from India and Morocco by Sharma et al. (2013) and Juan et al. (2008), respectively; while the rest of the countries showed aflatoxin contamination in the range of 0.045–110 μg/kg.

Prevalence of Aflatoxins in Spices

Spices are used as food additives since ancient times owing to their nutritional, coloring, flavoring, and preservative characteristics. The value of global production of spices is estimated to be more than US $ three billion/year. The steps involved in the processing of spices provide opportunities for the growth of fungus and ultimately

Table 2 Global prevalence of aflatoxins in different types of fruits

Country	Product	Aflatoxin type	Positive sample/ total sample	Range/mean (μg/kg)	Reference
Turkey	Roasted hazelnut	Total AFs	5/60	0.06–11.2/3.94[a]	Kabak (2016)
	Dried fig	Total AFs	16/130	0.06–28.2/3.80[a]	
Brazil	Cashew nuts	Total AFs	24/70	0.60–31.50/–	Milhome et al. (2014)
Greece	Dried vine fruits	AFB_1	6/26	0.045–0.60/0.15	Kollia et al. (2014)
Malawi	Nut-based foods	AFB_1	43/55	0.10–40.60/6.28	Matumba et al. (2014)
Saudi Arabia	Nuts	Total AFs	70/264	1.00–110/8.10	El tawila et al. (2013)
India	Nuts	Total AFs	27/58	0.5–136,000/1001	Sharma et al. (2013)
Pakistan	Dried apricot	Total AFs	4/20	0.50–10.80/4.55	Luttfullah and Hussain (2011)
	Dried fig	Total AFs	5/10	0.50–12.50/5.81	
Turkey	Pistachio	Total AFs	48/95	0.007–7.72/…	Set and Erkmen (2010)
Morocco	Walnut	AFB_1	6/20	<0.006–2500/360	Juan et al. (2008)
		Total AFs	6/20	<0.015–4320/730	
	Pistachio	AFB_1	9/20	<0.006–1430/158	
		Total AFs	9/20	<0.015–1450/163	

[a]Mean of positive samples only; *AFs* aflatoxins, *AFB_1* aflatoxin B_1

for the production of aflatoxins particularly in tropical and subtropical regions. Red chili is the second most consumed spice followed by black pepper and is also one of the food commodities which are most susceptible toward the production of aflatoxins. Aflatoxins might contaminate the red chili in fields, but the maximum production occurs during the drying and storage of chilies. Some other spices which are at high risk for the production of aflatoxins are nutmeg, turmeric, black pepper, and ginger. On the other hand, cinnamon, cloves, cumin, and *Mentha* are the spices which are reported to have fewer incidences of aflatoxin contamination indicating that these spices possess elevated antimicrobial activities (Kabak and Dobson 2017).

The prevalence of aflatoxins in different spices is presented in Table 3. From a total of 1372 samples of spices, 424 samples (30.9%) were found contaminated with aflatoxins. The range of aflatoxins in different spices was <0.02–79.71 μg/kg. Mean maximum aflatoxin concentration (15.50 μg/kg) was recorded for red pepper samples from Iran, while mean minimum concentration (0.45 μg/kg) was recorded for spices samples from China.

Table 3 Global prevalence of aflatoxins in different types of spices

Country	Product	Aflatoxin type	Positive sample/ total sample	Range/mean (μg/kg)	Reference
Nigeria	Ginger	Total AFs	66/120	0.11–9.52/0.54[a]	Lippolis et al. (2017)
		AFB_1	66/120	0.11–8.76/0.46[a]	
		AFB_2	44/120	0.13–1.01/0.09[a]	
Iran	Black pepper	Total AFs	5/40	<0.04–3.21/2.01	Barani et al. (2016)
	Red pepper	Total AFs	36/36	4.26–30.20/15.50	
Italy	Spices	AFB_1	20/130	0.59–5.38/0.31	Prelle et al. (2014)
China	Spices	Total AFs	53/480	<0.26–27.52/0.45	Zhao et al. (2013)
Turkey	Red chili	Total AFs	33/46	<0.04–37.38/4.27[a]	Ozbey and Kabak (2012)
	Black pepper		7/23	<0.04–0.46/0.24[a]	
Malaysia	Dried chili	Total AFs	52/80	<0.02–79.71/4.56	Jalili and Jinap (2012)
Spain	Paprika	Total AFs	38/64	<0.06–7.25/…	Santos et al. (2010)
	Chili		14/35	<0.06–2.49/…	
Brazil	Paprika	AFB_1	58/70	0.09–7.30/3.40[a]	Shundo et al. (2009)
Korea	Spices	Total Afs	12/88	0.08–4.46/…	Cho et al. (2008)
Ireland	Spices	AFB_1	20/130	<0.10–27.50/…	Riordan and Wilkinson (2008)
	Chili powder	Total AFs	10/30	<0.10–27.50/3.23	

[a]Mean of positive samples only; *AFs* aflatoxins; *AFB_1* aflatoxin B_1

Prevalence of Aflatoxins in Animal Fodder

Aflatoxin contamination in animal feed samples is reported from all around the globe. The most common causes of aflatoxin contamination in animal feed samples are lack of preventive measures before harvest, improper storage, and transportation facilities. During winter season fresh fodder is available; therefore, the chances of aflatoxin contamination above permissible limits are low, while in winter season stored fodder or commercial Vanda is used which might contain aflatoxins above permissible limits due to extensive storage time period and improper storage conditions. In developing countries the leftover bread samples are being sold to the street hawkers which after some day's collection sell these breads to the dairy farmers. By the time the bread reaches to the dairy farmers, it gets heavily contaminated, and therefore the chances of aflatoxin contamination are increased to several folds. The consumption of fungal-contaminated fodder by the dairy animals results in aflatoxin-contaminated milk; on the other hand, aflatoxins being lipophilic in nature are also stored in the liver and other fat tissues of animals which ultimately find their way to humans through food chain (Ismail et al. 2016).

Table 4 Global prevalence of aflatoxins in different types of animal fodder

Country	Product	Aflatoxin type	Positive sample/ total sample	Range/mean (μg/kg)	Reference
Pakistan	Vanda	AFB_1	32/50	<0.50–42.39/29.30	Chohan et al. (2016)
	Silage		5/23	<0.50–21.56/9.98	
	Cottonseed cake		17/25	<0.50–185.97/111.94	
Ethiopia	Dairy feed	AFB_1	156/156	7–419/97	Gizachew et al. (2016)
Italy	Soy feed	Total AFs	32/36	1–5.90/3.00	Gutleb et al. (2015)
Croatia	Dairy feed	AFB_1	73/325	1–304.6/8.41	Pleadin et al. (2015)
Spain	Barley	Total AFs	123/123	0.0002–0.75/0.14	Ibáñez-Vea et al. (2012)
Malaysia	Animal feed	Total AFs	8/42	<0.06–101.90/26.75[a]	Khayoon et al. (2010)
Tunisia	Sorghum	Total AFs	58/93	0.025–54.50/9.90	Ghali et al. (2009)
Kuwait	Animal feed	Total AFs	67/84	0.64–19.90/–	Dashti et al. (2009)

[a]Mean of positive samples only; *AFs* aflatoxins, *AFB_1* aflatoxin B_1

The prevalence of aflatoxins in different types of animal fodder is presented in Table 4. From a total of 957 samples of different types of animal feed, 571 samples (59.7%) were found contaminated with aflatoxins. The level of aflatoxins was found in the range of 0.002–419 μg/kg. Mean maximum aflatoxin level (111.94 μg/kg) was reported in cottonseed cake samples from Pakistan, while mean minimum level (0.14 μg/kg) was reported in barley samples from Spain.

Preventive Measures

Aflatoxins are not only limited with serious toxicological effects but are also directly linked with food security issues as the destruction of food through mold attack will ultimately bring food insecurity. Furthermore, aflatoxin prevalence above permissible limits affects global food trade and results in huge losses as a number of food consignments mostly coming from developing countries are rejected by the developed countries every year. Aflatoxin-producing mold species might contaminate the crops at each and every stage before their consumption, i.e., pre- and postharvest

contamination; therefore, preventive measures should be adopted right from the farms till the consumption of food. Aflatoxin control includes preventive measures to inhibit the growth of aflatoxin-producing fungal species and corrective/remedial actions to decontaminate the aflatoxins or to limit their production.

The preventive measures for aflatoxins are of two types, i.e., pre- and postharvest approaches. Aflatoxin-resistant crop varieties should be introduced particularly for the crops such as maize and peanuts which are more susceptible toward aflatoxin-producing molds. Aflatoxin-resistant germplasm should be identified by the biotechnologists; furthermore, conventional techniques of aflatoxin control such as crop rotation, timely sowing, and harvesting techniques are also necessary to minimize the chances of aflatoxin contamination. Aflasafe® (IITA) and Afla-guard® (Syngenta) are the recently introduced biocontrol systems which contain atoxigenic strains of aflatoxins; these biocontrol systems can control the production of aflatoxins above 90%. The postharvest management of aflatoxins includes storage at low temperature and at a_w below 0.80. Infected grains/other food items should be removed from the healthier food commodities through physical sorting or UV-/infrared-based sorters (Verheecke et al. 2016).

The most widely studied remedial technique against aflatoxins if already produced in food or feed items is the biological control. A number of strains of bacteria, yeast, and mold are reported to have potentials to degrade aflatoxins. A mixture of three different types of bacteria was reported to degrade 100% AFB_1 in pistachio nuts (Chen et al. 2015). More than 90% AFB_1 degradation was reported by Das et al. (2014) using two different strains of an edible mushroom (*P. ostreatus*), after an incubation at 30 °C for 15 days. Hackbart et al. (2014) also reported 100% AFB_1 degradation in potato dextrose agar medium at 30 °C after 5 days by using an *Ascomycota* (*Trichoderma reesei* QM941) and a *Zygomycota* (*Rhizopus oryzae* CCT7560).

Aflatoxin curative actions also include adsorption as well as physical and chemical reductions. A number of clay mineral mixtures are used for the binding of aflatoxins; the first commercially approved adsorbent for aflatoxins is Mycofix® (Biomin, Herzogenburg, Austria). Microbes are also used as aflatoxin adsorbent and therefore have the potential to reduce the bioaccessibility of aflatoxins. In one of our studies, we achieved 92% AFM_1 decontamination in artificially contaminated (spiked) milk samples at the level of 0.1 μg/L by using heat-killed cells of *Saccharomyces cerevisiae* (10^{10} cfu/mL). The possible mechanism behind the binding of aflatoxins with heat-killed cells is a non-covalent bonding of aflatoxins with microbial cell walls. However, this binding is weak as aflatoxins were by washing with phosphate saline buffer (PSB) solution, and therefore these mixtures are not used commercially up to now. Physical and chemical reduction methods for aflatoxins include ozonation, extrusion, nixtamalization, etc. These methods provide partial reductions in aflatoxin content and also do not fulfil the safety and economic parameters and therefore are not commercially employed up to now (Ismail et al. 2017; Udomkun et al. 2017).

Conclusions

Aflatoxins have become a serious threat for human life especially in the developing countries. Aflatoxins not only cause toxicity but are also producing global food insecurity. More than half of the world's population is exposed to the threat of aflatoxins. Aflatoxins are reported in many plant-based food commodities; however some plants are more susceptible than the others. Maize and barley in cereals, nuts in fruits, red chili in spices, and commercially prepared fodder of long storage life need special attention as they are more susceptible *to aflatoxins*. *Resistant crop varieties, proper crop management, controlled storage, and safe* transportation are necessary to limit the production of aflatoxins. To ensure aflatoxin-free plant foods, farmers and processors should be properly educated regarding the safe handling of food commodities. Although much research has been done in the field of aflatoxin degradation, further research to find safe and effective strategies for the degradation of aflatoxins is needed.

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Potential Roles for Endophytic Fungi in Biotechnological Processes: A Review

B. Shankar Naik

Introduction

Endophytic fungi are the microbes that grow intra- or intercellularly in the tissues of higher plants without causing symptoms on the plants in which they live and have proven to be rich source of bioactive natural products (Tan and Zou 2001). Almost every host plant studied so far is associated with some microorganism (Arnold et al. 2000; Shankar Naik et al. 2008). The host-endophyte symbiosis is a balanced antagonism between endophytic virulence and host defence response (Schulz and Boyle 2005). The endophyte gets benefit from the host plant by receiving organic nutrients, shelter, and guaranteed transmission to the next host generation and in turn provides host resistance to insects, drought, herbivore nematodes, and pathogens (Clay 1988; Redman et al. 2002). The endophyte-host association also plays an important role in structuring the plant communities by affecting the colonization, competition, co-existence, and soil nutrient dynamics (Lemons et al. 2005). Plants have several mechanisms to limit the growth of endophytes including producing a variety of toxic metabolites (Tanaka et al. 2002; Shankar Naik et al. 2006), but over a long period of co-evolution, endophytes have gradually formed a variety of tolerant mechanisms towards host metabolites by producing secondary metabolites, exoenzymes, and mycotoxins (Costa et al. 2000; Schulz et al. 2002). Several workers have reviewed that endophytes produce diverse secondary metabolites related to terpenes, flavonoids, alkaloids, quinines, cyclohexanes, and hydrocarbons; many of these compounds showed antimicrobial, antioxidant, antineoplastic, antileishmanial, and anti-proliferative activity and cytotoxicity (Shankar Naik et al. 2006; Wei et al. 2007; Zhou et al. 2009; Wang and Dai 2011) (Table 1). These secondary

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Table 1 Secondary metabolites produced from endophytic fungi

Metabolite	Endophytic fungi	Host plant	Activity	References
Colletotric acid	*Colletotrichum gloeosporioides*	*Artemisia mongolica* Fisch. ex Bess.	Antibacterial and antifungal (*Helminthosporium sativum*)	Zou et al. (2000)
Nodulisporic acids	*Nodulisporium* spp.	*Bontia daphnoides* L.	Anti-insecticidal	Bills et al. (2012)
Volatile antimicrobials	*Muscodor albus*	*Cinnamomum zeylanicum* Nees (cinnamon tree)	Antimicrobial	Strobel et al. (2001)
Naphthalene	*Muscodor vitigenus*	*Paullina paullinioides* (Liana)	Insect repellent against stem sawfly	Daisy et al. (2002)
Diplopyrone	*Diplodia mutila*	*Quercus suber* L. (cork oak)	Phytotoxic	Evidente et al. (2003)
Leucinostatin A	*Acremonium* spp.	*Taxus baccata* L.	Anti-oomycetes and anticancerous	Strobel et al. (1998)
Pestalotiopsis A and B	*Pestalotiopsis microspora*	*Taxus brevifolia* Nutt. (Pacific yew)	Anti-carcinogenic	Pulici et al. (1996)
Taxol	*Taxomyces andreanae*	*Taxus brevifolia* Nutt. (Pacific yew)	Anti-carcinogenic	Rodrigues (1996)
Taxol	*Tubercularia* spp.	*Taxus mairei* (Chinese southern yew)	Anticancerous (P388 cells, KB cells)	Wang et al. (2000)
Taxol	*Pestalotiopsis microspora*	*Taxus wallichiana* (Nepalese yew)	Anti-carcinogenic	Strobel et al. (1996)
Pestacin	*Pestalotiopsis microspora*	*Terminalia morobensis* Coode	Antioxidant, antifungal	Harper et al. (2003)
Cryptocin	*Cryptosporiopsis quercina*	*Tripterygium wilfordii* Hook. f.	Antifungal	Li et al. (2000)
Subglutinols A and B	*Fusarium Subglutinans*	*Tripterygium wilfordii* Hook. f.	Immunosuppressive	Lee et al. (1995)
Nodulisporic acids	*Hypoxylon pulicicidum* sp.		Biological control (insecticide)	Bills et al. (2012)
Gibberellins and indole acetic acid	*Paecilomyces formosus*	*Cucumis sativus* (cucumber)	Plant defence/plant growth	Khan et al. (2012)
Anthracenedione derivatives	*Guignardia* sp.	Mangrove plant	Potential anticancer activity	Zhang et al. (2010)

(continued)

Table 1 (continued)

Metabolite	Endophytic fungi	Host plant	Activity	References
Camptothecin Periconicin A and B Phomol	Colletotrichum sp.	*Artemisia annua*	Antifungal	Guo et al. (2008)
Pyrrolidine A and B	*Acremonium zeae*	*Zea mays*	Antifungal	Guo et al. (2008)
Sordaricin	*Xylaria* sp.	*Garcinia dulcis*	Antifungal	Pongcharoen et al. (2008)

metabolites find wide-ranging applications in medicine, agriculture, and industry (Gunatilaka 2006). The increasing levels of drug resistance by diverse plant and human pathogens forced microbiologists to find novel antimicrobial metabolites from microbial origin (Molina et al. 2012). In addition, they have the potential to decompose environmental pollutants and improve the soil micro-environment (Xiao et al. 2010; Wang and Dai 2011). A few recent studies revealed that endophytes affect litter decomposition rates (Purahong and Hyde 2011), stimulate soil carbon sequestration, and alter the flux of greenhouse gases (CO_2 and N_2O) from soil to the atmosphere. They play a role in leaf senescence and survive in decomposing leaf litter as saprophytes (Purahong and Hyde 2011). The endophyte-host symbioses alter the concentration of sugars and water and modulate their oxidation balance, phytohormone signalling, and other metabolic pathways. Endophytes produce various alkaloids such as lolines, ergot alkaloids, lolitrems, and peramines (Saikkonen et al. 2013). The use of endophytic fungi might be a novel approach and important source for degradation of toxic pollutants which include hydrocarbons, polychlorinated biphenyls (PCBs), polyaromatic hydrocarbons (PAHs), radionuclides, and metals. Russell et al. (2011) demonstrated the ability of endophytic fungal degradation of synthetic polymer polyester polyurethane (PUR) by production of serine hydrolases. The *Pestalotiopsis microspora* isolate was uniquely able to grow on PUR as the sole carbon source under both aerobic and anaerobic conditions. These non-saprobic fungi open a door to investigate on other fungal endophytes with biodegradable potential.

Endophytes as Producers of Novel Enzymes

Generally, fungal endophytes have the ability to utilize various organic compounds (carbon) which enables them in degradation of structural components such as glycose, oligosaccharides, cellulose, hemicelluloses, lignin, keratin, pectin, lipids, and proteins (Lumyong et al. 2002; Urairuj et al. 2003; Tomita 2003; Kudanga and Mwenje 2005) present in leaf litter and wood (Osono and Takeda 2001) by producing various extracellular enzymes which include pectinase, cellulase, lipase, proteinase, phenol oxidase, and lignin catabolic enzymes (Tan and Zou 2001; Bischoff

et al. 2009). These enzymes have potential in degradation of several macromolecule compounds into small molecules which could allow them to survive and reproduce despite plant defence mechanisms (Zikmundova et al. 2002). Lignocellulose is one of the most common biopolymers in nature and is composed mainly of cellulose, hemicelluloses, and lignin. Cellulose is a linear polymer of glucose units which can be hydrolysed by the action of endoglucanases, cellobiohydrolases, exoglucohydrolases, and β-glucosidases. Hemicellulose is a heterogeneous and branched polymer of pentoses, hexoses, and uronic acids. Complete enzymatic hydrolysis of xylan, the major polymer found in hemicelluloses, requires endo-β-1,4-xylanase, β-xylosidase, and several accessory enzymes, such as α-L-arabinofuranosidase, α-glucuronidase, α-galactosidase, acetylxylan esterase, and ferulic acid esterase. Degradation of lignocellulosic material has great importance for many industrial processes, and enzymatic hydrolysis has received attention due to its potential as an environmentally friendly process besides its enormous hydrolysis specificity (Chen et al. 2011) (Table 2).

Endophytic fungi also play an important role in degradation of plant debris. *Alternaria*, *Phoma*, and *Phomopsis* isolated from *Colophospermum mopane* (Jordaan et al. 2006; Wang and Dai 2011) exhibited lignocellulolytic activity. Wood-inhabiting fungal endophytes of Chilean tree species *Drimys winteri* and *Prumnopitys andina* were isolated and assayed for lignocellulolytic enzyme production and wood biodegradation. In *D. winteri*, an endophytic basidiomycete identified as *Bjerkandera* sp., and a deuteromycete classified as sterile fungi, whilst in *P. andina,* an unidentified basidiomycete, and also a sterile fungi able to develop a non-selective white-rot wood decay (Oses et al. 2006). Cellulose hydrolysis is achieved by endoglucanases and cellobiohydrolases, collectively termed cellulases. Hydrolysis of hemicellulose, a mixed polymer, occurs via the action of xylanases, mannanases, and other hydrolytic enzymes with broad substrate specificity. Cellulases are a complex enzyme system, comprising endo-1,4-b-D-glucanase, exo-1,4-b-glucanase, and D-glucosidase. These enzymes, together with other related enzymes, viz. hemicellulases and pectinases, are among the most important group of enzymes that are employed in the processing of lignocellulosic materials for the production of feed, fuel, and chemical feed stocks (Pandey et al. 1999). Cellulases and xylanases however find applications in several other areas, like in textile industry for fibre treatment and in retting process (Pandey et al. 1999). Cellulases can be used in the textile industry for bio-stoning and bio-finishing of cellulosic fibres, and hemicellulases can be applied for bio-bleaching of kraft pulps. Furthermore, in the food industry, cellulases and hemicellulases can be used for extraction and clarification of fruit and vegetable juices, and in the animal feed industry, these enzymes can promote an increase in the nutritive quality of feed and also in production of lignocellulosic ethanol (Pandey et al. 1999). Two species of *Acremonium* were able to produce cellulases and hemicellulases in submerged culture (SC) and in solid-state fermentation (SSF), using different carbon source (Almeida et al. 2011).

Xylanases have potential application in food, feed, paper, pulp, and textile industries (Pandey et al. 1999). These enzymes degrade plant fibres made of xylan hemicellulose producing xylose monomers. One of the most important xylanase

Table 2 Enzymes produced from endophytic fungi from different host plants

Endophytic fungi	Source plant	Enzymes	References
Colletotrichum sp.	*Cinnamomum iners*, *Camellia sinensis*	Cellulase, mannanase, protease, xylanase	Moy et al. (2002)
Phomopsis sp.	*Garcinia cowa* *Trichilia connaroides* *Cinnamomum iners*	Cellulase, mannanase, xylanase	Moy et al. (2002)
Colletotrichum sp. AHU9748	*Abelmoschus esculentus*	B-Galactosidase, rhamnogalacturonan lyase, acetylesterase	Grünig et al. (2008)
Acephala applanata	*Conifer roots*	Amylases, laccases, proteinases	Reddy et al. (1996)
Acremonium zeae	*Zea mays*	Xylanase	Bischoff et al. (2009)
Acremonium sp.	*Acrostichum aureum*	Amylase, cellulase, lipase	Maria et al. (2005)
Acremonium typhinum	*Poa ampla*	Proteinase	Sieber et al. (1991)
Acremonium terrícola *Cladosporium cladosporioides* *Fusarium lateritium* *Nigrospora sphaerica* *Penicillium aurantiogriseum* *Pestalotiopsis guepinii* *Xylaria* sp. *1*	*Opuntia ficus-indica* Mill. *(Cactaceae)*	Cellulase, protease, xylanase	Bezerra et al. (2012)
Alternaria chlamydospora	*Acanthus ilicifolius*	Cellulase, lipase, protease	Maria et al. (2005)
Alternaria sp. *Fusarium* sp.	*Acrostichum aureum*	Amylase, cellulase, lipase, protease	Maria et al. (2005)
Bjerkandera sp.	*Drimys winteri*	Cellulase, phenoloxidase	Oses et al. (2006)
Cladosporium sphaerospermum *Phoma tropica* *Phomopsis archeri* *Tetraploa aristata* *Xylaria* sp. *2*	*Opuntia ficus-indica* Mill. *(Cactaceae)*	Protease, xylanase	Bezerra et al. (2012)
Colletotrichum musae	*Musa cavendish*	*Acid phosphatase*	Bryant et al. (2007)
Cylindrocephalum sp.	*Alpinia calcarata* (Haw.) Roscoe	*Amylase*	Sunitha et al. (2012)
Discosia sp.	*Calophyllum inophyllum*	*Amylase*	Hegde et al. (2011)
Melanconium apiocarpum	*Alnus viridis*	*Laccase, amylase, cellulase*	Guo et al. (2008)

(continued)

Table 2 (continued)

Endophytic fungi	Source plant	Enzymes	References
Monodictys castaneae	*Opuntia ficus-indica* Mill. *(Cactaceae)*	*Xylanase*	Bezerra et al. (2012)
Monotospora sp.	*Cynodon dactylon*	*Laccase*	Weihua and Hongzhang (2008)
Mortierella hyalina	*Osbeckia stellata*	*Cellulase, lipase, protease, xylanase*	Bhagobaty and Joshi (2012)
Mycelia sterilia YY-*5*	*Rhus chinensis* Mill.	*Laccase*	Lumyong et al. (2002)
Neotyphodium lolii Epichloe festucae	*Poa ampla*	*þ − 1,6-glucanase*	Wang et al. (2006)
Neotyphodium sp.	*Poa ampla*	*Chitinase*	Saranpuetti et al. (2006)
Paecilomyces variabilis	*Osbeckia chinensis*	*Amylase, lipase, protease, xylanase*	Bhagobaty and Joshi (2012)
Penicillium sp.	*Camellia caduca Schima khasiana*	*Cellulase, lipase, protease, xylanase*	Bhagobaty and Joshi (2012)
Penicillium sp.	*Centella asiatica*	*Cellulase*	Devi et al. (2012)
Pestalotiopsis sp.	*Acanthus ilicifolius*	*Amylase, cellulase, lipase, protease*	Maria et al. (2005)
Pestalotiopsis sp.	*Manglietia garrettii*	*Cellulase, mannanase*	Moy et al. (2002)
Phoma sp.	*Garcinia cowa*	*Cellulase, mannanase, protease*	Moy et al. (2002)
Preussia minima Alternaria sp.	*Eremophila longifolia*	*Amylase*	Zhang et al. (2010)
Talaromyces flavus	*Potentilla fulgens*	*Lipase, protease, xylanase*	Bhagobaty and Joshi (2012)

applications is the pretreatment of pulps, prior to bleaching in pulp and paper industries and jute fibre upgradation (Beg et al. 2001). These enzymes release lignin fragments by hydrolyzing residual xylan, and the pretreatment with xylanase reduces the usage of chlorine as the bleaching agent. Xylanases are also used for bread-making and beer production. In recent years, lignin-degrading enzymes have been extensively studied because of their potential biotechnological applications in various industrial sectors. These applications include biotransformation of lignocellulosic biomass to feeds, fuels, and chemicals, bio-pulping, bio-bleaching of paper pulps, decolorizing and detoxifying kraft bleach effluents, degradation of highly toxic environmental chemicals, biosensor, cosmetics, food, and others (Maciel et al. 2010). Ligninolytic enzyme is a big family of various isoforms of extracellular enzyme, of which lignin peroxidase, manganese-dependent peroxidase, and laccase are the three major classes. These enzymes are directly involved not only in the degradation of lignin in their natural lignocellulosic substrates but also in the degradation of various xenobiotic compounds such as polycyclic aromatic hydrocarbons

(PAHs) (Pointing 2001). White-rot fungi are regarded as the most efficient producers which secrete ligninolytic enzymes in nature. An endophytic *Pestalotiopsis* sp. is able to produce laccase under submerged fermentation (SF) and solid-state fermentation (SSF) with various lignocellulosic by-products as substrates (Chen et al. 2011). Laccases (benzenediol, oxygen oxidoreductases) are glycosylated polyphenol oxidases which degrade lignin efficiently (Kunamneni et al. 2008). These enzymes have important applications in pulp and paper industry, animal biotechnology, biotransformation, and detoxification of phenolic pollutants (Kunamneni et al. 2008).

Proteases [serine protease, cysteine (thiol) protease, aspartic protease, and metalloprotease] are the most important class of enzymes that catalyse total hydrolysis of protein and have been studied extensively since the advent of enzymology (Oliviera et al. 2010). They find application in a number of biotechnological processes, viz. in food processing and pharmaceuticals, leather industry, and detergent industry (Joo et al. 2003). The amylase family of enzymes has been well characterized through the study of various microorganisms. Amylases break down starch to give a variety of products including glucose, maltose, and dextrin which can be used for the synthesis of a number of industrially important compounds such as citric acid, ethanol, glutamic acid, lactic acid, lysine, and sorbitol (Maarel et al. 2002). These enzymes have found applications in the processed food industry, fermentation technology, and textile and paper industries (Pandey et al. 1999). A root endophytic fungus *Piriformospora indica* was able to show amylase activity on soluble starch (Kumar et al. 2012).

Lipases (triacylglycerol hydrolase) are hydrolytic enzymes capable of cleaving the ester bond of triacylglycerol and catalyse ester synthesis in vitro by shifting equilibrium of reaction (Contesini et al. 2010; Fernandes et al. 2007; Li and Zong 2010; Mohamed et al. 2011). These enzymes can be obtained from several organisms; however, microorganisms are most promising for this purpose (Fernandes et al. 2007; Salihu et al. 2012). Lipases have a wide array of industrial applications in the production and processing of detergents, oils, fats, and dairy products. In addition, they are also used in the preparation of therapeutic agents (Gog et al. 2012). Within the broad application field of lipases, synthesis of biodiesel is attracting great interest (Hasan et al. 2006). Among the microorganisms, yeasts have been used widely for the production of these enzymes, with special emphasis on the genus *Candida* sp. (Sharma et al. 2001; Salihu et al. 2011). It was shown that partially purified enzyme containing lipolytic activity, produced by submerged fermentation in a lower-cost cultivation medium by endophytic yeast *Candida guilliermondii*, can be used as a catalyst for the production of methyl oleate using methanol as substrate (Oliveira et al. 2014).

Chitin, a linear homopolymer of β-1,4-linked N-acetylglucosamine, is a constituent of the exoskeleton of insects and shells of crustaceans and forms the basic structural component of the fungal cell wall. Enzymes that degrade this insoluble polymer are chitinolytic enzymes or chitinases. Fungal chitinases play a major role in the ecosystem by degrading and cycling carbon and nitrogen from chitin (Kellner and Vandenbol 2010). Chitinases of fungi are also being studied for their potential

in biocontrol of phytophagous nematodes and plant pathogenic fungi (Gan et al. 2007). Plants also produce chitinases as a defence response to infection by pathogens (Regalado et al. 2000). Chitinases have many desirable properties and find use in control of microbes and tumours, wound healing, wastewater treatment, and drug delivery (Aoyagi et al. 2007; Hamman 2010). Chitinases are produced by many fungi including those associated with plants such as mycorrhiza and pathogens, bacteria, and endophytes (Rajulu et al. 2011).

Volatile Hydrocarbons from Endophytic Fungi

Many endophytic fungi are known to produce a wide spectrum of volatile organic compounds with potential energy applications which have been described as mycodiesel (Strobel 2014a, b). Many of these mycodiesel hydrocarbons are terpenes (pinene and bisabolene) which are actively being investigated as potential "drop-in" biofuels for replacing diesel and aviation due to their high energy densities (Wu et al. 2016). The genus *Muscodor* especially *M. albus* has evoked the general interest for searching volatile organic compounds from endophytes around the world (Sharma et al. 2001). Further, *Gliocladium roseum* (now *Ascocoryne sarcoides*) was isolated from *Eucryphia cordifolia* able to produce class I alkanes that are found in all diesel fuels (Tomsheck et al. 2010; Strobel et al. 2008). Endophytic *Gliocladium roseum* grown on cellulose substrate and a number of isolates belonged to *Nodulisporium* sp. produce a plethora of volatiles having fuel potential (Ahamed and Ahring 2011; Mends et al. 2012; Hassan et al. 2013). These include *Daldinia* spp., *Hypoxylon* spp., and *Annulohypoxylon* spp. (Hassan et al. 2013) and produce 1, 8 cineole along with ketones and hydrocarbons such as terpenoids, cyclohexanes, benzene derivatives, esters, ketones, and straight-chained ones (Tomsheck et al. 2010, Mends et al. 2012, Strobel 2014a, b). Similarly, an endophytic *Phomopsis* sp. growing in rain forests of South America (Singh et al. 2011) and *Phoma* sp. isolated from the roots of *Larrea tridentata* were found to produce several hydrocarbons with fuel potential (Strobel et al. 2011).

In ecosystems, plants compensate for their immobility by releasing volatile substances into the atmosphere and from roots into the soil constituting about 1% of secondary plant metabolites. These volatile substances promote plant communication and interaction with the surrounding environment (Schalchli et al. 2016). Volatile compounds are typically lipophilic liquids with high vapour pressures. These are lethal to a wide variety of plant and human pathogenic fungi and bacteria and are also effective against nematodes and certain insects (Strobel 2003). *M. albus* grown in richer media produce volatile compounds with higher inhibitory and killing effect capacity of the test organisms. 1-Butanol, 3-methyl-, acetate was the most biologically active, reducing growth of *Pythium ultimum*, *Rhizoctonia solani*, *Tapesia yallundae*, *Xylaria* sp., *Sclerotinia sclerotiorum*, *Cercospora beticola*, and *Fusarium solani* (Strobel et al. 2001). The fungal volatiles are known to stimulate or enhance soilborne biocontrol agents (Wheatley 2002). Fungal pathogens

Fusarium oxysporum and *S. sclerotiorum* are inhibited by the volatiles produced by the endophytic fungi *Colletotrichum truncatum* isolated from oilseed crop *Jatropha curcas* (Kumar and Kaushik 2013). The volatiles of *M. albus* are useful for the control of postharvest plant diseases, known as "mycofumigation" (Stinson et al. 2003). Some endophytic fungal volatiles effectively inhibit or kill the most common postharvest fruit pathogens (Park et al. 2010). The volatiles produced by endophytic fungi have been used to replace methyl bromide (MeBr), a traditional soil fumigant that is now being banned in much of the world because it depletes the ozone layer. In addition, the VHCs of *M. crispans* killed several human pathogens, including *Yersinia pestis*, *Mycobacterium tuberculosis*, and *Staphylococcus aureus* (Mitchell et al. 2010). Another endophyte, *M. fengyangensis*, killed the pathogen *Escherichia coli* (Zhang et al. 2010). Fumigation with VHCs produced by *M. albus* caused mortality of codling moth adults and neonate larvae (Strobel et al. 2010; Schalchli et al. 2016).

Biotransformation Mediated Through Endophytic Fungi

Biotransformation can be defined as the use of biological systems to produce chemical changes on compounds which are not easily prepared by the chemical methods (Borges et al. 2009b, b). In this process a molecule can be modified by transforming functional groups with or without degradation of carbon skeleton. Such modification results in the formation of novel and useful products (Borges et al. 2009b; Pimentel et al. 2011). Moreover, biotransformation process allows the production of enantiomerically pure compounds by region and stereoselectivity, thus eliminating the need for complicated separation and purification steps (Borges et al. 2008). Further, it can be carried out under mild conditions like ambient temperature and without the need of high pressure and extreme conditions. Besides, the reaction occurs under ecologically acceptable conditions with lower emissions of industrial residues and production of biodegradable residues and products, thus reducing the environmental problems (Pimentel et al. 2011). For this reason biotechnology using microbial cultures and/or their enzymatic systems alone has received increasing attention as a method for the conversion of lipids, monoterpenes, diterpenes, steroids, triterpenes, alkaloids, and lignans to produce novel bioactive molecules with potential for pharmaceutical and food industries (Bianchini et al. 2015). Microorganisms are able to catalyse a broad spectrum of chemical reactions. They also exhibit high substrate tolerance by accepting a large variety of compounds. Fungi have been used extensively for biotransformation studies (Borges et al. 2009a). Biotransformation can be effectively used in detoxification of toxic substances. The biotransformation of the phytoanticipins by four endophytic fungi isolated from *Aphelandra tetragona* was reported. Endophytic *Fusarium sambucinum* detoxified 2-benzoxazolinone (BOA) and 2-hydroxy-1,4-benzoxazin-3-one (HBOA) to N-(2-hydroxyphenyl)malonamic acid. Other endophytes such as *Plectosporium tabacinum, Gliocladium cibotii*, and *Chaetosphaeria* sp. transformed HBOA to 2-hydroxy-N-(2-hydroxyphenyl)

acetamide, N-(2-hydroxyphenyl)acetamide, N-(2-hydroxy-5-nitrophenyl)acetamide, N-(2-hydroxy-3-nitrophenyl)acetamide, 2-amino-3H-phenoxazin-3-one, 2-acetylamino-3H-phenoxazin-3-one, and 2-(N-hydroxy)acetylamino-3H-phenoxazin-3-one (Zikmundova et al. 2002). Molina et al. (2012) evaluated antimicrobial and biotransformation of terpenes by endophytic fungi isolate from *Dipteryx alata* Vog. Three fungal strains bioconverted α-pinene into verbenol compound with great industrial interest. Endophytic *Fusarium verticillioides* isolated from maize (*Zea mays* L.) detoxified 2-benzoxazolinone into 2-aminophenol and 2-acetamidophenol (Glenn et al. 2003). Gonda et al. (2016) studied decomposition of diclofenac, diflunisal, ibuprofen, mefenamic acid, and piroxicam using nine identified strains of endophytic and epiphytic fungi (from *Ascomycota*) isolated from a medicinal plant, *Plantago lanceolata* leaves. Endophytic *Aspergillus nidulans* and *Bipolaris tetramera* effectively decreased the concentration of NSAIDS in model solutions. The stereo- and regioselective synthesis of target compounds is one of the most important subjects in synthetic organic chemistry. The biotransformation of exogenous substances has been widely used and studied for the synthesis of chiral compounds (Hamada et al. 2003). Endophytic fungi have been employed to change the three-dimensional conformation of compounds because of their effective biotransformation enzymes. Borges et al. (2007) reported stereoselective biotransformation of thioridazine yielding major human metabolites thioridazine-2-sulfoxide and thioridazine-5-sulfoxide from *Asteraceae* plant hosts.

The microbial stereoselective biotransformation of flavans has been achieved with endophytic *Diaporthe* sp. isolated from *Camellia sinensis*. The endophytic fungus stereoselectively oxidized the C-4 position of (+)-catechin and (−)-epicatechin to give the corresponding 3,4-cis-dihydroxyflavan derivatives and oxidized (−)-epicatechin-3-O-gallate and (−)-epigallocatechin-3-O-gallate into 3,4-dihydroxyflavan derivatives. It was found that this microorganism promotes the 2R substitution in the same direction as the configuration of 3-hydroxyl function (Augusta et al. 2005).

Efforts have been made to study bio mimetic systems has been to demonstrate parallels between the phase I reactions of drugs and other xenobiotics in mammalian and microbial systems. This kind of study could be accomplished by using either isolated enzyme systems or whole intact organisms (Pupo et al. 2008). Some biocatalysts can accomplish reactions at sites that are difficult to access by organic synthesis, e.g. the selective functionalization of nonactivated positions in organic molecules such as the hydroxylation of aliphatic chains.

Propranolol (Prop) is a non-cardioselective β-adrenergic blocking agent that is widely used in the treatment of cardiovascular diseases. One fungus, *Glomerella cingulata*, isolated from *V. arenaria* can transform Prop into a more potent derivative (Borges et al. 2009b, b). In a similar study, endophytic *Phomopsis* sp., *Glomerella cingulata*, *Penicillium crustosum*, *Chaetomium globosum*, and *Aspergillus fumigatus* were able to biotransform propranolol (Prop) (Borges and Bonato 2011). Aphelandrine, a macrocyclic polyamine alkaloid found in the roots of different species of the genus *Aphelandra* (Acanthaceae), was metabo-

lized by several endophytes isolated from the roots of *A. tetragona* (Werner et al. 1997). *Fusarium moniliforme*, one of the most common endophytic fungi associated with corn (*Zea mays* L.), was able to metabolize 6-methoxy-benzoxazolinone and 2-benzoxazolinone (Benzoxazinone, a class of phytoanticipins that occurs in the Gramineae, Acanthaceae, Ranunculaceae, and Scrophulariaceae families) into N-(2-hydroxy-4-methoxyphenyl) and N-(2- hydroxyphenyl)malonamic acids, respectively (Yue et al. 1998; Borges et al. 2009b). An endophytic fungal strain was able to produce lepidimoide, an allelopathic substance, by using polysaccharides from *Abelmoschus esculentum* Moench (okra), which were added to the culture medium as a carbon source (Tanaka et al. 2002). Antimalarial alkaloids quinine, quinidine, cinchonidine, and cinchonine were biotransformed into their 1-N-oxide derivatives by endophytic *Xylaria* sp. isolated from *Cinchona pubescens* (Shibuya et al. 2003). Verza et al. (2009) reported the biotransformation of the tetrahydrofuran lignan, (−)-grandisin, by the endophytic fungus *Phomopsis* sp., obtained from *Viguiera arenaria*, led to the formation of a new compound which showed a trypanocidal activity similar to the natural precursor. Betulinic acid and betulonic acid are natural triterpenes found in many plants that exhibit important biological properties, e.g. antineuroblastoma and antiviral activity. The endophytic fungi *Arthrobotrys*, *Chaetophoma*, and *Colletotrichum dematium* showed mild and selective oxidation reactions that could convert betulinic acid to many oxygenated derivatives (Bastos and Magan 2007). The endophytic fungus *Coelomycetes* AFKR-3 isolated from young stems of yellow moonshed plant (*Arcangelisia flava* (L.) Merr.) has shown the capability to biotransform berberine (antimicrobial compound) into its 7-*N*-oxide derivative. This fungus can also biotransform the protoberberine alkaloid palmatine into a new derivative palmatine 7-*N*-oxide in liquid medium of glucose-yeast extract-peptone (Agustaa et al. 2014). Endophytic fungus *Umbelopsis isabellina* isolated from medicinal plant *Huperzia serrata* was found to transform ursolic acid, a pentacyclic triterpene, into 3b-hydroxy-urs-11-en-28,13-lactone, 3b,7b-dihydroxy-urs-11-en-28,13-lactone, and 1b,3b-dihydroxy-urs-11-en-28,13-lactone (Fu et al. 2011). The fungus *Aspergillus flavus* isolated as endophytic of the plant *Paspalum maritimum* Trin. was evaluated for its potential application in biotransformation reactions. The compounds chalcone, 3,4,5-trimethoxychalcone, and 2,3,4,4-tetramethoxychalcone were biotransformed, respectively, in dihydrochalcone (4), 3,4,5-trimethoxydihydrochalcone, and 2,3,4,4-tetramethoxydihydrocha lcone (Corrêa et al. 2014). The biotransformation of the major saponins in *Panax notoginseng*, including the ginsenosides by endophytic *Fusarium*, *Nodulisporium*, *Brevundimonas*, and *Bacillus*, was reported (Luo et al. 2013). Polyphyllin VII (PPL7) was biotransformed by endophytes from the medicinal plant *Paris polyphylla* Smith, var. yunnanensis. This produced a new compound, ZH-2, with pharmacological activity in vitro and in vivo. ZH-2 was more potent than PPL7 in selectively killing more chemoresistant than chemosensitive breast cancer cells (He et al. 2016).

Conclusion

Endophytic fungi have proved to be sources of economically and therapeutically important enzymes; however, endophytes have only recently been prospected for enzymes. New and more extensive studies using the well-known methods of fungal cultivation to optimize the production of enzymes are necessary. Recently, endophytes have gained attention and usefulness in biotransformation processes. Fungi have catalysed chemical transformations in a broad range of substrates yielding novel chemical entities that could be used as leads for drug design. Fungi have also been used as reliable models to study drug metabolism, an essential step in the drug development process. Modern, genetic, and genomic methods have led to a more comprehensive elucidation of some biochemical pathways for secondary fungal metabolite production and their functional diversity. The fungal genomic era has shown great and unexpected fungal ability for the biosynthesis of natural products. Genomic fungal sequences have revealed important information about novel gene clusters; however, collaborative work among chemists, mycologists, and geneticists is essential for better correlating the genomic information to the secondary metabolites and their functions.

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Vitamin E

Umaiyal Munusamy and Siti Nor Akmar Abdullah

Abbreviations

DMPBQ	2-methyl-6-phytylbenzoquinol
DMGGBQ	2, 3-dimethyl-5-geranylgeranyl benzoquinol
GGDP Reductase	geranylgeranyl diphosphate reductase
GGDP	Geranylgeranyl diphosphate
HGA	Homogentisate
HGGT	Homogentisate geranylgeranyl transferase
HPP	p-hydroxyphenylpyruvate
HPPD	p-hydroxyphenolpyruvate dioxygenase
HPT	Homogentisate phytyltransferase
MGGBQ	2-Methyl-6-geranylgeranylbenzoquinol
PDP	Phytyl diphosphate
PQ-9	Plastoquinol-9
PrBQMT	2-methyl-6-prenylbenzoquinol methyltransferase
TC	Tocopherol cyclase
γ-TMT	Gamma-tocopherol methyltransferase

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Introduction

Plants produce a wide range of metabolites that are beneficial to human as food, medicines and industrial raw materials (Dudareva and Pichersky 2000; Oksman-Caldentey and Saito 2005). Plant-derived vitamins are of great interest because of their impact on human health (Asensi-Fabado and Munne-Bosch 2010). α-tocopherol and α-tocotrienol (component of vitamin E and chemically known as tocochromanols) (Horvath et al. 2006) are produced by two metabolic pathways, the shikimate and non-mevalonate pathways (Collakova and DellaPenna 2001). In plants, vitamin E plays various roles in plant growth and development (Horvath et al. 2006), while, in human and animals, vitamin E can protect membranes from photooxidation involving several signal transduction pathways (Ren et al. 2011). In addition, α-tocotrienol is considered as a promising anticancer agent due to its potent effects against a wide range of cancers, as a cholesterol-reducing agent and as a better neuroprotective agent compared to α-tocopherol (Ling et al. 2012; Viola et al. 2011). Given the important functions of α-tocopherol and α-tocotrienol in plants, humans and animals, in addition to its accumulation in plants, studies on its biosynthetic pathways in plants have been actively pursued. These studies resulted in the identification of the different enzymes involved in the production of α-tocochromanols (Fujita et al. 2009; Abbasi et al. 2007).

Vitamin E

Discovery of Vitamin E

In 1922, Evans and Bishop had discovered vitamin E when a substance from lettuce and alfalfa leaves was believed to maintain fertility in rats (Duracevic et al. 2010). In 1924, the term vitamin E for the unknown fertility factor was coined by Sure in analogy to the previously identified vitamins A, B, C and D (Patel et al. 2011). In 1936, according to Evans due to its role in fertility, this compound was named tocopherol from the Greek word “tocos” meaning birth and “phorein” meaning to bear (Gray 1996). While in 1937, Emerson had discovered four different forms, α, β, γ and δ-tocopherol in wheat germ oil (Tan 2005). In 1938, the α-tocopherol structure was elucidated by Fernholz, and only in 1963, Schudel elucidated the structure for α-tocotrienol. The identification of the chromanol ring with vitamin E activity leads to the designation of the term tocochromanols, a group of substances encompassing tocopherols and tocotrienols (Yoshida et al. 2007). The α-tocopherol had become the most important family of the vitamin E (Setiadi et al. 2003) and as a universal constituent of all higher plants (Horvath et al. 2006).

Availability of Vitamin E

The content of the tocochromanols differs between tissues (Crowell et al. 2008), species, subspecies, varieties or even between different cultivation techniques (geographic, climate, season, feeding) (Kruk et al. 2008; Sundl et al. 2007; Ryynanen et al. 2004). Most plant-derived foods especially fruits and vegetables contain low to moderate levels of vitamin E (Adzim Khalili et al. 2010). Due to the abundance of plant-derived foods in our diets, a significant and consistent source of vitamin E can be obtained (Chen et al. 2006). α-tocopherol exists in all plant species, green algae and cyanobacteria (Dormann 2007). The tocopherol levels vary in plants during the different development stages (Sen et al. 2007) and in response to a variety of abiotic stress conditions (Collakova and DellaPenna 2003b) such as light (Kruk et al. 2008), temperature, drought and salt stress (Collakova and DellaPenna 2003a). In addition, variations in the level of tocochromanols obtained can occur due to differences in processing procedure, storage time and sample preparation (Chen et al. 2006; Chun et al. 2006).

Leave Tissues

The most abundant form of tocochromanols found in the leaves is α-tocopherol, but α-tocotrienol is not detected in this tissue (Voll and Abbasi 2007). In addition, α-tocopherol can be abundantly found in mature leaves where the level of photosynthesis is higher (Kruk et al. 2008; Abbasi et al. 2007). It is also found in the thylakoid membranes of chloroplasts to protect the photosynthetic apparatus against oxidative stress and damages by protecting polyunsaturated fatty acids from lipid peroxidation (Chen and Bergman 2005; Koch et al. 2002).

Grains

A significant amount of tocochromanols are predominantly found in seeds (Zingg 2007). Cereal grains such as wheat, rice and barley contain both tocopherols and tocotrienols (Chen and Bergman 2005; Gopala Krishna et al. 1997). However, cereals such as oat contain more tocotrienols than tocopherols (Ryynanen et al. 2004). γ-Tocopherol is found mainly in plant seeds (Dormann 2007). Raw cashew nuts contain 0.29 and 1.10 mg/100 g of dry matter of α-tocopherols and γ-tocopherols, respectively (Gomez-Caravaca et al. 2010). Another study showed that γ-tocopherol is predominant in dicot seeds and usually contributes to more than 90% of the tocochromanols pool, while tocotrienols prevail in cereal endosperm (Rippert et al. 2004).

Fruits

Tocochromanols level changed during fruit ripening stage (Sakouhi et al. 2008; Abushita et al. 1997). The α-tocopherol contents in the fruit pericarp of the yellow and red pepper varieties of different origins showed different levels of α-tocopherol. It shows that red pepper fruits contained noticeably higher amount of tocopherol than the yellow pepper fruits (Koch et al. 2002). In oil palm fruits, tocotrienol can be found in mesocarp tissues (Nurniwalis et al. 2008) and also from kernel (Wan Omar et al. 2008).

Oils

In plant-derived oils, the oil is the major source of vitamin E (Corley 2009; Lau et al. 2008; Rodriguez Posada et al. 2007). Palm oil is a major commercial source of tocopherols and tocotrienols (Hunter and Cahoon 2007), but tocotrienol is the major component in palm oil (Sen et al. 2007). Olive oil is highly appreciated for its good taste, as well as for its nutritional properties which mainly related to α-tocopherol (Sakouhi et al. 2008). Silva et al. (2001) have demonstrated the presence of tocotrienols in *Iryanthera*. Raw cashew nut oil contains β-tocopherol (132.98 mg/100 g) and δ-tocopherol at much lower concentration (0.63 mg/100 g) (Gomez-Caravaca et al. 2010). Corn oil contains mainly γ-tocopherol, and soybean oil contains a high amount of δ-tocopherol (Zingg 2007). Tocopherol content in oilseed rape or canola ranges between 180 and 370 mg/kg (Endrigkeit et al. 2009). Salad oil contains tocopherols, provides added nutritional value and stabilises the fatty acid present in the oil (Sivakumar et al. 2005; Savidge et al. 2002; Bonnie and Choo 2000).

Potential Role of Vitamin E

The antioxidant activity varies among tocochromanols α-tocopherol > γ-tocopherol > δ-tocopherol > β-tocopherol (Kinen et al. 2000). They are also useful in nonbiological systems such as foods, cosmetics and pharmaceuticals (Oh et al. 2009; Panahi et al. 2003). Recent studies showed that tocotrienol has higher antioxidant potential than tocopherol (Bardhan et al. 2011). They usually can be found in soybean oil and palm oil (Hunter and Cahoon 2007). The γ and δ forms of tocopherols and especially tocotrienols confer the greatest degree of oxidative stability in vegetable oils that are exposed to prolonged high temperatures. This characteristic is particularly important for the performance of vegetable oils in food processing and bio-based lubricants (Yang et al. 2011).

Plant

Tocopherols are usually found in the membranes because of their lipophilic characters. The primary task of vitamin E in plant is as an antioxidant agent (Traber and Atkinson 2007) which protects membranes by preventing free radical damage (Dutta and Singh 2011), against photoinhibition (Fryer 1992), and helps in providing an optimal environment for photosynthesis (Sen et al. 2007). The main function of α-tocopherol is to react rapidly in a non-enzymic manner to scavenge the lipid peroxyl radicals before they are able to react with the lipid substrate (Strzalka et al. 2009; Ching and Mohamed 2001). α-tocopherol is most efficient at providing protection against peroxyl radicals in a membrane environment (Rippert et al. 2004). It also prevents lipid peroxidation during seed dormancy, germination and in early seedling (Horvath et al. 2006).

Human

Oxidation that damages the cell components is the starting point in several human diseases (Cha-Sook et al. 2005; Silva et al. 2001). α-tocopherol has been reported to possess non-antioxidant function which helps in protection against diseases. α-Tocopherol has an inhibitory effect on protein kinase C through activation of protein phosphatase (Azzi et al. 2002). It also explains the anti-atherosclerotic and antitumour effect of vitamin E in most cells (Barella et al. 2004). While at the cellular level, α-tocopherol will also act as a gene regulator in the upregulation of mRNA or protein synthesis (Rimbach et al. 2010; Gonzalez et al. 2007), it modulates the activity of several enzymes involved in signal transduction, perhaps through influencing protein-membrane interactions (Joseph et al. 1998). This in turn reduces the release of reactive oxygen species that will affect gene expression. In addition, α-tocopherol stabilises the structure of membranes (Bradford et al. 2003), to modulate the immune response (Muir et al. 2002) by enrichment of monocytes and neutrophils which leads to the reduction of the adhesion to human endothelial cells (Azzi 2004) and as a participant in electron transport chains (Kang and Pervaiz 2012). α-Tocopherol may affect the process of cellular trafficking especially in the intracellular traffic of enzymes and vesicles, membrane fusion and the release of the contents of vesicles (Arita et al. 1997). Tocotrienols in human are shown to have neuroprotective effects, to inhibit cholesterol synthesis by lowering LDL (Imsanguan et al. 2008; Ryynanen et al. 2004) and to reduce the growth of breast cancer cells in vitro (Nesaretnam et al. 1995). In addition, vitamin E also contributes in enhancing male fertility (Gasior et al. 2009; Catoni et al. 2008) and limiting the incidents of generative human disease such as heart disease (Misuna et al. 2008; Leong and Shui 2002; Pryor 2000), cancer (Miyazawa et al. 2009; Bermudez et al. 2007; Weber

et al. 1997), cataracts, neurological disorders (Ueda et al. 2009; Sachdev et al. 1999) and inflammation (Azzi and Stocker 2000; Upritchard 2000). Besides that, studies by Campbell et al. (2008) and Nesaretnam et al. (2004) also showed that δ-tocotrienols are effective in the inhibition of cancer cell growth and α- and γ-tocotrienol are able to inhibit solid tumour growth of carcinoma 180 and also Lewis lung carcinoma cells (Silva et al. 2001).

Animal

Vitamin E supplementation is also required for ruminants (Rippert et al. 2004) for prevention of various diseases and protection of integrity of tissues (Mcdowell et al. 1996). Pigs, when supplemented with vitamin E, are more red and less brown (O'Sullivan et al. 2002). Fish fed diets containing vitamin E increase the concentration of vitamin E in their tissues (Huang et al. 2004). Livestock producers, especially dairy cows' producers, will need higher levels of α-tocopherol to produce more nutritious milk (McDowell et al. 1996). Fish containing tocotrienol had a positive impact on the seafood quality in prolonging shelf life, enhancing the nutritional value in the seafood and also maintaining the colour of the seafood (Ng et al. 2008).

Vitamin E Deficiency

Plant

Vitamin E does not play the same role in plants as in human or animals because plant is a vitamin E producer. A very rare vitamin E deficiency observed in plant happens only during stress such as with extreme low temperature (Havaux et al. 2005), very high light (Porfirova et al. 2002) and oxidative stress induced by metals (Collin et al. 2008).

Human

In human, vitamin E deficiency is never caused by a poor diet (Brigelius-Flohe and Traber 1999). Instead, it is caused by three specific situations such as the inability of a person to absorb dietary fat (Traber and Sies 1996), prematurely born person (Kaempf and Linderkamp 1998) or very low birth weight infants (Kositamongkol et al. 2011). In elderly people, vitamin E deficiency causes Parkinson's disease and heart disease (Serafini 2000). In addition, it is also seen in individuals with rare disorders of fat metabolism such as abetalipoproteinaemia, a rare inherited disorder of fat metabolism that results in poor absorption of dietary fat including vitamin E (Peretti et al. 2010). A rare genetic condition caused by mutations in the gene of the tocopherol transfer protein will also cause vitamin E deficiency because these

individuals have an extremely poor capacity to absorb vitamin E (Ouahchi et al. 1995). Most of the symptoms of vitamin E deficiency will lead to the loss of the antioxidant protection it offers to cells. This protective effect also keeps vitamins A, B and C from oxidising to an inactive form, and when vitamin E is lacked, deficiency of these vitamins will also occur (http://www.scienceclarified.com).

Animal

Vitamin E deficiency in animal will cause nutritional muscular dystrophy (Abutarbush and Radostits 2003) and infertility in animals (Oda and El-Maddawy 2012). Other signs of vitamin E deficiency are related to damage of cell membranes (Evans 2000) and leakage of cell contents to external fluids. The diets of animals with low vitamin E will also cause skeletal myopathy (Hill et al. 2001), liver necrosis (Johnson et al. 2002) and neurodegenerative (Mohammed et al. 2007).

Structure and Chemistry

Tocochromanols are amphiphilic compound (Rippert et al. 2004; Collakova and DellaPenna 2001), and its isomers can be distinguished based on the number and positions of methyl groups on the chromanol ring (Dormann 2007). Four different forms (α, β, γ, δ) of tocochromanols (tocopherol and tocotrienol) exist (Chun et al. 2006; Rocheford et al. 2002). Tocopherols consist of a polar chromanol head group and a non-polar isoprenoid-derived tail (Fig. 1). The chromanol head group is

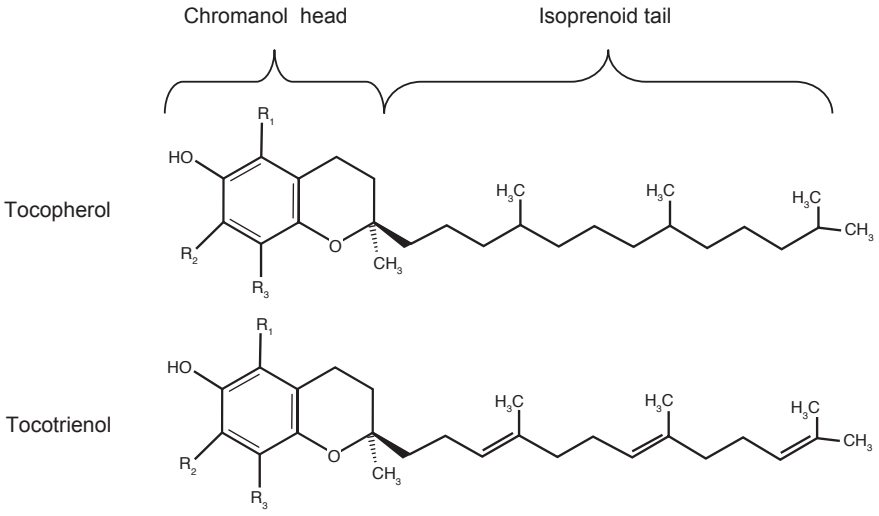

Fig. 1 General molecular structures of tocochromanols

bicyclic with three positions on the benzoquinone ring which can be methylated (Dormann 2007). The isoprenoid chain of tocopherol is fully saturated (Sen et al. 2007), whereas in tocotrienols, it contains three trans double bonds. The number and location of the methyl groups of the chromanol ring play an important role in influencing the biological activities of all the tocopherol and tocotrienol isoforms (Marsin Sanagi et al. 2006).

Biosynthesis of Tocochromanols in Plants

Tocochromanols are exclusively synthesised in the plastids of higher plants (Kumar et al. 2005), and the biosynthetic enzymes are found in the chloroplast. Tocochromanols are synthesised from precursors derived from two pathways: (a) shikimate pathway and (b) non-mevalonate pathway (Lee et al. 2007) (Fig. 2). This is the simplest biosynthesis route that can account for tocopherols and tocotrienols. However recent literature had shown that no cyclase enzyme that is specific to the tocotrienol biosynthetic pathway (Zbierzak et al. 2010). Based on Fig. 3, the condensation of PDP and HGA by HPT (Savidge et al. 2002) will produce intermediate product for tocopherol production which is MPBQ (Tian et al. 2007). Basically HGA serves as precursors for the synthesis of tocopherols, tocotrienols and also plastoquinol-9 (PQ-9) in chloroplasts of plants. The reaction also occurs when PDP is replaced by geranylgeranyl diphosphate (GGDP). Condensation of HGA and GGDP by homogentisate geranylgeranyl transferase (HGGT) will produce MGGBQ (Venkatesh et al. 2006). For tocopherols, the intermediate product of MPBQ will be methylated by PrBQMT to produce DMPBQ (Collakova and DellaPenna 2003b), while for tocotrienols, MGGBQ (2-methyl-6-geranylgeranylbenzoquinol) will be methylated by PrBQMT (prenylbenzoquinol methyltransferase) to produce DMGGBQ (2,3-dimethyl-5-geranylgeranyl benzoquinol) (Chen et al. 2006) (Fig. 4). From this step, the pathway will branch to transform DMPBQ and DMGGBQ into γ-tocochromanols (Sadre et al. 2006) by TC through cyclisation and methylation. Here, TC is involved in the formation of the chromanol ring structure in vitamin E biosynthetic pathway (DellaPenna 2005) (Fig. 5). This γ-tocochromanols will be further methylated by γ-TMT to produce α-tocochromanols (Sirikhachornkit et al. 2009) (Fig. 6). The remaining MPBQ and MGGBQ will be cyclised by TC to produce δ-tocochromanols, and further methylation by γ-TMT will produce β-tocochromanols (Collakova and DellaPenna 2003b) (Fig. 7). γ-TMT catalyses the final step in the synthesis of β-tocochromanols by using δ-tocochromanols as substrate (Hunter and Cahoon 2007) (Fig. 8). Enzymes that are involved in the production of vitamin E are summarised in Table 1.

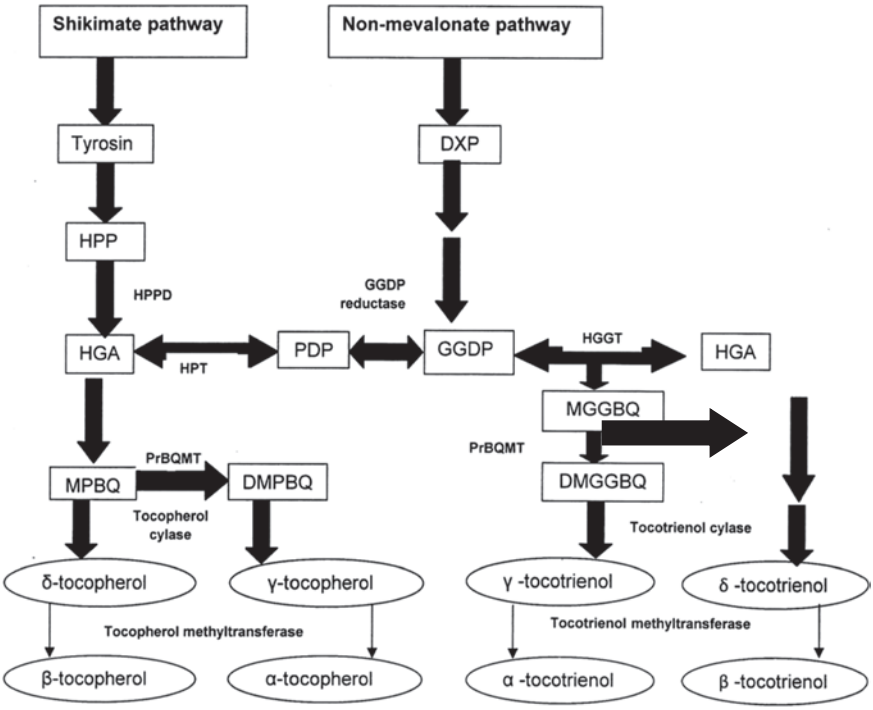

Fig. 2 Shikimate and non-mevalonate pathway for synthesis of α-tocochromanols in plants were based on Dormann (2007), Hunter and Cahoon (2007) and Cheng et al. (2003). Homogentisate acid (HGA) and phytyl diphosphate (PDP), derived from cytosolic aromatic amino acid metabolism and plastidic deoxyxylulose 5-phosphate pathway (DXP), respectively. PDP and GGDP (geranylgeranyl diphosphate) serve as substrates for the synthesis of α-tocochromanols (α-tocopherol and α-tocotrienol). Reactions between HGA and PDP or HGA with GGDP are carried out by homogentisate phytyltransferase (HPT) or homogentisate geranylgeranyl transferase (HGGT). The intermediate products 2-methyl-6-phytylbenzoquinol (DMPBQ) and 2,3-dimethyl-5-geranylgeranyl benzoquinol (DMGGBQ) will be further cyclised and methylated to produce α-tocochromanols. MPBQ and MGGBQ (2-methyl-6-geranylgeranyl benzoquinol) serve as substrate for the synthesis of β-tocochromanols. Enzymes in BOLD and UPPER CASE are specific to the synthesis of the subsequent intermediate products: p-hydroxyphenolpyruvate dioxygenase (HPPD), geranylgeranyl diphosphate reductase (GGDP reductase), 2-methyl-6-prenylbenzoquinol methyltransferase (PrBQMT), tocopherol cyclase (TC), and γ-tocopherol methyltransferase (γ-TMT). Substrates in upper case are specific to the synthesis of the subsequent intermediate products: p-hydroxyphenylpyruvate (HPP); 2-methyl-6-phytylbenzoquinol (MPBQ); and 2, 3-dimethyl-5-geranylgeranyl benzoquinol (DMGGBQ). Phytyl diphosphate (PDP) + Homogentisate (HGA) <=> 2-Methyl-6-phytylbenzoquinol (MPBQ) + Diphosphate + CO_2

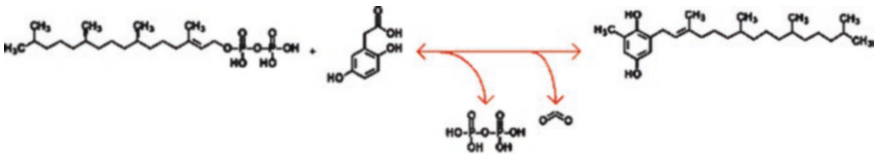

Fig. 3 Condensation reaction between PDP and HGA by HPT. 2-Methyl-6-phytylbenzoquinol (MPBQ) + S-Adenosyl-L-methionine <=>2,3-Dimethyl-5-phytylbenzoquinol (DMPBQ) + S-Adenosyl-L-homocysteine

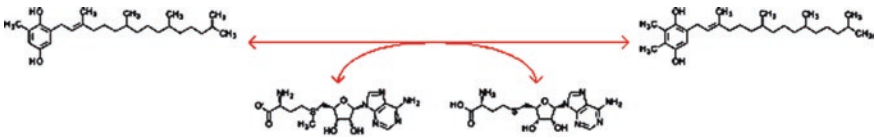

Fig. 4 Methylation reaction of MPBQ to produce DMPBQ. 2,3-Dimethyl-5-phytylbenzoquinol (DMPBQ)/(DMGGBQ) <=> γ-tocochromanols

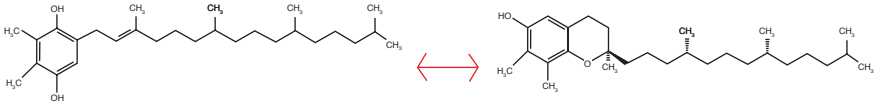

Fig. 5 Chromanol ring structure formation by tocopherol cyclase. S-Adenosyl-L-methionine + γ-tocochromanols <=> S-Adenosyl-L-homocysteine + α -tocochromanols

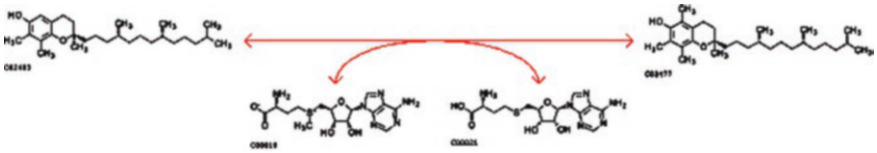

Fig. 6 Production of α-tocochromanols. 2-Methyl-6-phytylbenzoquinol (MPBQ)/(MGGBQ) <=> δ-tocochromanols

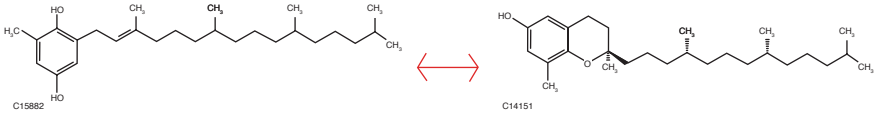

Fig. 7 Production of δ-tocochromanols. δ-tocochromanols + S-Adenosyl-L-methionine <=> β-tocochromanols + S-Adenosyl-L-homocysteine

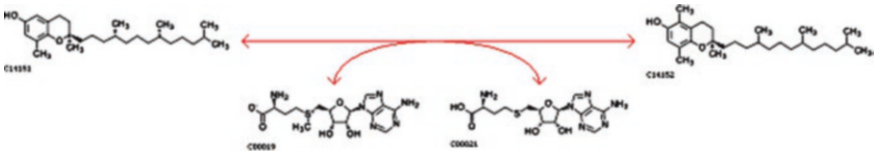

Fig. 8 Production of β-tocochromanols

Table 1 The different enzymes associated with tocochromanols biosynthesis

Enzymes	Reference
Homogentisate phytyltransferase (HPT)	Crowell et al. (2008) Venkatesh et al. (2006)
Tocopherol cyclase (TC)	Hass et al. (2006)
Tocopherol methyltransferase (γ-TMT)	Hass et al. (2006), Endrigkeit et al. (2009)
Homogentisate geranylgeranyl transferase (HGGT)	Horvath et al. (2006)
4-Hydroxyphenylpyruvate dioxygenase (HPPD)	Crowell et al. (2008), Raclaru et al. (2006)

Conclusion

In the vulnerable population of developing countries, nutrient deficiency will lead to health disaster. Elimination of nutrient deficiency has always been the priority agenda in the international organisations; however, it still remains as an unsolved major issue. Nutrient deficiency comprises many elements, and the element that was studied here is vitamin E. In this chapter, it was described that vitamin E consists of eight components which are α, β, γ and δ tocopherol and tocotrienol. The importance and the source of this component were described thoroughly in this chapter.

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Bioengineered Plants Can Be an Alternative Source of Omega-3 Fatty Acids for Human Health

Nita Lakra, Saquib Mahmood, Avinash Marwal, N. M. Sudheep, and Khalid Anwar

Introduction

Omega-3 fatty acids, also called as long-chain polyunsaturated fatty acids (PUFAs), belong to the family of lipids, immediately after its acquaintance to human benefits, which resulted in the revolution in several branches of health and medicines (Mozaffarian and Wu 2011; Schmidt and Dyerberg 1994). The omega name corresponds to the location of double bond with respect to the methyl group present in the fatty acid molecule. Alpha-linolenic acid (ALA) is short-chain omega-3 fatty acid, which polymerizes to form long-chain omega-3 fatty acids such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). This polymerization is quite low in the human body; hence, such long-chain omega-3 fatty acids need to be obtained/supplemented from diet. We reviewed the scientific literature on omega-3 fatty acids obtained from various sources and its health benefits to provide clinically relevant evidence-based information to ethnobotanists and physiologist. The purpose of this chapter is to summarize the current state of knowledge focusing on the health benefits of the long-chain omega-3 PUFA obtained naturally from plants and via transgenic approach, and in addition, reflecting the proteomics and genomics approach of these beneficial compounds will be highlighted.

Nita Lakra and Saquib Mahmood equally contributed to this work.

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Sources of Omega-3 Fatty Acids

A dietary source of omega-3 fatty acids ranges from plant kingdom to animals and microorganisms. These sources were divided in two major categories, namely, marine-based and land-based sources, which include oily fishes and other sea animals, dinoflagellates, protists and many plant species as listed in Table 1.

Marine-Based Source

Animal sources of omega-3 fatty acids living in marine water include oily fishes and dinoflagellates and protists. Marine animals especially fishes generally do not produce omega-3 fatty acids naturally (Albert et al. 1998), in fact these fishes obtain PUFA via marine microorganism through the ocean food chain which get accumulated in the form of fish oils (Lee et al. 2008). Even eggs and meat too contain a good amount of docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). Microalgae and species of lower fungi accumulate a high percentage of EPA in the lipid fraction. Currently, marine fishes are the primary dietary sources of these polyunsaturated fatty acids or can be commercially obtained as supplements, which are available over the counter as pharmaceutical preparations (Kris-Etherton et al. 2002; Saravanan et al. 2010). Production and processing of fish oils is a costly manufacturing process, aimed at removing colour pigments, contaminants (dioxins, furans and/or polyaromatic hydrocarbons) and volatile components responsible for the oil's odour and flavour (Bimbo 2011). There is concern regarding the sustainability of fish, due to its decreasing populations from decades of overfishing (Pauly et al. 2005). Environmental pollution has resulted in the accumulation of dioxins and heavy metals in fish and must be tested to rule out dangerous levels of pollutants (Yokoo et al. 2003). Due to potential contamination, the benefits of obtaining PUFAs from fish are being questioned (Yokoo et al. 2003). It is cheaper and easier to remove oil from flaxseed, which is a renewable material, whereas fish is a diminishing source.

Land-Based Source

On the other hand, plant sources are rich in omega-3 fatty acids especially α-linolenic acid which varies as high as 63% depending on the plant source such as flaxseed (50%) and walnut (10%). Finding plant-based sources of the essential omega-3 fatty acid could provide a sustainable, renewable and inexpensive source of omega-3 fatty acid, compared to fish oils. Sacha inchi seeds have a unique fatty acid composition containing a large amount of unsaturated fatty acids (about 85% polyunsaturation), comprised of approximately 34% linoleic acid and 51% linolenic acid

Table 1 Various sources of omega-3 fatty acids

Scientific name	Common name	Type	Reference
Cannabis sativa	Hemp	Plant	Da Porto et al. (2012)
Juglans cinerea	Butternuts	Plant	Gharibzahedi et al. (2012)
Perilla frutescens	Perilla	Plant	Asif (2011)
Ficus carica	Fig seed oil	Plant	Ulbricht et al. (2006)
Carya illinoinensis	Pecan nuts	Plant	Toro-Vazquez et al. (1999)
Linum usitatissimum	Flaxseed	Plant	Cunnane et al. (1993)
Actinidia deliciosa	Kiwifruit seed	Plant	Bahramsoltani et al. (2014)
Corylus avellana	Hazel nuts	Plant	Bernardo-Gil et al. (2002)
Rubus occidentalis	Black raspberry	Plant	Parry and Yu (2004)
Vaccinium vitis-idaea	Lingonberry	Plant	Bere (2007)
Juglans regia	Persian walnuts	Plant	Amaral et al. (2003)
Camelina sativa	Camelina	Plant	Lu and Kang (2008)
Salvia hispanica	Chia seed	Plant	Ayerza et al. (2002)
Portulaca oleracea	Purslane	Plant	Liu et al. (2000)
Hippoglossus hippoglossus	Halibut	Seafood	Falk-Petersen et al. (1986)
Oncorhynchus tshawytscha	Salmon	Seafood	Erdal et al. (1991)
Xiphias gladius	Swordfish	Seafood	Smida et al. (2009)
Etrumeus teres	Herring	Seafood	Ersoy and Celik (2009)
Pollachius pollachius	Pollock	Seafood	Jensen et al. (2012)
Rastrelliger brachysoma	Mackerel	Seafood	Ahmad et al. (2016)
Gadus morhua	Cod	Seafood	Izquierdo (1996)
Engraulis encrasicolus	Anchovies	Seafood	Kaya and Turan (2010)
Ethmidium maculatum	Menhaden	Seafood	Durand and Seminario (2009)
Tuna thynnus	Tuna	Seafood	Saito et al. (1997)
Lopholatilus chamaeleonticeps	Tilefish	Seafood	Cladis et al. (2014)
Sardina pilchardus	Sardines	Seafood	Khoddami et al. (2009)
Perna canaliculus	Mussel	Seafood	Murphy et al. (2003)
Euphausia superb	Krill	Seafood	Bottino (1975)
Saccostrea cucullata	Rock oyster	Seafood	Mclean and Bulling (2005)
Loligo vulgaris	Squids	Seafood	Arts et al. (2001)
Arctocephalus forsteri	Seal	Seafood	Koep et al. (2007)
Thraustochytrium sp.		Protist	Burja et al. (2006)
Schizochytrium sp.		Protist	Barclay et al. (1994)
Crypthecodinium sp.		Protist	Ward and Singh (2005)
Phaeodactylum sp.	Diatom	Microalgae	Yongmanitchai and Ward (1991)
Monodus sp.		Microalgae	Vazhappilly and Chen (1998)
Mortierella sp.		Fungus	Shahidi and Wanasundara (1998)

(Guillen et al. 2003). Flaxseed oil is a rich source of *n*-3 and has been proven to have good bioavailability when used in bulk or emulsion in several studies.

Alternative Sources for Omega-3 Fatty Acids

For improving human health and allaying fear of fish stock collapse and risk of contamination, alternative sources of long-chain omega-3 (n-3) polyunsaturated fatty acids (LC3PUFA) could be one way to address these issues. A number of alternative sources of PUFA are available. ALA-rich oils would be appropriate supplements for vegetarians and vegans. Recent advances in processing techniques in the food industry genetically modified the traditional oils such as soybean and canola to produce enriched quantities of LC3PUFAs (Gillingham et al. 2011; Lemke et al. 2010). Functional foods may be whole, fortified, enriched and enhanced foods that provide health benefits beyond the provision of essential nutrients (Hasler 2002; Berntssen et al. 2010). The European market for functional foods is estimated to be valued in excess of two billion US dollars (Menrad 2003). Enriched or functional foods containing LC3PUFA from plant origin may offer an alternative to supplementation for vegetarians who account for around 6% of the population (Food Standards Agency 2008). Algae oils are a relatively recent innovation in the food industry, and these are contaminant-free and could be used to provide a direct, vegetarian source of DHA (Breivik 2007).

Omega-3 Fatty Acids in Health and Disease Control

There is now strong scientific evidence highlighting that an adequate LC3PUFA status is a key factor in the maintenance of health and can reduce the risk of chronic and inflammatory diseases (Welch et al. 2010). Clinical trials that have been conducted on volunteers subjected to diet rich in PUFA and lower in carbohydrates revealed remarkable upshot on all lipoproteins and circulating lipids, counting noteworthy dwindle in the high-density lipoprotein cholesterol (HDL-C) ratio, which corresponds to strong predictor of cardiovascular disease (CVD) risk (Mensink and Katan 1992; Mozaffarian 2012). Similarly in a study on 80,000 women for a time span of 20 years, increased PUFA consumption was associated with significantly lower coronary heart disease (CHD) risk when replacing carbohydrates (Oh et al. 2005; Mente et al. 2009).

Consumption of plant- and microalgae-based omega-3 polyunsaturated fatty acids helps in lowering the risk of sudden cardiac death (SCD), myocardial infarction (MI), heart failure (HF) and atrial fibrillation (AF) (Lavie et al. 2009; Kromhout et al. 1985; Yokoyama et al. 2007). Mechanisms of action of omega-3 fatty acids revealed clear insight of its benefits on hemodynamic function (cardiac mechanics), cardiac function (including antiarrhythmic effects), arterial endothelial function and

prevent heart attack as well as decrease the overall risk of cardiovascular disease (Kris-Etherton et al. 2003). Docosahexaenoic acid and omega-3 fatty acid are necessary for the sound growth and better development of the brain and eye in children as well as for continuation of normal brain function in adults (Birch et al. 2007). Deficiencies of DHA are associated with deficits in learning. DHA deficiencies are associated with attention deficit hyperactivity disorder, unipolar depression, foetal alcohol syndrome, aggressive hostility, cystic fibrosis, adrenoleukodystrophy and phenylketonuria (Horrocks and Yeo 1999). Literature revealed further benefits of omega-3 fatty acids in the prevention and treatment of rheumatologic, gastrointestinal, respiratory illnesses and bone disorders (Sakaguchi et al. 1994; Ambrosone et al. 1998). Omega-3 polyunsaturated fatty acids (omega-3-PUFAs), such as eicosapentaenoic acid (EPA; 20:5 Δ5,8,11,14,17) and docosahexaenoic acid (DHA; 22:6 Δ4,7,10,13,16,19), have great impact on several diseases including hypertension, arthritis, cancer and other inflammatory and autoimmune disorders, but significant benefits are reported related to the disease of cardiovascular disorder (Fig. 1). Benefits of DHA and EPA in human health have been extensively evaluated through many studies (Kang and Weylandt 2008; Takahata et al. 1998). Omega-3 fatty acids have been shown to help DHA and EPA inhibit the proliferation of vascular smooth muscle cells, thus contributing to the prevention of atherosclerosis disease (Horrocks and Yeo 1999). DHA also plays a vital role in thraustochytrid cell life (Jain et al. 2007).

Omega-3 fatty acids have been shown to increase platelet responsiveness to subtherapeutic anticoagulation therapies, including aspirin. DHA is present in large amounts in neuron membrane phospholipids, where it is involved in proper function of the nervous system. PUFAs populate cellular membranes and serve as precursors for hormonelike eicosanoids. It also helps in decreasing the risk of lung, prostate and breast cancer (Freeman et al. 2006; Veierod et al. 1997). Omega-3 fatty acids are also associated with lower blood pressure, improve endothelial function, decrease triglyceride and remnant lipoprotein levels and decrease risk for thrombosis and arrhythmias (Kris-Etherton et al. 2003). EPA and DHA have been linked to

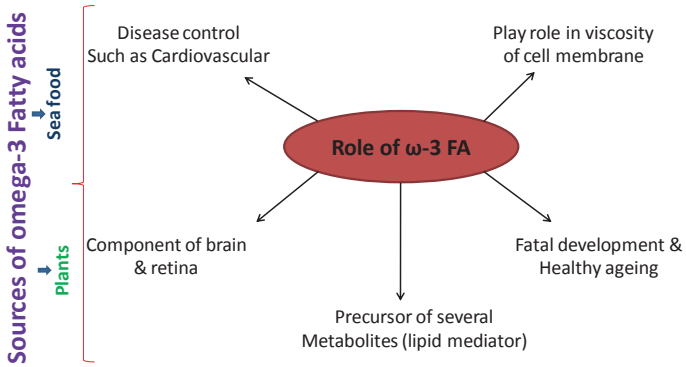

Fig. 1 Role of omega-3 fatty acids

promising results in prevention, weight management and cognitive function in those with very mild Alzheimer's disease and have neuroprotective properties against dementia, and lack of omega-3 PUFA in dietary supplements leads to the risk of cognitive decline or Alzheimer's disease (AD) (Fotuhi et al. 2009).

Omega-3 Polyunsaturated Fatty Acid Regulates Various Proteins

Several mechanisms have been proposed by which n-3 and n-6 PUFA exert their biological actions (Massaro et al. 2008; Adkins and Kelley 2010), however, the information available is scanty on explicitly define the proteins and pathways involved in the actions of PUFA. PUFAs form bioactive mediators which act on different receptors and proteins in the body (Wada et al. 2007). Proteins are important mediators of biological activities in all living cellular units. Proteome is comprised of all expressed proteins encoded by the genome of a cellular system (Schweigert 2007). Ahmed et al. (2014) showed that n-3 PUFAs regulate several proteins involved in lipid metabolism, carbohydrate, citric acid cycle and protein synthesis (Fig. 2). Study suggested an important functional role of dietary n-3 PUFA in regulating proteins involved in lipids, glucose metabolism and protein synthesis. The findings showed that high dietary n-3 PUFA reduced the expression of regucalcin, adenosine kinase and aldehyde dehydrogenase. On the other hand, diets high in n-3 PUFA increased the expression of apolipoprotein A-I, S-adenosylmethionine synthase, fructose-1, 6-bisphosphatase, ketohexokinase, malate dehydrogenase, GTP-specific succinyl CoA synthase, ornithine aminotransferase and protein disulfide isomerase-A3 (Ahmed et al. 2014).

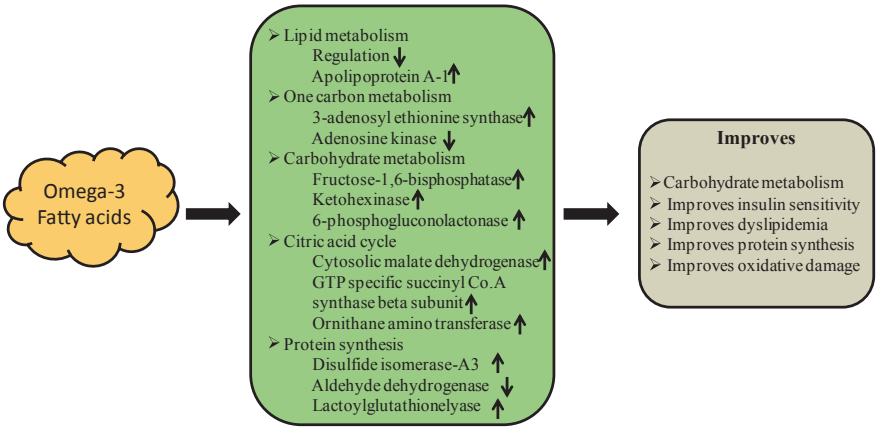

Fig. 2 Effect of omega-3 fatty acids on the regulation of various metabolic pathways. Source: Ahmed et al. Nutrition & Metabolism 2014, 11:6

Omega-3 Polyunsaturated Fatty Acid Biosynthetic Pathways

PUFAs constitute a large group of fatty acids containing long-chain carbonic molecules that include omega-3-fatty acids (Lozach 1986). Omega is the position of the first double bond when counted from the ethyl end, and the number "3" refers to the number of carbon atoms at that position from the methyl end. Eicosapentaenoic acid (EPA, C20:5, n-3) and docosahexaenoic acid (DHA, C22:6, n-3) are two typical omega-3 fatty acids. The prefixes "docosa" and "eicosa" are of Greek descent, meaning the 22 and 20 C-atoms present in DHA and EPA (Lozach 1986).

In plant cells, fatty acid biosynthesis occurs in the plastids with a successive concatenation of 2 carbon units resulting in production of the 16 or 18 carbon long fatty acids that predominate in cellular membranes. A soluble fatty acid desaturase is present in the plastid stroma for conversion of 18:0 into 18:1, where the number before the colon represents the total number of carbons in the fatty acid chain and the number after the colon indicates the number of double bonds. The 18:1 fatty acid is subsequently available for further desaturation by one of two parallel pathways operating in either the plastid or endoplasmic reticulum (ER). For instance, 18:1 may be converted to 18:2 in plastids by a membrane-bound fatty acid desaturase called FAD6, or the 18:1 may be exported from the plastids to the ER for conversion to 18:2 by a structurally related enzyme called FAD2. The FAD2 and FAD6 enzymes are similar at the polypeptide sequence level, with the exception that the FAD6 protein contains a longer N-terminal sequence that is characteristic of a chloroplast transit peptide. In a similar fashion, 18:2 may be converted into 18:3 in plastids by the FAD7 or FAD8 enzymes, which are encoded by two closely related genes in *Arabidopsis* or can be exported to the ER for conversion to 18:3 by the FAD3 enzyme. This latter group of enzymes (FAD7/FAD8 and FAD3) is referred to as omega-3 fatty acid desaturase, since they introduce a double bond at the omega-3 position of the fatty acid structure. Thus the FAD6 and FAD2 enzymes, which produce 18:2, and the FAD7/FAD8 and FAD3 enzymes, which produce 18:3, all play central roles in production of the PUFAs that are present in all plant species. The ER-localized desaturase FAD2 and FAD3 are also involved in the production of PUFA components of seed oils (Ohlrogge and Browse 1995), given the importance of these fatty acids to human nutrition and in determining stability of oils during cooking or other food applications (Bocianowski et al. 2012; Hu et al. 2006; Tian et al. 2014; Yang et al. 2012).

Alpha-linolenic acid (ALA) is an essential fatty acid and the substrate for the synthesis of longer-chain, more unsaturated omega-3 fatty acids, eicosapentaenoic acid (EPA), docosapentaenoic acid and docosahexaenoic acid (DHA), which are associated with human health benefits. The biosynthetic pathway includes a series of desaturation, elongation and beta-oxidation reactions, with the rate-limiting enzyme considered to be that catalysed by delta-6 desaturase (Fig. 3). SDA (stearidonic acid) is an intermediate in the pathway of EPA and DHA biosynthesis (Fig. 3), being the product of ALA desaturation by delta-6 desaturase. Since D6D is rate limiting for conversion of ALA to EPA, SDA is potentially another better substrate

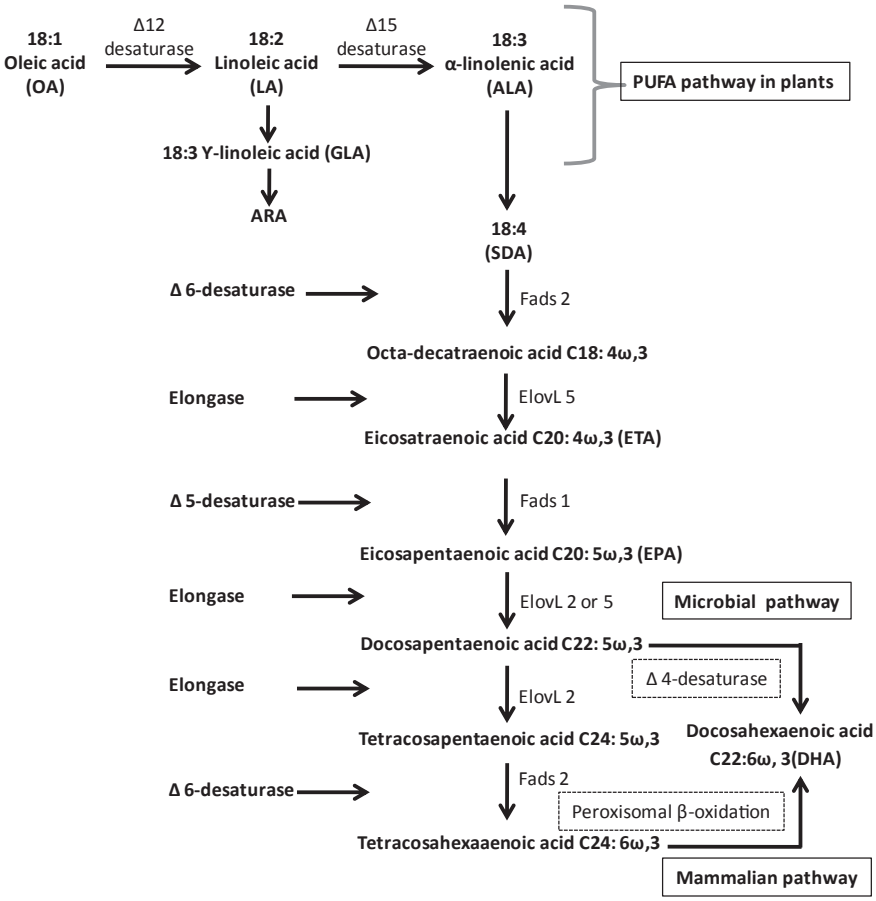

Fig. 3 Biosynthetic pathway of omega-3 fatty acids in plants and animals

for the biosynthesis of omega-3 PUFAs. There are few natural sources of SDA; it is found in *Echium* oil, where it contributes about 9–16% of fatty acids (Berti et al. 2007, Bioriginal 2014). Levels of SDA have been substantially increased in soybean oil by genetic modification (Ursin 2003).

Various different biosynthetic pathways for DHA and EPA have been deduced in marine microbes earlier (Venegas-Calerón et al. 2010; Petrie and Singh 2011). The traditional route by which diatoms and microalgae synthesize EPA, DHA and other omega-3 LC-PUFAs is via a series of alternating desaturation and elongation steps acting on long-chain (C18) polyunsaturated substrates, and this process is aerobic process. This process can be furthered into two distinct types of primary biosynthetic activities, the predominant "Δ6-pathway", in which introduction of a Δ6-desaturation into a C18 substrate is the first committed step, followed by C2 elongation and further Δ5-desaturation. Another pathway is alternative or "Δ8-pathway", which is a less common route, and this pathway starts with the C2

elongation of the C18 substrate, followed by two successive desaturation reactions (Δ8, Δ5) (Ruiz-Lopez et al. 2015). For the synthesis of DHA, this is initiated by the C2 elongation of EPA and further (Δ4) desaturation.

Animals, including humans, lack the Δ-12 and Δ-15 desaturase enzymes, which are essential for the synthesis of omega-3 and ω-6 fatty acids. As such they are not capable of building long-chain omega fatty acids and must acquire them from their diets. Humans and other animals obtain some DHA and intermediate products such as EPA through bioconversion of α-linolenic acid (18:3, omega-3) and some from direct consumption of DHA itself (Fig. 3; Innis 2003). Omega-3 fatty acids are typically synthesized from α-linolenic acids acquired from dietary plant sources and are hence regarded as biological sources of EPA and DHA. Further metabolism of α-linolenic acids is characterized by the action of the Δ-6 desaturase enzyme followed by the action of the elongase enzyme for the addition of a carbon atom to the molecular chain, leading to action by Δ-5 desaturase to form EPA. Earlier studies revealed the occurrence of the desaturation process of the long-chain fatty acids in endoplasmic reticulum (Innis 2003).

In the last decade, several genes have been identified from a range of different microbial sources for all these activities. Some prokaryotic and eukaryotic microorganisms (marine) take alternative route for synthesis of EPA or DHA that is anaerobic or polyketide synthase (PKS) pathway. Several genes of this pathway have been identified and characterized. In this pathway, malonyl-CoA is converted into omega-3 PUFAs by using processive polyketide synthase-like enzymatic system that is independent of any fatty acid intermediate formation (Metz et al. 2001). It was first described in *Shewanella pneumatophori* strain SCRC-2378, a marine bacteria, the complex consists of eight different PKS protein domains (Yazawa 1996).

Metabolic Fate of Alpha-Linolenic Acid in Humans

ALA in human nutrition becomes important in terms of long-term dietary intake, and its advantage over omega-3 fatty acids from fish is that the problem of insufficient vitamin E intake does not exist with high intake of ALA from plant sources. The ALA-rich lipids are gradually digested, first by gastric lipase and then by pancreatic lipases. The liberated free fatty acids are readily absorbed and incorporated into lymph chylomicrons, largely in the form of phospholipids. Chylomicron TAG is hydrolysed by lipoprotein lipase expressed on the endothelium; adipose tissue lipoprotein lipase is upregulated in the postprandial period which results in targeting of meal fatty acids for storage. In tissues, both ALA and LA can be converted into fatty acids of longer and more unsaturated chain via common pathway of alternate desaturation and elongation (Bezard et al. 2003; Nelson and Ackman 1998). However, in humans, the conversion of ALA to longer-chain essential fatty acids is very inefficient, (Bezard et al. 2003) possibly because of slow desaturation of ALA in the presence of excess LA in the tissues (Simopoulos 1991) and/or because of high susceptibility of ALA to oxidation (Pawlosky et al. 2001). It has been

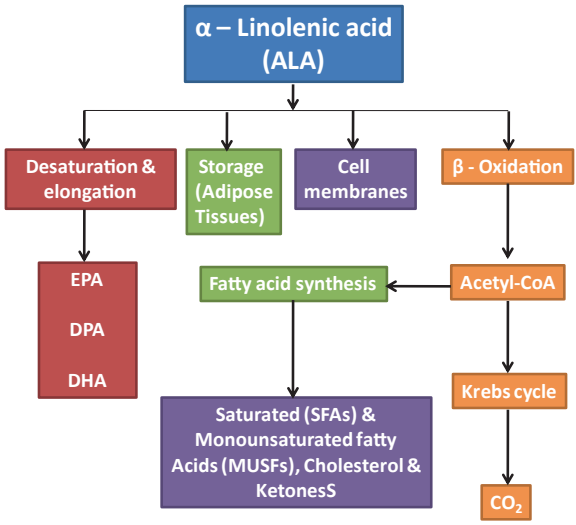

Fig. 4 Metabolic fate of omega-3 fatty acids in humans

postulated that the role of dietary ALA as a long-chain omega-3 precursor may become important in human nutrition in terms of long-term dietary intake.

Through these processes ALA from the diet can be made available to be incorporated into cell membranes and pools for storage (in adipose tissue), energy production (cell and tissue) or conversion to longer-chain omega-3 PUFAs (supposed to mainly occur in the liver). The metabolic fates of ALA are summarized in Fig. 4. ALA and SDA are converted to EPA and DHA through a series of elongation and desaturation processes occurring predominantly within the endoplasmic reticulum, excluding the last β-oxidation reaction which forms DHA and occurs in peroxisomes (Fig. 4). This pathway is believed to mainly occur within the liver (Nakamura and Nara 2004), but there is some evidence that the brain, retina and testis also have high expression of the genes encoding the relevant enzymes compared with other tissues (Agbaga et al. 2010; Sassa and Kihara 2014). D6D is encoded by the gene fatty acid desaturase 2 (Fads2) and has a preference for ALA over LA. SDA is converted to 20:4 omega-3 by the enzyme elongase-5, encoded by fatty acid elongase 5 (Elovl5). SDA is an intermediate in this metabolic pathway, being synthesized from ALA via the action of D6D (Fig. 4).

Metabolic Engineering of Pathways for Production of Omega-3 Polyunsaturated Fatty Acids (Omega-3 PUFAs) in Transgenic Plants

The increase in demand of these fatty acids as fish oil has led to putting enormous pressure on marine stock which is already in diminishing state (Cressey 2009). Increasing pollution of marine environment and an adequate lack of fish stock have

prompted the desperate search for an entirely new source of omega-3 polyunsaturated fatty acids, and the approach that meets the increasing demand should be sustainable and capable (Tocher 2009). Furthermore, product quality derived from fish oil is generally dependent on the season and location, and it can be affected by the ocean pollution. The process for purifying these fatty acids from fish oil itself is complicated as well (Lenihan-Geels et al. 2013).

New-generation GM crops are now also being developed for the production of recombinant medicines and industrial products, such as monoclonal antibodies, vaccines, plastics and biofuels. Alternative resources of n-3 PUFAs created by transgenic domestic animals would also be an economic approach. In a study, mfat-1 transgenic cattle expressed a *Caenorhabditis elegans* gene, mfat-1, encoding an n-3 fatty acid desaturase. Fatty acid analysis of tissue and milk showed that all of the examined n-3 PUFAs were greatly increased and simultaneously the n-6 PUFAs decreased in the transgenic cow. Kang et al. reported that expression of a humanized fat-1 gene (hfat-1), encoding an n-3 fatty acid desaturase from *Caenorhabditis elegans*, in mice resulted in a significant increase of n-3 fatty acids as well as a sharply decreased ratio of n-6/n-3 fatty acids in the hfat-1 transgenic mice (Kang et al. 2004). n-3 fatty acids in the milk of transgenic mice were also significantly increased.

Another alternative and novel sources of omega-3 fatty acids can be green manufactured from marine algal or algae-like microbial oils, which could eliminate many of the taste and odour problems associated with fish and discard the shortcomings of fish oil-based process. The process of culturing the algae or algae-like microorganism to accumulate the oil rich in omega-3 fatty acids was defined as "Omega-3 Biotechnology" (Gupta et al. 2012). Currently, the most common algae or algae-like microorganism used for the production of DHA belongs to the marine members of the families Thraustochytriaceae and Crypthecodiniaceae. Thraustochytrids include the genera *Schizochytrium* and *Ulkenia*, whereas dinoflagellate *Crypthecodinium* is a genus of the family Crypthecodiniaceae (Barclay et al. 1994; Borowitzka 2013; Klok et al. 2014).

For the last three decades, it has been continuously observed that the focus of research has shifted towards genetic engineering of crop plants to increase the possibility of rapid achievement of goal for enhancing nutritional value of crop plants. The genetic engineering technique allows altering genomic composition of the higher plants that changes their metabolism and physiology that leads to plants with improved yield quality (Mullet 1990). This technique allows using vast array of useful genes and also introduction of different desirable genes in single event in plants. Genetic engineering practices have been utilized to modify the plants genetically for enhancement of nutritional values of plants, to improve quality of the crops and for several other reasons such as increase the adaptability of plants in adverse conditions while maintaining the yield quality (Lemaux 2008). The site of the o3 desaturase reaction is the endoplasmic reticulum or chloroplasts (Heinz 1993), and a seed-specific o3 desaturase cDNA of perilla has recently been cloned and characterized (Chung et al. 1999). Additionally, mutants deficient in α-linolenate synthesis were isolated in linseed and *Arabidopsis* (Stymne et al. 1992; Browse et al. 1993).

Perilla oil is one of the important oil resources, because more than 60% of the total fatty acids in triacylglycerol (TG) is α-linolenic acid (Hilditch 1956).

Alpha-linolenic (ALA; 18:3 Δ9,12,15) is considered as essential fatty acids for higher animals including humans because they are essentially required in their diet but they lack the Δ12- and Δ15-desaturase activities that convert oleic acid (OA; 18:1 Δ9) to ALA. In vivo studies in humans show that only ∼5% of ALA is converted to EPA and <0.5% of ALA is converted to DHA (Williams and Burdge 2006). This low conversion efficiencies of ALA to omega-3-PUFAs lead to the lack of sufficient dietary omega-3-PUFAs which could compromise health. Obtaining of omega-3-PUFAs for fulfilling dietary consumption through oily fish, could risk the increasing the conditions such as cardiovascular disease, metabolic syndrome and obesity (Calder 2004; Nugent 2004; Williams and Burdge 2006; Poudyal et al. 2011). For increasing the intake of EPA and DHA to the sufficient amount through marine fishes, the adequate supply of marine fishes is difficult due to number of reasons such as, high degree of exploitation of marine fishes stock (Food and Agriculture Organisation of the United Nations, 2010), environment pollution of marine ecosystem (Domingo et al. 2007) and the marine fishes are poor producers of Omega-3 polyunsaturated fatty acids (omega-3-PUFAs) and the original source of omega-3-PUFAs are marine microalgae (e.g. diatoms), which are accumulating in fish lipids through food chain (Smith et al. 2011, b).

Countering the limited supply and increasing demand for omega-3-PUFAs, much research effort has focused on engineering oilseed plants to synthesize EPA and DHA. Such enormous potential to develop the use of terrestrial plant-based lipids is a significant approach; as such a substitution could relieve the aquaculture industry's pressures on forage fisheries. It would also lighten the human health problems associated with the presence of polychlorinated biphenyls and dioxins in fish feeds. Therefore, the concept of acquiring them from higher plants in commercial and significant quantities is particularly appealing. However, no oilseed species produces such products naturally, so there is an essential need to genetically engineer the agronomically viable oilseed species to synthesize these fatty acids.

Petrie et al. (2010, 2012) have recently described metabolic engineering of ω3 LC-PUFA in plants: after inserting seven biosynthesis genes of the docosahexaenoic acid (DHA) biosynthesis pathway from microalgae into the genome of *Arabidopsis thaliana*, they were able to obtain ω3 LC-PUFA levels in seeds similar to that observed in bulk fish oil. If applied to oilseed crops such as *Brassica napus*, this technology could potentially form the basis of a plant-based sustainable source to complement the existing marine fish oil supply.

In spite of identification and functional characterization of so many genes of the pathways involve in synthesis EPA and DHA, this whole process is not so straightforward as it might appear, for many limiting steps and for a number of reasons. The reconstitution of entire pathway for synthesis of EPA and DHA needs coordinated expression and activity of several genes, which vary in many transgenic systems. Apart from this, some other factors may hinder the synthesis of desired product such as the need of pre-existing substrate C18 di- or tri-unsaturated fatty acids for constituent enzymes in aerobic pathway. Similarly, in anaerobic pathway, the

requirement of malonyl-CoA is primary metabolites whose level is abundantly low. Moreover, the selective exclusion of EPA or DHA could take place in system where the capacity of EPA and DHA synthesis is introduced because the native enzymes of the pathways will be unfamiliar with these fatty acids in host system, or sometimes the "trapping" of biosynthetic intermediates in undesired metabolic dead ends could also take place due to activity of housekeeping enzymes (McCartney et al. 2004; Ruiz-Lopez et al. 2007). Considering all these functional characterizations of various genes of the entire pathways and the enzymatic activities they possess, an effort could be applied to reconstitute them through metabolic engineering in order to provide them the capacity to synthesize EPA and DHA in a heterologous system. Before the engineering of metabolic pathways for synthesis of (omega-3-PUFAs) in plant system, individual genes of pathways such as Δ6- and Δ5-desaturases were expressed and evaluated in plant system to know whether such activities are feasible in heterologous system or not after identification of all the biosynthetic genes needed for the synthesis and accumulation of omega-3-PUFAs by 2004 (Kinney 2006; Napier 2007). These events led the foundation of belief that engineering such pathways in plant system is feasible. First time in 2004, Qi et al. has shown the accumulation of EPA in the leaves of transgenic *Arabidopsis* plant, when they expressed genes of alternative pathway from algae *Isochrysis galbana* and *Euglena gracilis*. This event led to vegetative production and accumulation of EPA. Since all the plants ideally store target lipids in the form of triacylglycerol (TAG) that is terminal point in seed oil biosynthesis. Plant oils are rich in C18 fatty acids, including the essential fatty acids linoleic acid (18:2Δ9,12,n-6; LA) and α-linolenic acid ALA, but it is devoid of EPA and DHA (Saravanan et al. 2010). Next challenge was to produce and accumulate omega-3-PUFAs in plant seeds and this objective was achieved when the seed specific synthesis and accumulation was achieved by expression of genes fatty acyl-desaturases and elongases of conventional Δ6-pathway in transgenic tobacco (*Nicotiana tabacum*) and linseed (*Linum usitatissimum*), using the seed-specific promoters (Abbadi et al. 2004). In another attempt, a metabolic pathway was engineered in *Arabidopsis thaliana* using genes of alternating desaturation and elongation from using seed-specific promoters for the synthesis of DHA. DHA production up to 15% was observed in the seeds of transgenic plants (Petrie et al. 2012). Further works have been done on the optimization of pathways so that only desired fatty acids can be accumulated in seeds. The utilization of omega-3-specific desaturases that check the accumulation of unwanted omega-6 fatty acids and the application of acyl-CoA-dependent desaturases for the first committed (Δ6) step on the pathway led to further optimization and purity of the product (Domergue et al. 2005; Hoffmann et al. 2008; Cheng et al. 2010; Petrie et al. 2010). As a result, significant development took place in metabolic engineering of pathways, and a considerable level of omega-3-PUFAs such as EPA and DHA can accumulate in oil seeds of transgenic plants that is similar to what is found in marine fish oil.

The accumulation of EPA and DHA in model systems was achieved by using different approaches, and this was facilitated by identification of different genes from algae and other lower eukaryotes and the use of them in engineering metabolic

pathways in plant system. This metabolic engineering approach was further taken one step ahead, in order to direct the accumulation of omega-3-PUFAs in seeds of transgenic plants. After the successful engineering of metabolic pathway in order to synthesize EPA and DHA in model plants, this approach was further used in crop plants to address the challenges accompanied with generating a potential substitute in agriculturally important crop plants.

Later on, more complex metabolic engineering was done in *Brassica juncea* for the production of EPA in seeds, where there are series of transformation and expression of transgenes of alternating desaturation/elongation pathways. This led to the accumulation of arachidonic acid (20:4n-6, AA) in the n-6 and EPA. The plants seeds were so engineered that led to the conversion of ARA into EPA. This metabolic engineering also results into synthesis of DHA via Δ5 elongation and Δ4 desaturation reactions (Wu et al. 2005). Further, Ruiz-Lopez et al. (2014) have shown the significant increase in the level of EPA and DHA in the oil seeds of *Camelina sativa*, when a set of heterologous genes were expressed in *Camelina sativa*, which directs the synthesis of these fatty acids, and this also led to lowering the chances of accumulation of unwanted fatty acid intermediates. In another study, Petrie et al. (2014) has also shown the considerable accumulation of DHA level in seeds of *Camelina sativa*, when metabolic engineering of the pathway which directs the synthesis of DHA was achieved in *Camelina sativa*. In both studies, the significant increase in the level of DHA was achieved, while the level of EPA was low in the latter case. Until recently, modifications of oil composition could be achieved through traditional plant breeding, where natural diversity within closely related species could be exploited, or through mutagenesis. Transgenic technology widens the scope of modifications achievable in oil composition by allowing the introduction of a wider range of genetic elements than is otherwise possible. There has been considerable focus on the identification and analysis of enzymes and underlying genes involved in plant lipid biosynthesis. Coupled with efficient plant transformation systems for the key oilseed crops, canola and soybean, metabolic engineering of seed lipids in these two crops has become feasible. Genes encoding the fatty acid desaturase enzymes that catalyse these reactions have been identified and characterized from diverse sources including higher plants and fungi (17). Ursin (2003) has generated transgenic canola lines expressed in seeds, the Δ6 and Δ12 fatty acid desaturases isolated from the commercially grown fungus, *Mortierella alpina*, and the Δ15 fatty acid desaturase from canola (*Brassica napus*).

The fascinating observation of these studies in crop plants further validates the reconstitution of metabolic pathways performed in model plants in order to achieve the accumulation of DHA and EPA. Hence, working in crop plants like *Brassica juncea* and *Camelina sativa* delivers more opportunities in better manner for engineering the pathways in crop plants with the incorporation of heterologous genes in order to enhance the synthesis accumulations of target fatty acids.

Conclusion

Omega-3 polyunsaturated fatty acids (PUFAs) such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have been regarded as an important dietary component with multiple health benefits. It works through interaction with various proteins and act on cellular, molecular and physiological levels which have significant positive impact on improving health status of human beings. Currently, the primary dietary source of these fatty acids is marine fish, but the establishment of sustainable nonfish sources is important to overcome the fish-dependent limited supply of this critical long-chain unsaturated fatty acids.

The human body cannot naturally produce ALA, and its role in heart health is assigned primarily to its conversion to EPA and DHA in the human body. While the human body is able to synthesize EPA and DHA from ALA, the process is inefficient. An interesting approach for significant supply of omega-3 polyunsaturated fatty acids (PUFAs) is metabolic engineering of a crop plant with potential to synthesize these fatty acids. Multiple genes are required to construct the metabolic pathway for the synthesis of omega-3 polyunsaturated fatty acids (PUFAs) that work in coordination for the tissue-specific expression in transgenic plants. Genes involved in metabolic pathways were identified, and metabolic engineering was done to reconstruct the metabolic pathway in model plant system that led to the tissue specific synthesis and accumulation of EPA and DHA in transgenic model plants. This was followed by engineering of metabolic pathway in crop plants to generate the new source of these fatty acids in agriculturally important crop plants. *Brassica juncea* and *Camelina sativa* were engineered for the production of DHA and EPA, and the level of DHA was significantly increased in both cases, while the level of EPA was lower in the latter case. The interesting results achieved in these studies in crop plants encourage metabolic engineering of pathways in other crop plants to increase the level of DHA and EPA to the significant level. The whole study discharges more opportunities and encourages reconstruction of metabolic pathways in an effective manner in crop plants and the application of transgenic plants for the production of molecules which have multiple health benefits, and the need of the hour is to adapt and increase the agriculture-based production systems with low cost.

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Environmentally Friendly Plant-Based Natural Dyes: Extraction Methodology and Applications

Shahid Adeel, Fazal-Ur Rehman, Sana Rafi, Khalid Mahmood Zia, and Muhammad Zuber

Introduction of Natural Dyes

Nature has been bestowed with enormous color which add the beauty and influence the human. It is the sensation that inspires us through their presence in different items such as food, textile, decorative objects, printing (Silva et al. 2010). In the textile industry, dyes and pigment have a main role in imparting color and adding beauty to natural (wool, silk, cotton) and synthetic fabrics (nylon, polyester). In ancient times, people used natural dyes that were obtained from natural sources such as plant, animals, minerals, etc. and used for different purposes such as to decorate their cave, walls, their body, their fabrics, cosmetics, etc. Historically it is found that purple dye (Tyrian purple) obtained from murex shell has been utilized by Phoenicians and the Roman Empire. Madder, cochineal, and kermes for red and indigo and alkanet and marine algae for blue and purple have been used by Egyptian and ancient North African dyers (Garcia-Macias and John 2004; Guinot et al. 2006; Alam et al. 2007). However, their wide use went off in 1856 with the sudden discovery of synthetic color by W.H. Perkin called "mauve" that became an alternative to the Tyrian purple. This suddenly has caused the decline of natural dyes due to its excellent coloring properties, cost-effectiveness, brilliant shades, and high color depth. But recent studies revealed that use of synthetic dyes poses a series of health hazards to the environment due to their toxic wastes and complex structures which are nondegradable and disturb ecobalance. Consumers are more conscious about their health and are demanding the eco-friendly and sustainable products. For the reasons it has also been found that natural dyes are resuscitated and gained its

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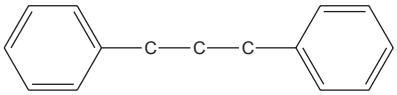
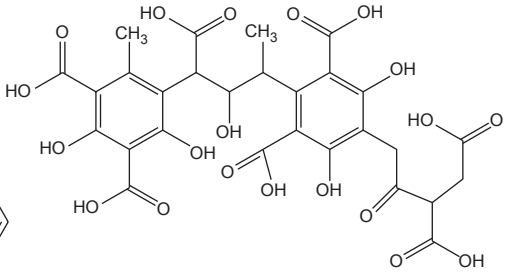
importance because of their biodegradable, biocompatible, and renewable nature and have owned with numerous bioactive components. However, with a lot of advantages, natural dyes have some limitation: colors obtained are non-reproducible, it is very costly to achieve bright shades, they give low color yield, and they have poor fastness properties. The need of auxiliaries and less technical information about their use has made it difficult to introduce them at commercial level. Researchers are finding new techniques to overcome their disadvantages via introduction of modern methods and improvement in conventional methods. Hence, the revival of natural dyes in applied walks of life is on the way either through conventional techniques or modern tools.

Environment Aspects

"Century of the environment" is the environment of 21 century and now people are much aware about toxic chemicals and want healthy environment. Release of harmful waste during manufacturing of synthetic dyes and their utilization in textile causes serious health issues; these effluents are not only damaging the environment but also causing global warming, ozone layer depletion, water pollution, disturbance in ecobalance, etc. (Zheng et al. 2011). Due to such hazardous effects, Germany, the USA, EU, the UK, etc. have banned the azo dye production that produces different 22 amines which are highly lethal. Traders, consumers, and suppliers are nowadays focusing on the utilization of eco-friendly products by analyzing their manufacturing, supply, application, and their waste effects to the environment (Banerjee and Sharma 2013). Most of the chemicals from textile industries which cause water pollution are difficult to remove. Because wastewater dyes have a complex structure and are very difficult to remove, for such purpose, different costly methods are employed, whereas natural dye waste has no such problem (Alsehri et al. 2014). Apart from these advantages, natural resources also act as treatment for effluent waste such as dried leaves of almond (*Terminalia catappa*) which produce natural dye and these leaves lower the pH level of water, absorb toxic chemicals from water due to the presence of flavonoids, humic acid, and tannin which act as cheapest source of adsorbent (Jenos et al. 2005; Demirbas 2009).



Flavonoid



Humic acid

Natural dyes that contain tannin can be utilized as a biomordant as well as green alternative to poisonous metal ions (Foo and Hameed 2010; Khan et al. 2012). Similarly walnut (*Juglans regia*) is not only used to dye fabric but also acts as an absorbent and removes basic dye (malachite green oxalate) from wastewater (Gunes and Atav 2017). Tannin that forms complex structure with metal ions that present as effluent in wastewater can be used as absorbent (Serrano et al. 2009).

Hence from a sustainable point of view, the natural colorants derived from plants, animals, and minerals are blessings for the ecosystem.

Classification

Natural dyes derived from natural sources such as plants, animals, minerals, and microorganisms have been classified on the basis of their:

1. Sources
2. Colors
3. Structures
4. Methods of applications

Here in this chapter we will just focus on the natural dyes based on their color (Fig. 1).

Color-Based Natural Dyes

Plants are the biggest source which provide the broad spectrum of shades which are isolated from different parts such as stem, bark, leaves, shoot, flowers, fruit, etc. These colors depend on different constituents present in different sources (Christie 2013). Some of the colors which are derived from plants, animals, and minerals are discussed below in detail.

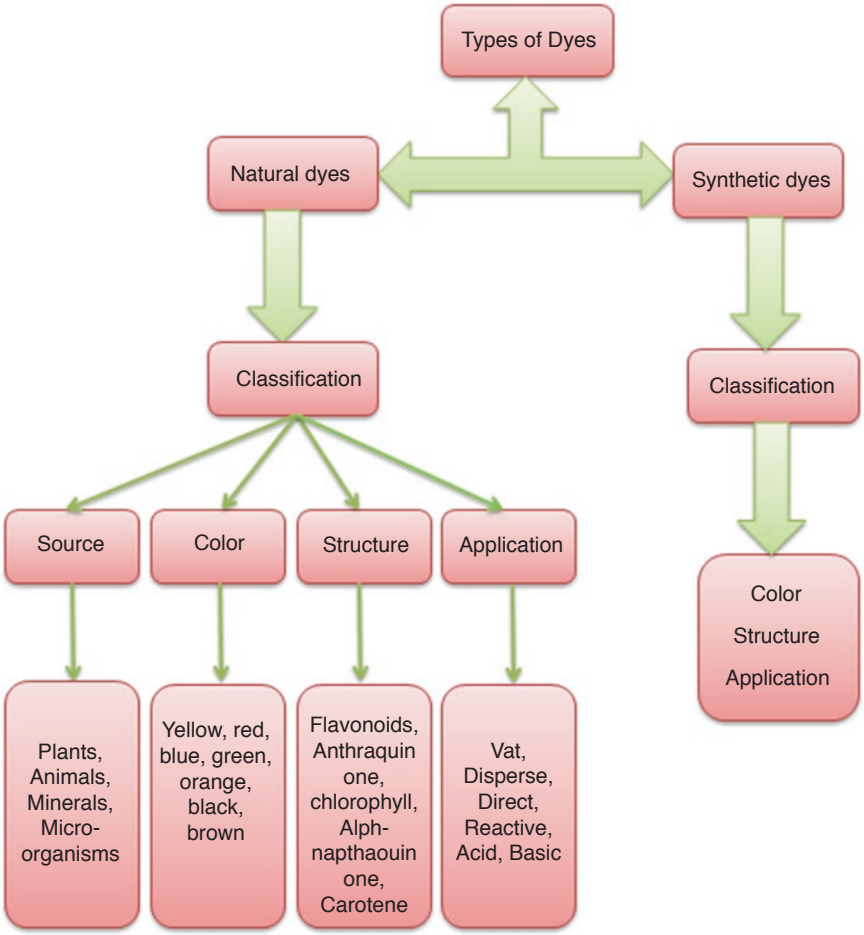
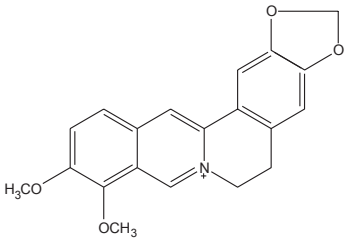


Fig. 1 A view of natural dyes classification

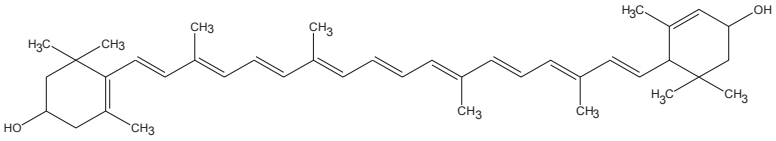
Yellow

Yellow color is abundantly present in the nature where about 28 number of yellow dyes are especially obtained from plants mainly depends on methoxy and hydroxyl substituted flavonoid structure i.e., isoflavones, flavones and flavonols. Other constituents responsible for the yellow color include alkaloids (such as berberine) and betaxanthin. Indian barberry (*Berberis aristata*), European barberry (*Berberis vulgaris*), Amur cork (*Phellodendron amurense*), and coptis root (*Rhizome coptidis*) have berberine structure and give a brilliant yellow color (Bart and Pilz 2011). Lutein present in marigold (*Tagetes erecta*), spinach (*Spinacia oleracea*), broccoli (*Brassica oleracea*), and cabbage (*Brassica oleracea*) and luteolin/butrin from dyer's rocket (*Reseda luteola*) also give a yellow color. Another plant species, i.e.,

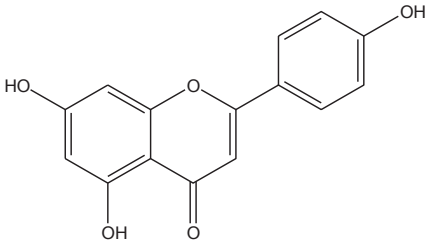
saw-wort (*Serratula tinctoria*), has been known since medieval times that yields a yellow color. All parts of saw-wort plant except the root contain flavonoids in excess amount mainly in the leaves which provide yellow color due to presence of luteolin and luteolin 7-O-glucoside (Guinot et al. 2009).



Berberine

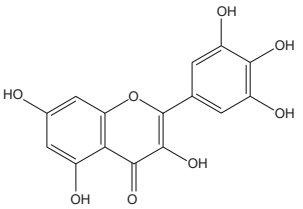


Lutein

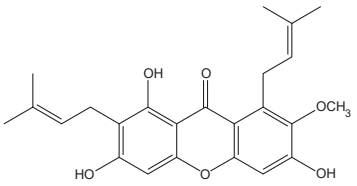


Luteolin

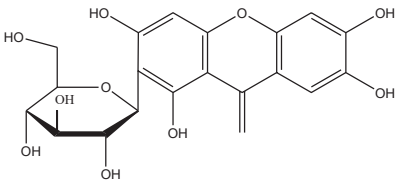
Similarly, Chinese bayberry (*Myrica rubra*) roots contain myricetin as flavonoid derivative and give a yellow color. Rutin from Japanese pagoda tree (*Sophora japonica*) flowers, mangostin from mangosteen (*Garcinia mangostana*), nictanthin from *night-flowering jasmine* (*Coral jasmine*), mangiferin from mango (*Mangifera indica*), isorhanmetin and quercetin from delphinium (*Delphinium zalil*) flowers, and carotenoids from African boxthorn (*Lycium ferocissimum*) and tomato (*Solanum lycopersicum*) give a light-yellow to dark-yellow color (Win and Swe 2008; Kiumarsi et al. 2017).



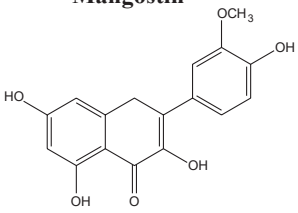
Myricetin



Mangostin



Mangiferin

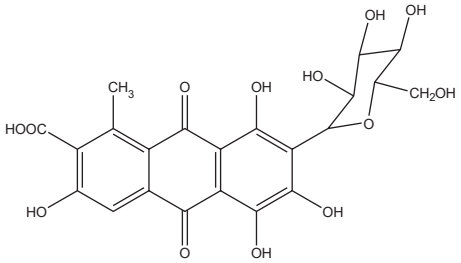


Isorhamnetin

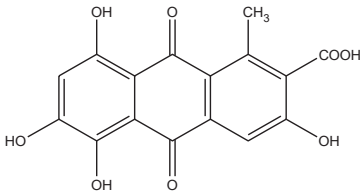
Natural minerals responsible for the yellow pigment include yellow ochre ($Fe_2O_3 \cdot H_2O$), raw sienna ($Fe_2O_3 \cdot MgO$), orpiment (As_2O_3), and litharge.

Red

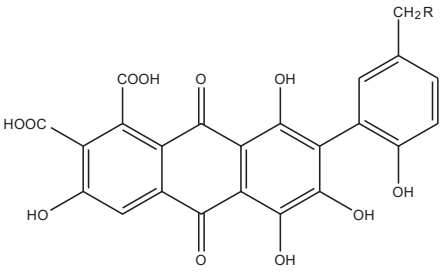
The red color is present in bark, root, and stem of different plants, and is also present in the dry bodies of insects that contain anthraquinone derivative dye. The red color has very good fastness to light and washing. Some of the common examples includes madder (*Rubia tinctorum*), lac (*Kerria lacca*), kermes (*Kermes ilicis*), cochineal (*Dactylopium coccus*), etc. (Ammayappan and Shakyawar 2016). Alizarin present in Indian madder (*Rubia cordifolia*) gives a red color. Carthamin and lycopene present in red bell peppers (*Capsicum annuum*), beetroot (*Beta vulgaris*), strawberries (*Fragaria ananassa*), cherries (*Prunus serotina*), and tomatoes (*Solanum lycopersicum*) also give a red color (Cheng et al. 2017).



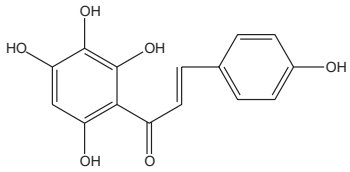
Carminic acid from Cochineal



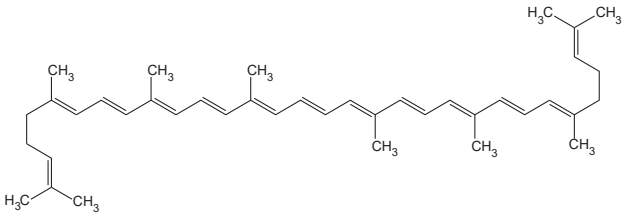
Kermesic acid from Kermes



Laccaic acid from Lac



Carthamin

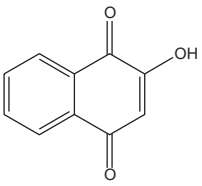


Lycopene

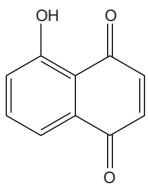
The red color is obtained from natural mineral sources such as red ochre (Fe_2O_3) and red lead (Pb_3O_4).

Orange

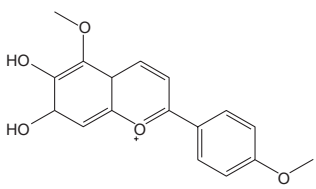
This color basically depends on naphthoquinone, aurones, carotenoids, and anthocyanidin structure, e.g., leaves of henna (*Lawsonia inermis*) contain lawsone, walnut (*Juglans regia*) contain juglone, leaves of Chikarot (*Bignonia chica*) contain carajurin, and carrots (*Daucus carota*) have β-carotene as main coloring component (Kiokias et al. 2016).



Lawsone



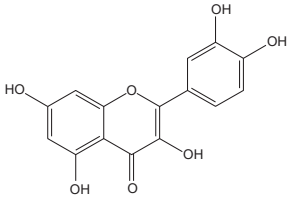
Juglone



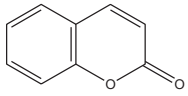
Carajurin

Brown/Black

Black and brown colors obtained from tannin-rich plant sources have also good fastness properties and have the ability to make firm bonding with natural fibers such as cotton, wool, and silk, e.g., harda (*Terminalia chebula*), custard (*Annona cherimola*), apple (*Malus domestica*), etc. Acacetin and quercetin obtained from the bark of babul/kikar (*Acacia nilotica*) provides light-brown to dark-brown color (Mohsin et al. 2016). Dyer's sumach (*Cotinus coggygria*) has been applied in textile and leather dyeing that yields brownish black color. Ober (*Albizia coriaria*) contains coumarin and tannin as major coloring component for brown color. Brown color is also obtained from coconut coir (Cocos nucifera) fibre and is used to dye cotton fabrics as reported by Jabar and Abayomi (2015).



Fisetin

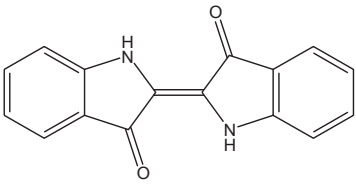


Coumarin

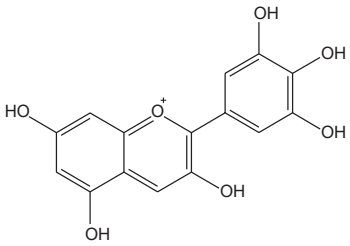
Black pigment is obtained from charcoal black, lamp black, ivory black, bone black, graphite, black chalk, and terre noire.

Blue/Purple

Blue colors have good color fastness to light and washing based on indigoid, anthocyanin, betacyanin structure e.g., woad (*Isatic tinctoria*), indigo (*Indigofera tinctoria*), Dyer's knotweed (*polygonum tinctorium*), Chinise indigo (*Persicaria tinctoria*), Chinese rain bell (*Strobilanthes flaccidifolius*) etc. (Laitonjam and Wangkheirakpam 2011). Similarly sea snails (*Nassarius mutabilis*), Murex (*Bolinus brandari*), cochineal (*Dactylopius coccus*), logwood (*Haematoxylum campechianum*) etc. are also examples of purple dye (Ahn et al. 2012). Blue pea flowers (*Clitoria ternatea*) have been used to provide blue colors (Sinha et al. 2012).



Indigo

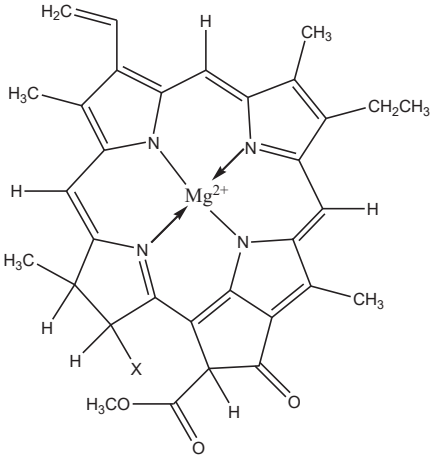


Anthocyanin

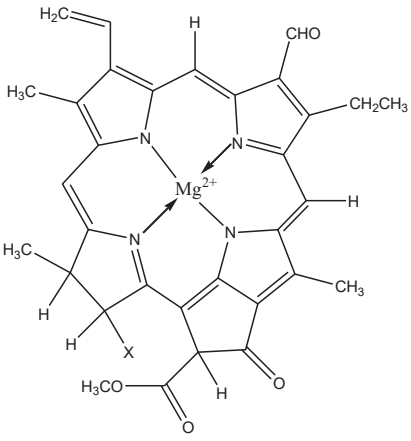
Ultramarine blue and azurite [$Cu_3(CO_3)_2(OH)_2$] are mineral sources that give blue pigment.

Green

Green color is abundantly present in the leaves of the plants as leaves are rich in chlorophyll that is responsible for this color. Chlorophyll is further divided into two groups, i.e., chlorophyll "a" and chlorophyll "b." They differ in their structure because chlorophyll "a" has a methyl group in its ring and gives a blue/green color, while chlorophyll "b" contains formyl group and gives green/yellow colors. Brimstone (*Morinda lucida*) leaves have been used to get green dye due to presence of chlorophyll.



Chlorophyll a



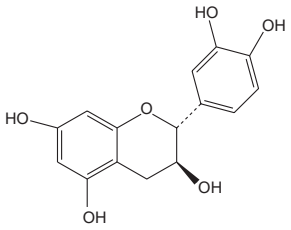
Chlorophyll b

Green pigment is also obtained from mineral sources such as malachite [$Cu_2{\cdot}(OH)_2{\cdot}CO_3$], Vedgiris [$Cu(CH_3COO)_2$], and terre verte.

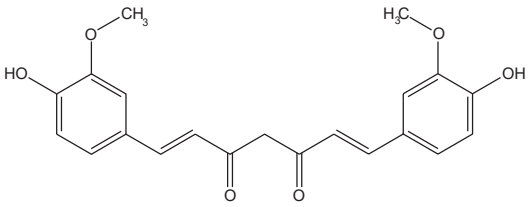
Functional Properties of Natural Dyes

Antimicrobial Characteristics

Textiles especially natural fibers are the main reason for growth of harmful microbes (bacteria, fungi) as these provide suitable environment such as oxygen, moisture, and nutrients. These microbes then transfer to the human skin when they come in contact and cause severe allergic response, dermal infection, and foul smell (Rajendran et al. 2011). Various antimicrobial agents such as polybiguanides, triclosan, nanoparticles of noble metals, quaternary ammonium compounds, etc. are used in textile finishing process to protect the fabric from their growth (Simoncic and Tomsic 2010). However these antimicrobial agents are not effective and cause severe skin disorder and carcinogenic effect during their handling. A recent study exposed the use of natural dyes for coloration as well as for eco-friendly antimicrobial finishing due to the presence of active constituents present in different parts of sources, e.g., curcumin from turmeric rhizomes (*Curcuma longa*), naphthoquinone from tegu (*Salvator merianae*), and catechin from cutch (*Acacia catechu*) bark (Dev et al. 2009; Prusty et al. 2010).



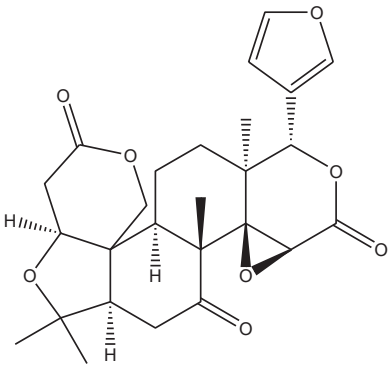
Catechin



Curcumin

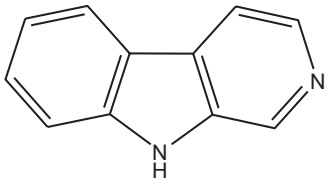
Rhubarb (*Rheum emodi*)-dyed wool fabric contains an anthraquinone-based structure that has the ability to show maximum antimicrobial activity against bacterial strains *Escherichia coli* and *Staphylococcus aureus* and fungal strains *Candida albicans* and *Candida tropicalis* (Khan et al. 2012). It is also stated that

biomordant used in natural dyeing process instead of metallic mordant not only increased the fixation of dye molecule on the fabric but also showed antimicrobial properties, as this contains tannin and polyphenols that have a high ability to increase the inhibition zone against microbes (Prabhu and Teli 2014). Fenugreek (*Trigonella foenum*), *tulsi* (*Ocimum sanctum*), clove (*Eugenia caryophyllata*), and nerium (*Nerium oleander*) contain flavonoids, tannin, phlobatinins, alkaloids, reducing sugars, and terpenoids as bioactive compounds, respectively. Similarly limonoids as active compound that are present abundantly in lilac (*Melia azedarach*) showed antimicrobial property against *S. aureus*, *S. epidermidis*, *B. cereus*, *E.coli*, *K. pneumonia*, *S. flexneri*, and *P. vulgaris*.



Limonoid

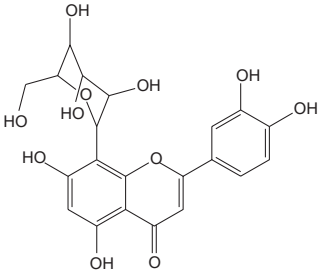
Nenaah (2010) extracted β-carboline from harmal (*Peganum harmala*) seeds and found significant antifungal activity. Other examples which include almond (*Terminalia catappa*), jackfruit (*Artocarpus heterophyllus*), teak (*Tectona grandis*), mulberry (*Morinda citrifolia*), lebbek (*Albizia lebbeck*), and asoka tree (*Saraca asoca*) show remarkable antimicrobial activity against various bacterial and fungal species (Prusty et al. 2010; Baliarsingh et al. 2012).



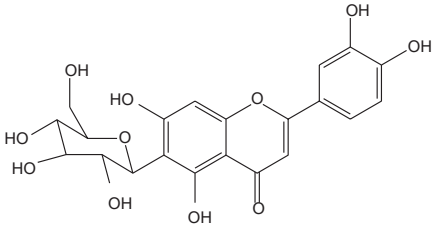
β-Carboline

Antioxidant Characteristics

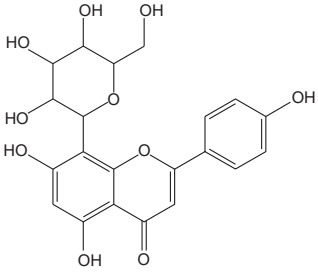
Antioxidant activity prohibits auto-formation of free radicals that may damage the living cells by several oxidant species, e.g., H_2O_2,. It causes DNA damage, protein degradation, and lipid peroxidation that can lead to brain damage, heart attack, and cancer. These severe oxidants may come from textile industries when fabric is treated with several oxidized reagents. Various antioxidant supplements are available to prevent an oxidant attack, but they are not as efficient as they should be. Extensive researches and reports reveal that plant-derived natural colorants contain polyphenol constituents such as flavonoids and tannin that have antioxidant properties, e.g., orientin, homoorientin, vitexin, and isovitexin as flavonoid derivatives from bamboo plants (*Bambusa vulgaris*) (Guo et al. 2013).



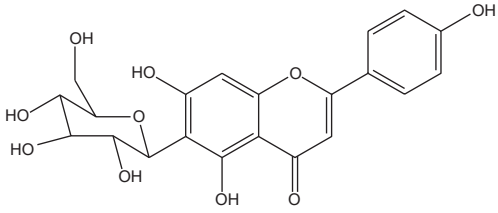
Orientin



homoorientin

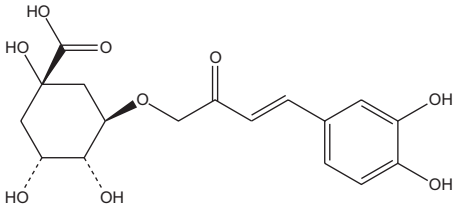


Vitexin



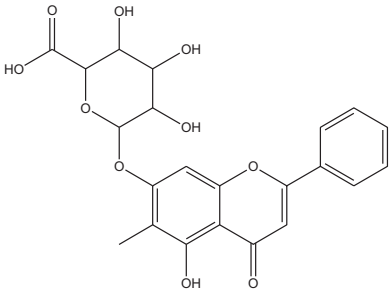
Isovitexin

Nahak and Sahu (2010) found that neem (*Azadirachta indica*) and mahaneem (*Melia azedarach*) contain phenolic compounds as major active species that have the ability to act as an antioxidant. Shahid et al. (2017) dyed silk fabric with honeysuckle (*Lonicera japonica*) and got light yellow-brown shades. They found that honeysuckle contains chlorogenic acid which is the main bioactive component responsible for the antioxidant property of the dyed sample.

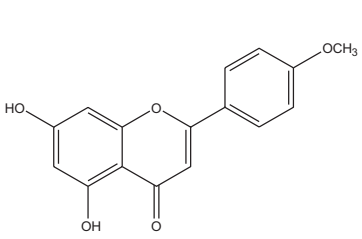


Chlorogenic acid

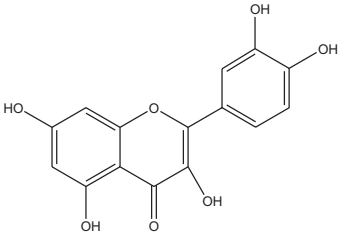
Similarly, Hwang and Hong (2016) examined the artificial silk "viscose rayon" dyed with gallnut (*Quercus infectoria*) extract and concluded that the fabric showed remarkable antioxidant property. Other examples of natural dye sources that have the ability to neutralize the free radicals that accelerate cellular aging are harmal (*Peganum harmala*) (Khlifi et al. 2013), carotenoids from tomato (*Solanum lycopersicum*) (Baaka et al. 2017), baicalin from skullcap (*Scutellaria baicalensis*) (Zhou et al. 2016), acacetin and quercetin from kikar (*Acacia nilotica*) (Rather et al. 2017), etc.



Baicalin



Acacetin



Quercetin

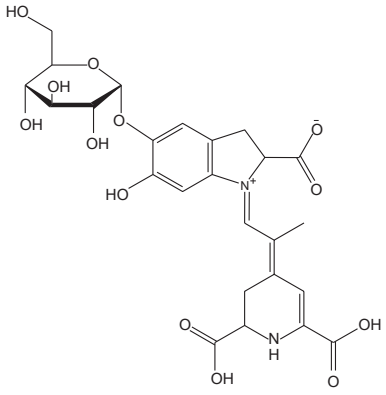
Deodorant Characteristics

Sweating which occurs due to the high temperature of the surrounding is a cooling response of the human body to maintain body temperature. This sweating contain various constituents such as carbohydrate, proteins, sulphur compounds, ketone etc. that are easily biodegradable by bacteria present in the atmosphere and release ammonia gas and acetic acid that is the main cause of unpleasant odor. Nowadays people are more sensitive about their health and want a clean and fresh environment (Cistea and Vilarem 2006). Recently it is observed that fabric dyed from natural sources absorbs odor-causing gas and keeps the body fresh, e.g., peony (*Paeonia suffruticosa*), clove (*Syzygium aromaticum*), gardenia (*Gardenia jasminoides*), coffee sludge (*Coffea canephora*), gallnut (*Quercus infectoria*), etc. (Lee et al. 2015). It was concluded from the studies of Lee et al. (2017) that dyed cotton, wool, and silk fabric with myrrh (*Commiphora myrrha*) and pine cone (*Pinus albicaulis*) extract exhibit noteworthy deodorizing property against ammonia and acetic acid. Hwang et al. (2008) dyed cotton, wool, and protein fabric with sickle senna (*Cassia tora*), pomegranate (*Punica granatum*), coffee sludge (*Coffea canephora*), and gardenia (*Quercus infectoria*) using different mordants and found that these natural dyes help to suppressed the unpleasant smell and keep the fabric fresh. Similar results have been found on natural fabric dyed with cork tree (*Quercus suber*), wood fern (*Dryopteris crassirhizoma*), makino (*Chrysanthemum boreale*), and wormwood (*Artemisia annua*) by Lee et al. (2010). Jung (2016) also examined deodorizing properties of gallnuts (*Quercus infectoria*), palm (*Areca catechu*), and pomegranate (*Pomegranate*) dyed on silk fabric. Jang and Jung (2016) also investigated sage weed (*Salvia plebeian*) which enhanced the deodorizing property of cotton fabric and made the environment hygienic. All these studies showed that colorants extracted from the plants can be used as substitutes of synthetic deodorants.

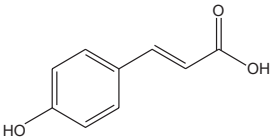
UV Protection Characteristics

UV radiation is an invisible light that comes from sunlight having a wavelength range of 200 to 400 nm. However, many advanced developments have led to ozone depletion which enables UV radiation to hit the surface of the earth straightforwardly and pose harmful effects on living organisms by disturbing their metabolism and causing skin cancer. Laborers, athletes, farmers, and fishermen are more susceptible to such rays as they have to spend more time under the sun. For such reason, many sunscreen lotions and creams and modified fabrics with enhanced functional properties have been commercially available to protect the skin against UV

radiation. However, recently, the focus shifted on to dyed textiles that absorb UV radiation and prevent their transfer to the human skin (Grifoni et al. 2011). Many researchers found that natural dyes have structures, i.e., flavonoid, curcumin, anthraquinone, etc., that are able to absorb such rays and provide shield to the skin. It was observed that raw silk fabric has bad UV protection property but when dyed with curcumin-containing natural dye showed remarkable anti-UV property. Curcumin obtained from rhubarb (*Rheum emodi*) and yellow gardenia (*Gardenia jasminoides*) when dyed on silk fabric makes the fabric highly UV protective. Similarly honeysuckle (*Lonicera periclymenum*) which contains chlorogenic acid when used to dye wool fabric behaves as excellent natural UV protective agent (Hou et al. 2012). Chattopadhyay et al. (2013) dyed wool fabric with eucalyptus (*Eucalyptus globulus*) leaves and found remarkable UV protective source (Mongkholrattanasit et al. 2011a, b). The studies of Hou et al. (2013) show that wool fabric dyed with natural dye obtained from orange peel (*Citrus sinensis*) showed higher UPF value as compared to the wool fabric dyed with synthetic dye. Similarly juglone extracted from Caucasian walnut (*Pterocarya fraxinifolia*) when dyed on wool fabric gives best result against UV radiation (Ebrahimi and Gashti 2015). Bonet-Aracil et al. (2016) found red, black, and green tea (*Camellia sinensis*) as anti-UV agents due to the presence of catechin component after dyeing of cotton fabric. Betalains obtained from pokeweed (*Phytolacca berries*) and flavonoids and hydroxycinnamic acid from curry plant (*Helichrysum italicum*), wild madder (*Rubia peregrine*), daphne (*Daphne gnidium*), lavender (*Lavandula stoechas*), and artichoke (*Cynara scolymus*) have shown fantastic anti-UV properties (Grifoni et al. 2014; Liu et al. 2014).



Betalains



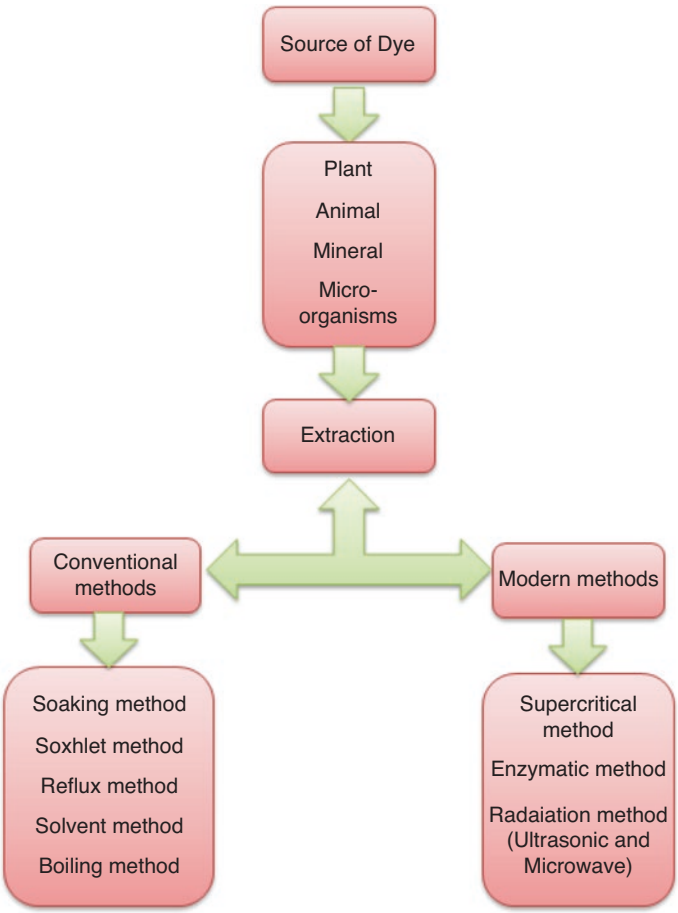
Hydroxycinnamic acid

Use of Sustainable Extraction and Dyeing Methodology in Natural Dyeing Process

There are two major methods for extraction of natural dyes on which its sustainability is revived owing to its extraction yields and good color characteristics. These methods include:

1. Conventional method
2. Modern method

Here is a flow sheet diagram of the extraction method.



Conventional Method

The pretreatment of fabric such as scouring, bleaching, and mercerization to increase the dyeing capability can cause pollution to the environment and consume water (Khandegar and Saroha 2013). The extraction of natural dye material with conventional methods such as soaking, immersion Soxhlet method, refluxing method, boiling method, and mechanical shaking has been used based on their component polarity and thermal stability due to which these methods require huge amount of solvent and time. Refluxing method has been utilized to extract the colorant from madder plant. Similarly, dye component from eucalyptus (*Eucalyptus globulus*) leaves such as quercetin and rutin has been extracted using reflux method (Mongkholrattanasit et al. 2010). Soxhlet method has been used in extracting colorant from pomegranate peel (*Punica granatum*). However these will not provide the effective color yield and color strength on fabric and also consume a lot of energy and labor.

Modern Methods

Technology basis extraction helps to reduce the water consumption and pollution to the environment by modifying the surface of the fabric and also extracted colorant effectively. These advanced methods include solid-phase microextraction, compressed gas, supercritical fluid extraction, pressurized liquid extraction, and radiation technology. It was found that compressed gas has been applied for the extraction of natural colorant such as carotenoid dye that gives high yield (Kulkarni et al. 2011). Supercritical fluid method contains compressed CO_2 gas. In this method, the material is dissolved with gas and converted into dissolved dye which is then absorbed on fabric effectively where the gas is recovered after dyeing process thus acts as sustainable extraction and dyeing method.

But the role of radiation-induced extraction and dyeing is gaining widespread popularity due to their rapid, clean, and uniform efficiency (Adeel et al. 2017).

The radiation method includes:

1. Gamma radiation
2. Plasma radiation
3. Microwave radiation
4. Ultraviolet radiation
5. Ultrasonic radiation

Gamma Radiation

Gamma radiation comprises of high energy of about 100 KeV having high frequency of 10^{19} Hz. These radiations have promising effect in extraction of natural dye, to modify fabric surface to make them highly hydrophilic and to improve their dye ability and provide high productive yield (Bhatti et al. 2012). The dye from red calico (*Alternanthera bittzickiana*) leaves is extracted by using different doses of gamma radiation where exposed cotton fabric has given excellent shades and fastness properties (Khan et al. 2014).

Microwave Radiation

Similarly, microwave rays can be a potential alternative to the conventional method of extraction and dyeing due to its less consumption of energy, solvent, and time of processing. It gives high color yield mainly because of its heating effect that is generated by the dipole rotation of the solvent that ultimately rises the temperature of the solvent to solubilize the coloring compound. Dabiri et al. (2005) used this technology to extract the coloring component such as purpurin and alizarin from madder (*Rubia tinctorum*). Similarly, mulberry (*Morinda citrifolia*) root consists of an anthraquinone-based structure that has been effectively extracted using microwave-assisted extraction.

Ultrasonic Radiation

Ultrasonic radiation is one of the effective methods in extracting coloring component from natural dyeing materials by generating a phenomenon of cavitation which is the expansion and collapse of microbubbles in the liquid media (Rumeau et al. 2004). Its role in extraction and dyeing of various natural and synthetic fabrics has been gaining much popularity due to its eco-friendly nature. Weng and Sheu (2000) used ultrasonic method to extract the anthraquinone-based dye from the Rhei Rhizoma (*Rheum palmatum*). Similarly, lac (*Kerria lacca*) dye has been extracted using ultrasonic treatment, and its performance has been compared with the result obtained by conventional method (Kamel et al. 2005). Natural dye from false daisy (*Eclipta prostrata*) has been used to dye cotton fabric using ultrasonic method that has given good dye uptake on fabric (Vankar et al. 2007). Sivakumar et al. (2011) extracted dye from green wattle (*Acacia decurrens*) bark, marigold (*Calendula officinalis*) flower, pomegranate (*Punica granatum*) rind, four-o'clock plant (*Mirabilis jalapa*) flowers, and cockscomb (*Celosia argentea*) flowers using sonicator technique and found effective color yield.

Plasma Technique

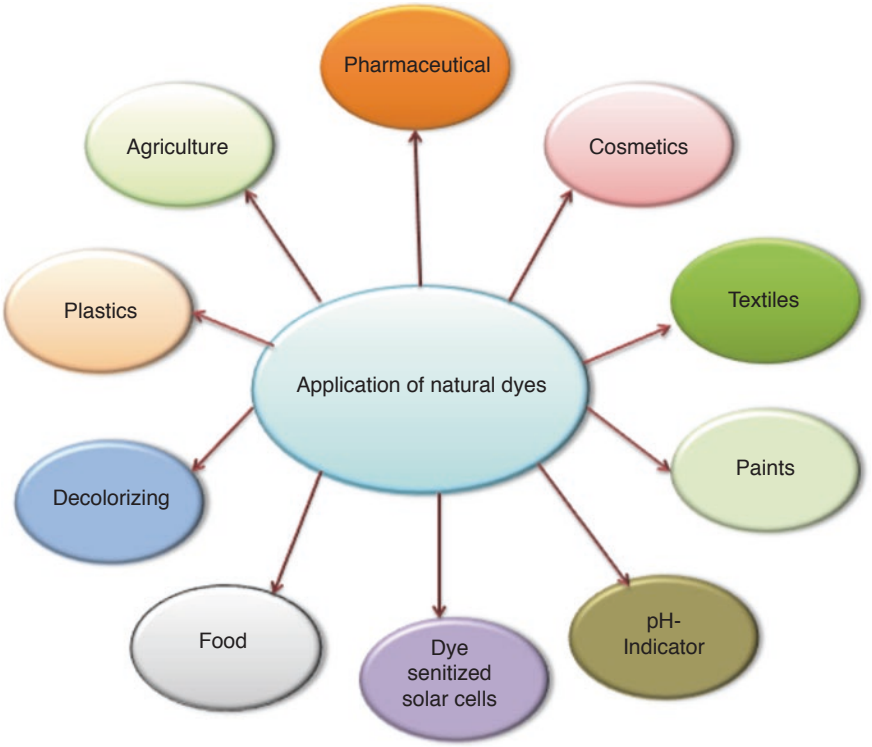
Plasma being ionized gases consist ions, electrons, neutrals, excited molecules, and photons. Cold plasma or low temperature plasma and atmospheric pressure plasma using oxygen, nitrogen, air, argon, helium or fluorine as gas source is an example of plasma technique that has the ability to modify the surface of the fabric without affecting the interior structure of the fabric and cause surface roughness, etching, coating, cleaning, oxidation, surface activation (Ren et al. 2017). This treatment helps to increase the absorbance of water, wetting property, wicking property, dyeing ability, capillary action, and adhesion of dye component to the fabric. Plasma technique also helps in improving the functional properties of the fabric such as antimicrobial, antioxidant, anti-UV, and flame-retardant properties. It is the most effective technique that consumes less water, energy, and chemicals and considers the environment factor (Ozguney et al. 2017). Gorjanc et al. (2016) used low-pressure plasma on cotton and bamboo yarn and dyed with Japanese knotweed (*Fallopia japonica*) and found remarkable dye ability and color strength. They found that this treatment increases the hydrophilicity of the fabric that increases the dye ability of the fabric. Similarly, Haji et al. (2016b) pretreated wool fabric with oxygen plasma to reduce the structural scale of fabric and increase the dye ability of wool fabric. The fabric was then dyed with grape (*Vitis vinifera*) leaves which were found to have good color strength. The same result has been found on oxygen plasma-treated wool fabric dyed with cotton pods (*Gossypium hirsutum*) (Haji et al. 2016, b). Dave et al. (2016) dyed atmospheric plasma-treated leather with natural dyes Eco Garnet brown, Eco Hill brown, Eco Turkey Red, and Eco smoky gray and achieved best results.

Ultraviolet Radiation

UV is an electromagnetic radiation whose wavelength ranges from 100 to 280 nm. Ultraviolet radiation has also played a role in the textile industry with various beneficial aspects such as involving a dry process and being inexpensive, time- and energy-efficient, and chemically effective particularly in wool, polyester, and cotton fabrics (Rehman et al. 2017). UV rays are utilized to change the surface of the fabric by improving its wetting, wicking, dyeing, antifelting, and hydrophobic properties. These rays also encourage to generate the polar group (carboxylic acid) on the fabric structure that makes the fabric hydrophilic and improves the interaction between the dye molecule and fabric. These rays improve the dye exhaustion that leave the less coloring component in the waste water and act as ecofriendly source to promote natural dyeing (Micheal and El-Zaher 2005). In a nutshell, the modern tools are now being welcomed to make the dyeing process more sustainable and eco-friendly.

Application of Natural Dyes

Natural dyes have a broad area of application due to its eco-friendly nature. These applications include textile, cosmetics, painting, inks, agriculture, dye-sensitized solar cells, decolorizing, pH indicator, pharmaceutical, etc. Some of these are discussed here in detail.

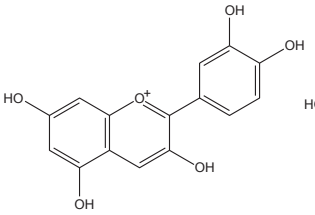


pH Indicator

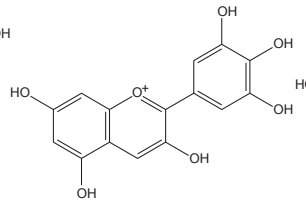
Indicator is a substance that tells us an end point in any acid-base titration as well as the visual colorimetric changes. Various synthetic indicators have been used such as phenolphthalein, methylene blue, methyl red, methyl orange, etc. But these synthetic ones are costly and impose pollution to the environment (Calogero et al. 2012; Zajko and Klimant 2013). Similarly in the food industry, its role is vital. These pH sensors mainly comprise of two part one is solid support and second is dye which is embedded on this film and whose color is change with varying of pH (Golasz et al. 2013). For such film preparation, various natural biodegradable polymers, i.e., chitosan, pectin, starch, and agar, have been used. Many examples

disclose the usage of plants as a natural pH indicator (Khan and Farooqui 2011; Chen and Gu 2013). However, it was first reported by Sir Robert Boyle in 1664. The component in the plants such as tea (*Camellia sinensis*), grapes (*Vitis vinifera*) etc. when react with acid or base it change its color. Some comprises just two colors, one on acid or one on base, while others show whole spectra.

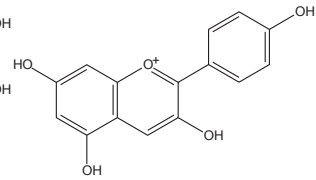
Anthocyanin is the most commonly used pH indicator as its color vigorously varies with the change of its surrounding environment due to the presence of a conjugated system present in its structure. These structures are cyanidin, delphinidin, pelargonidin, peonidin, and petunidin which change their structure when pH changes.



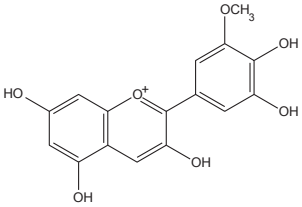
Cyanidin



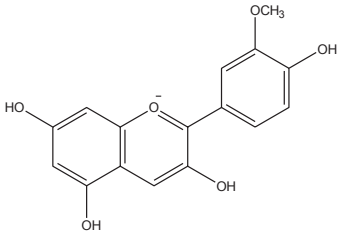
Delphinidin



Pelargonidin

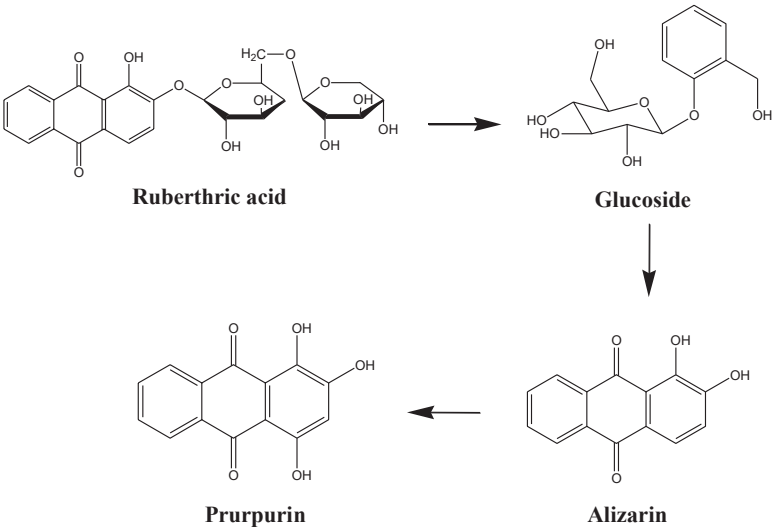


Petunidin



Peonidin

Anthocyanin is the subclass of the flavonoid group that gives blue, red, and purple color to the flower and leaves (Chandrasekhar et al. 2012). Many examples revealed the application of anthocyanin-containing natural dyes as pH indicator (Macial et al. 2015). One of the examples includes red cabbage (*Brassica oleracea*) that contains anthocyanin as main coloring component and gives different colors at different pH such as red to blue at low pH and green at high pH (Pourjavaher et al. 2017). Anthocyanin present in China rose (*Hibiscus rosa*), Mart. ex choisy (*Ipomoea fistulosa*), and blue pea (*Clitoria ternatea*) has been used as natural pH indicator. Similarly the dye obtained from *Acalypha wilkesiana* leaves is applied as pH indicator (Bhise et al. 2014). Madder (*Rubia tinctorum*) root also changes its structure as pH changes which ultimately gives different colors, e.g., ruberthyric acid to glucose to alizarin to purpurin.



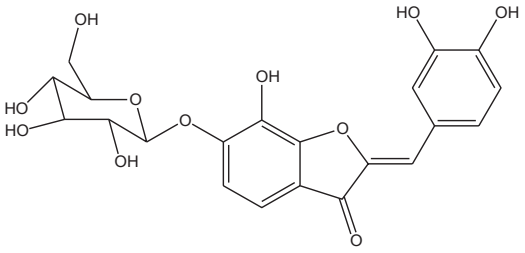
However it gives a red color in acidic pH. Similarly, sawdust gives a yellow color at low pH and red color at high pH. Carminic acid obtained from cochineal (*Dactylopius coccus*) gives yellow at low pH and purple at high pH. Curcumin from Indian curry powder (*Murraya koenigii*) gives yellow at neutral pH and red at alkaline pH. Chayroot (*Oldenlandia umbellata*) gives red at pH 10, yellow on pH 5–6, and green on pH acidic. Tea gives a dark color in basic media and lighter in acidic media. Other examples used as natural pH indicator obtained from natural sources are turmeric (*Curcuma longa*), jack tree heartwood (*Artocarpus heterophyllus*), ratanjot (*Alkanna tinctoria*), beetroot (*Beta vulgaris*), jungle flame (*Ixora coccinea*), etc. Hence natural dyes can be successfully used as alternative to synthetic indicator.

Dye-Sensitized Solar Cells

Solar energy is considered to be a free, clean, and renewable source of energy that fulfills the energy demand of global manufacturing system and can be used as alternative to the dropping energy source (Smestad and Steinfeld 2012). This energy has been utilized by manufacturing high efficient and low-cost photovoltaic cells in which electrons are excited to the high energy level by the photons and then collected to use as electrical energy. However their fabrication process was very complex and very costly and has been advanced by using dye-sensitized solar cells which are third-generation photovoltaic cells that have the same function with low-cost and high efficient results (Gratzel 2003). Here dyes are fixed on the TiO_2- or ZnO_2-based

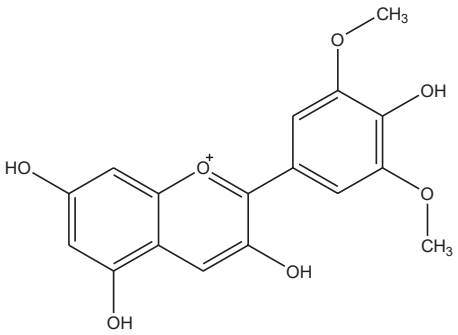
film surface and absorb photons from the sun, and these photons excite the electrons of dye molecule transferred to the conduction band (Gray 2003). Most of the synthetic sensitizers, for example, ruthenium complex dye (Ru polypyridyl complexes) and organic donor acceptor dyes (coumarin dye, cyanine dye, indoline dye), have been used, but these dyes contain high heavy metals that pose harmful effects to the environment (Patrocinio and Murakami Iha 2010). For such reason natural dyes are considered to be the main substituted, cheapest, sustainable, and acceptable efficient source of manufacturing dye-sensitized solar cells that are obtained from leaves, bark, flower, stem, root, shoot, and fruit of the plants. These sources contain various functional groups such as anthocyanin, flavonoids, carotenoids, cyanins, tannins, and chlorophyll that have been utilized as DSSC (Zhou et al. 2011).

Flavonoid-based natural dyes require less energy for their electron excitation and thus can be utilized efficiently for such purpose. Flavonoids are present abundantly and can be extracted from several plant sources. Many examples give us information of the use of flavonoids in DSSC application, e.g., bitter leaves (*Vernonia amygdalin*) comprise flavonoid structure and have been utilized as DSSC by Boyo et al. (2012). Ekanayake et al. (2013) found that dabai (*Canarium odontophyllum*) contained pelargonidin, cyanidin, and maritimein which are flavonoid structures. They used this plant as DSSC application and found efficient result.



Maritimein

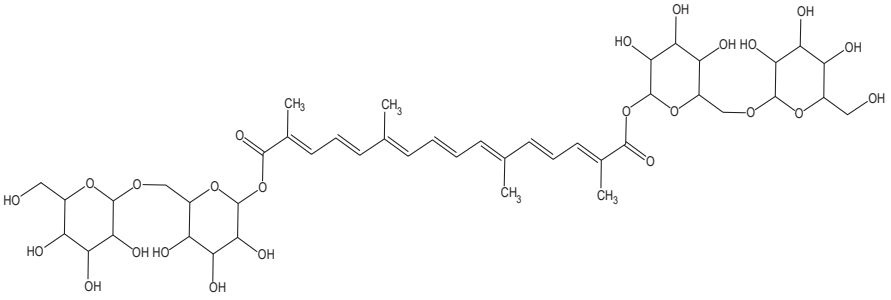
Anthocyanin-based natural colors have carbonyl and hydroxyl groups that make them water-soluble. Most of the natural dye-based solar cells are mainly comprised of anthocyanin (San Esteban and Enrriquez 2013). Different researchers used anthocyanin-containing plant species as dye-sensitized solar cells. Anthocyanin structures extracted from box-leaved barberry (*Berberis buxifolia*) and Brazilian grape tree (*Myrtus cauliflora*) and applied to fabricate dye-sensitized solar cells. Cyanidin and delphinidin from rosella (*Hibiscus sabdariffa*) and turnatin from blue pea (*Clitoria ternatea*) have been practiced by Wongcharee et al. (2007) as sensitized solar cell. Similarly malvidin-3-fructoside present in grapefruits (*Citrus paradise*) has been studied for sanitization purpose (Szostak et al. 2015).



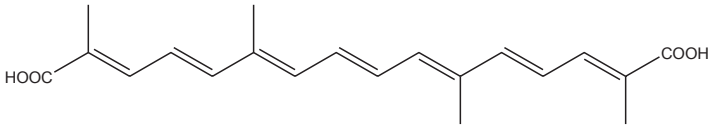
Malvidin

Other examples that contain anthocyanin and have been successfully applied as sensitized solar cells are mulberry fruit (*Morus alba*) (Chang and Lo 2010), yellow kopsia (*Kopsia flavida*) bark, red cabbage (*Brassica oleracea*) (Li et al. 2013), and Malabar melastome (*Melastoma malabathricum*) fruit pulp (Singh et al. 2014). It was also concluded that cocktail dye (mixing of different dyes) has enhanced the conversion efficiency by increasing the absorption intensity, e.g., blue berries (*Cyanococcus angustifolium*), purple cabbage (*Brassica oleracea*), and grapes (*Vitis vinifera*) contain chlorophyll and anthocyanin groups that have been used as cocktail dye-sensitized solar cells (Syafinar et al. 2015).

Carotenoid based natural dyes have also play an important role in photo sanitizer that has been divided into two groups i.e. xanthophyll (Oxygen containing) and carotene (no oxygen). It is observed that carotenoid groups that contain carboxyl group have a major contribution in the conversion of solar energy into electrical energy (Wang et al. 2007). Two natural colorants such as crocin and crocetein obtained from saffron (*Crocus sativus*) have been used as carotene source in sanitization.



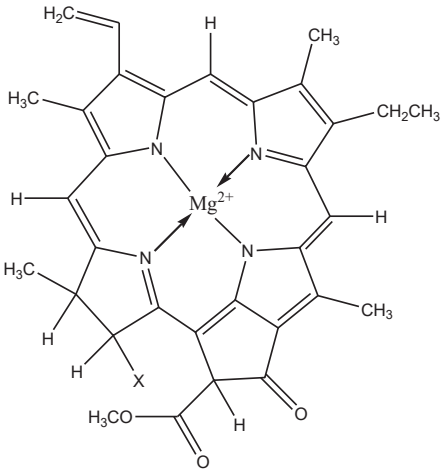
Crocin



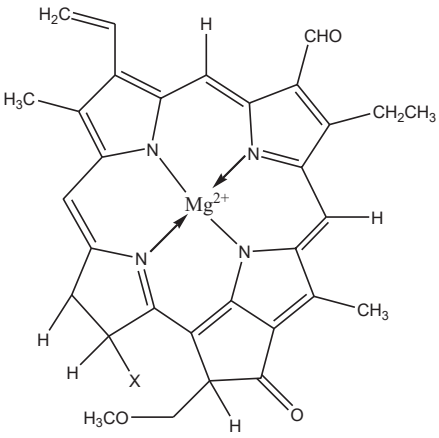
Crocetein

Similarly, Eka et al. (2013) studied the effect of chicken egg banana (*Musa aromatic*) and citron (*Citrus medica*) fruits on conduction band film as these contain carotenoids as main pigment. Cocktail dye used by Shanmugam et al. (2013) has obtained carotenoid from ivy gourd (*Coccinia grandis*) and anthocyanin from red frangipani (*Plumeria rubra*) flowers and is utilized for sanitization purposes.

It is considered that chlorophyll is one of the economical sources of application in DSSC (Amao and Komori 2004). Both chlorophyll a and chlorophyll b take part in photosensitization. Chang and Lo (2010) and Chang et al. (2013) obtained chlorophyll from pomegranate leaves (*Punica* granatum) and wormwood (*Artemisia absinthium*) and analyzed their conversion efficiency.

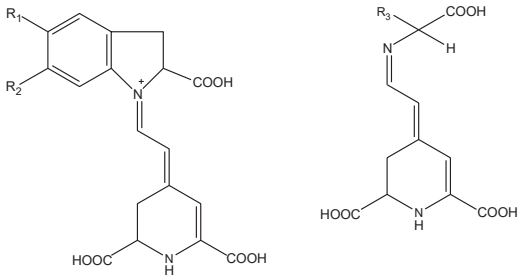


Chlorophyll a



Chlorophyll b

Betalains are coloring components present in fruits, roots, and flowers of different plant species. Betalains are further classified into two groups such as betacyanin (red violet pigment) and betaxanthin (yellow orange pigment) that have also been studied by different research scholars in DSSC fabrication.



Betacyanin and Betaxanthin

Betalains were extracted from red root (*Beta vulgaris*) that has been practiced for photosensitization and observed with good conversion efficiency compared to anthocyanin group (Sengupta et al. 2015). Similarly, Hernandez-Martinez et al. (2013) extracted a coloring component from *Beta vulgaris* by using tetraethyl orthosilicate which helps in increasing the conversion efficiency. On the other hand, prickly pear (*Opuntia ficus-indica*) fruit, bougainvillea (*Bougainvillea glabra*) flowers, and red turnip (*Brassica rapa*) juice extract have been used by Calogero et al. (2010) and Hernandez-Martinez et al. (2011) to obtain cocktail dye by extracting betaxanthin and betacyanin from these plant species. Other examples include mulberries (*Morus alba*), blackberries (*Rubus fruticosus*), grapes (*Vitis vinifera*), eggplants (*Solanum melongena*), radicchio (*Cichorium intybus*), etc. that have the same role in manufacturing dye-sensitized solar cells.

Cosmetics

Every person especially women has the desire to look beautiful and attractive for sense of pleasure and to feel confident. They used different beauty products such as lotion, cream, lipstick, etc. that make them look active and fresh. One factor which increases the popularity of cosmetics nowadays is that women are becoming socialized and more conscious about fashion. However, the prolonged use of synthetic cosmetics causes severe side effects to their skin. But recently it has been studied that natural active ingredients used in cosmetics not only enhanced beauty but also gave healthy and functional benefits to the skin such as lip care, UV protection, and antiaging. Several examples of natural dyes that have been used in cosmetics include naphthaquinone from henna, carotenoids from saffron and peppers, chlorophyll from green leaves pomegranate, indigo, and curcumin from turmeric.

Henna, locally known as “mehandi,” contains lawsone as major coloring component. It is used significantly in cosmetics to color the palm of the hands, feet, nail and hair. It is also used in shampoos, hair rinses, and conditioners and helps to

grow hair. Other plants that contain lawsone are rhubarb, calendula, and chamomile that have the same application. Due to the antibacterial activity of sappan wood extract, it has been used in different cosmetics products such as creams, gels, lotion, and lipsticks. It also gives some aesthetic effect to soaps (Xu and Lee 2004). As sandalwood contains antioxidant property, it has been used as acne cream, facial scar cream, and talcum powder. It gives glowing effect to the skin. It also gives a purplish rose color to soaps and lipsticks (Manjunatha 2006). This plant contains anthocyanidin that shows antioxidant activity and betalains as main coloring components. It has wide application in hair color, shampoos, bleaching, facial moisturizer, and antiaging and anti-acne creams. *Adhatoda vasica* belongs to the *Acanthaceae* family. The extract obtained from vasaca is used as a skin cream. Aloe vera has several advantages and has been used to smoothen the skin in moisturizing lotions and creams and for treatment of skin burns. It has also been used as hair tonic. Rhizome of turmeric (*Curcuma longa*) contains antioxidant and anti-inflammatory characteristics and has been used in facial treatments, face creams, and balms. Extract of badam (*Prunus amygdalus*) is considered to increase the fairness of the skin. It also has the ability to protect the skin from sunburn. Some other examples of botanical sources such as pods of shikakai (*Acacia concinna*), flower of arnica (*Arnica montana*), leaves of birch (*Betula pendula*), and seed oil of mustard (*Brassica spp*.) have been used as hair cleanser, hair oil, and shampoo.

Conclusion

The revival of natural dyes in different fields is due to their renewable, cheap, and eco-friendly nature. A wide selection of natural colorants has been obtained from plants, animals, and minerals which are now being encouraged to use in cosmetics, fashion, solar cell industry, etc. Various techniques have been developed in order to get maximum colorants yield from these sources with enhanced hues. However to adopt these methodologies on a large scale is a big challenge which is entails the use of modern tools such as radiation-induced treatments for extraction as well as applications. The applications discussed in this chapter show the resurgence of a natural colorant with potential implementations in the industry.

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Assessment of Pesticide Residues in Vegetables of Telangana State

Syeda Azeem Unnisa

Introduction

The diverse climate of India ensures availability of all varieties of fresh fruits and vegetables. India ranks second in fruit and vegetable production in the world, after China. According to National Horticulture Database published by National Horticulture Board, during 2014–2015 India produced 169.478 million metric tonnes of vegetables under cultivation area at 9.542 mh (NHB 2015).

The chemical substances in pesticides are commonly used in modern agriculture practices to protect the crops from diseases and pests (Guler et al. 2010). Vegetables are important components of the human diet as it consists of calories (9%), magnesium (16%) and iron (19%) on the recommended intake value (Iqbal et al. 2009). Due to modern agriculture, vegetables are potential source of harmful and toxic pesticides, which has increased the concern about food safety worldwide (Radwan and Salama 2006). It is essential that pesticides should be controlled and reduces in precise level due to their relative toxicity to the human health and environment (Jiang et al. 2009). Pesticide is a mixture of substances or a substance for destroying, controlling and preventing pest, vectors or unwanted species interfering with the production, processing, storage, transport and marketing of food (FAO 2002). The residues of pesticide in vegetables are a major public health concern in developing countries as well as developed countries (Dikshit et al. 2003). The harmful pesticide residue which remains on edible portion of vegetables has become the great cause of concern (Boon et al. 2008).

In India, about 6000 tonnes of active ingredients are used by farmers to control pests of fruits and vegetables (Mohan and Gujar 2003). Pesticide residue level investigation in vegetables is a main concern of many scientists and researchers to evade possible risks of toxicity to human health (Osman et al. 2010). The pesticide

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residues can cause birth defects, depression, miscarriages, respiratory disorders and neurological deficiencies (Lehotay et al. 2007). The reason for investigation to assess pesticide residues in vegetables of Telangana State (Hyderabad and Ranga Reddy) is to know the concentration and its potential risk to the consumer health.

Study Area

The study was conducted for the assessment of pesticide contamination in vegetables of Telangana State. The sampling locations in Telangana State (Hyderabad and Ranga Reddy) are shown in Fig. 1. Hyderabad is the capital city of Telangana and Andhra Pradesh and is the sixth largest city in India. The annual mean temperature is 26 °C, whereas summers are hot with maximum temperatures of 40 °C, and winter has varying temperatures from 14.7 to 28.6 °C and with annual rainfall of 812.5 mm. In Hyderabad there are 12 no. of Rythu Bazars and 3 no. of vegetable market committees. Nearly 70–80% of vegetables are coming from the other state to city of Hyderabad.

Material and Method

Sampling

For the present study sampling was conducted for the period from July 2015 to August 2016. Three vegetables were selected for the assessment of various pesticide residues. Sixty samples of vegetables, namely, cauliflower, cabbage and brinjal, were collected from Telangana State (Hyderabad and Ranga Reddy) shown in Table 1. The

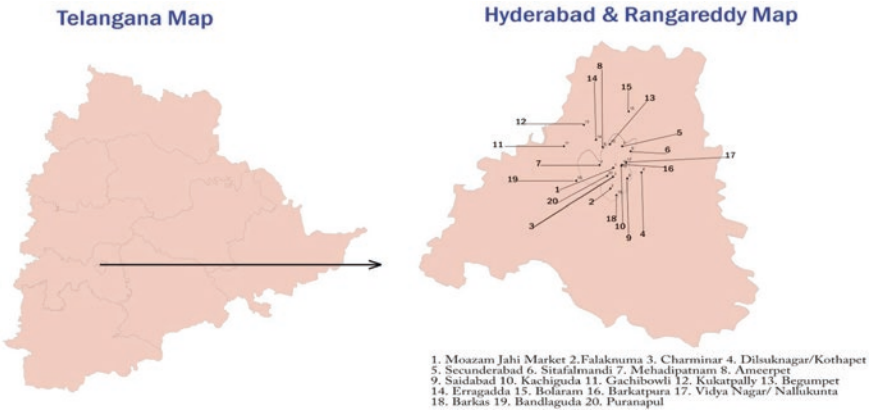

Fig. 1 Sampling points of vegetables in Telangana State (Hyderabad and Ranga Reddy)

Table 1 Sampling locations of vegetables in Telangana State (Hyderabad and Ranga Reddy)

Sample no	Location	Sample no	Location
1	Moazzam Jahi Market	11	Gachibowli
2	Falaknuma	12	Kukatpally
3	Charminar	13	Begumpet
4	Kothapet	14	Erragadda
5	Secunderabad	15	Bolarum
6	Sitaphalmandi	16	Barkatpura
7	Mehdipatnam	17	Nallakunta
8	Ameerpet	18	Barkas
9	Saidabad	19	Bandlaguda
10	Kachiguda	20	Purana pul

collected vegetable samples were stored at 4 °C until further extraction and analysis (Islam et al. 2009) by chopping and homogenizing 200 gm portion which was stored in glass stopper bottle (Cook 2002).

Extraction and Cleanup

10 gm aliquot from each sample was weighted and extracted with 20 mL ethyl acetate twice. The extract was kept in sonicator for 2 min at 40 ± 2 °C. Through suction pump assistance, the extract was filtered through a filter paper. After filtration the residues were washed with 10 mL ethyl acetate, and extract was transferred to separating funnel (Chahal et al. 1994). The organic phase was passed through anhydrous sodium sulphate and was evaporated on rotary evaporator until dry. In ethyl acetate residues were dissolved and cleaned up on SPE column containing 1 gm of C 18 preconditioned with 5 mL water and 3 mL acetonitrile. The extracted residues were eluted twice with hexane-ethyl acetate (5 mL) (1:1, v/v). Eluate was evaporated and dissolves in ethyl acetate and transferred to glass tube and concentrated under gentle stream of air to suitable volume. The final aliquot was determined by GC-ECD (Prasad 2001).

Gas Chromatography

The pesticide residues in vegetable samples were analysed using a chemito gas chromatograph equipped with ^{63}Ni ECD and NP detector (Model 5610) having capillary column (BPX-608-25 m × 0.32 ID × 0.46 µm) (Gupta et al. 1998).

Result and Discussion

The current research was undertaken to assess the concentration of various pesticide residues in the vegetables (cauliflower, cabbage, brinjal) in and around Telangana State (Hyderabad and Ranga Reddy).

Cauliflower

It is called as *Brassica oleracea* which belong to the family Brassicaceae. It is an annual plant that reproduces by seed, and the head (the white curd) is eaten. Commonly occurring disease is blackleg, clubroot, fusarium yellows, *Sclerotinia* blight, etc.

Table 2 indicates the pesticide residues detected percentage of contamination and residues range in cauliflower, cabbage and brinjal. The insecticides identified and quantified in cauliflower were endosulfan, quinalphos and methyl parathion. Twenty samples of cauliflower were analysed in duplicate for the presence of pesticide residues which revealed that six samples were contaminated with endosulfan (30%), four were contaminated with quinalphos (20%) and ten were contaminated with methyl parathion (50%). It is observed that cauliflower is found with pesticide residues which is exceeding the maximum residual limit (MRL) values as per the FAO/WHO (FAO/WHO 1986).

Table 2 Pesticide residue levels (μg/gm) found in vegetables collected from Telangana State (Hyderabad and Ranga Reddy)

S. no	Name of the sample	No. of samples studied	Detected pesticide name and (no. of samples contaminated)	Percentage of vegetable samples contaminated with pesticides	Pesticide levels (μg/gm)	MRL (μg/gm)	No. of samples above MRL (μg/gm)
1	Cauliflower (*Brassica oleracea*)	20	Endosulfan (6)	30%	ND- 1.66	0.35	6
			Quinalphos (4)	20%	ND- 0.50	0.29	3
			Methyl parathion (10)	50%	ND- 0.40	1.20	2
2	Cabbage (*Brassica oleracea* capitata group)	20	Chlorpyrifos (6)	30%	ND- 0.10	0.22	2
			Monocrotophos (14)	70%	ND- 0.30	0.08	4
3	Brinjal (*Solanum melongena*)	20	Methyl parathion (9)	45%	ND- 2.00	0.32	7
			Cypermethrin (5)	25%	ND- 0.56	0.25	4
			Monocrotophos (6)	30%	ND- 1.82	0.59	4

Cabbage

It is called as *Brassica oleracea* from capitata group. It is a leafy green biennial plant, grown as an annual vegetable crop. Cabbage is a good source of vitamin K, vitamin C and dietary fibre. Commonly occurring diseases are bacterial soft, black-leg fungus, *Alternaria* leaf spot, etc.

The insecticides identified and qualified in cabbage were chlorpyrifos and monocrotophos. Twenty samples of cabbage were analysed in duplicate for the presence of pesticide residues which revealed that 6 samples were contaminated with chlorpyrifos (30%) and 14 samples were contaminated with monocrotophos (70%). It is observed from the results that cabbage is found with pesticide residues which slightly exceed the (MRL) levels.

Brinjal

It is called as *Solanum melongena* which belongs to family Solanaceae which is a species of night shade grown for its edible fruit. Commonly occurring diseases are aphids, blossom-end rot, fruit rot, etc.

The insecticides identified and quantified in brinjal were methyl parathion, cypermethrin and monocrotophos. Twenty samples of brinjal were analysed in duplicate for the presence of pesticide residues which reveals that nine samples were contaminated with methyl parathion (45%), five samples were contaminated with cypermethrin (25%) and six samples were contaminated with monocrotophos (30%). It is clearly indicated that the brinjal samples have exceeded the MRL levels. As shown in Fig. 2, the MRL values of all pesticide residues in all vegetables like monocrotophos, methyl parathion, quinalphos, chlorpyrifos and cypermethrin were

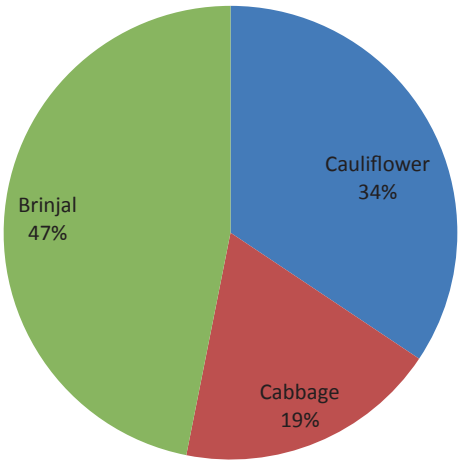

Fig. 2 Percentage of no. of vegetable samples above MRL (µg/gm) contaminated by various pesticide residues

slightly exceeded. The study results are consonance with earlier studies (Madan et al. 1996; Chahal et al. 1997).

There are several studies on pesticide residue contamination and health implications resulting from the intensive use of pesticides in agriculture (Bhanti and Taneja 2007; Chen et al. 2011). In present study 100% of the vegetable samples were assessed with 20 pesticide residues which were found at or exceeding MRL values. Individual vegetables like cauliflower, cabbage and brinjal showed presence of pesticide residues exceeding MRL values in terms of percentages 34%, 19% and 47%. This trend is in consonance with the reports of higher levels of contamination of brinjal, cabbage and cauliflower (Amoah et al. 2006; Kumari et al. 2003; Lozowicka et al. 2012).

A total compounds, covering organophosphorus, organochlorine and synthetic pyrethroid, were detected in the present study with various concentrations. The residues of the compounds detected with predominant in all vegetable groups indicating their widespread usage (Bhattachayya et al. 2009).

Conclusion

Analysis of total 60 fresh vegetable samples, collected from Telangana State (Hyderabad and Ranga Reddy), indicated presence of pesticide residues. It is evident from the results that none of the samples was found free from the contamination of pesticide residues. All the pesticides detected were above the MRL values in all vegetable samples which can pose adverse effects on health of consumers. The frequent and increase usage of pesticides on vegetables has enhanced potential risk of term effect on consumers. The indecent use of pesticides indicates unawareness of farmers and lack of effective legislation.

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An Insight to Micropropagation of Freshwater Aquatic Medicinal Plants

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Introduction

Freshwater aquatic plants include medicinal and ornamental species that are extensively used in folk medicines for their healing characteristics, planted in water bodies or aquarium for ornamental purpose and phytoremediation or maintaining water quality. Freshwater aquatic plants are selected based on their chemical or morphologic characteristics (Stodola 1980). They have limited and narrow demand by specific customers. These aquatic plants are in use for long time in different traditional medicinal systems like Ayurveda (Gonsalves 2010). Aquatic plants are gaining popularity due to their use as ornamental or medicinal purposes (Shahidullah 2007). These plants are collected from wild and used for medicinal purposes making some of the plants threatened to extinction. It is estimated that terrestrial or aquatic plants provide 80% of medicinal ingredients used in Indian traditional medicinal system. Owing to limited demand, they are rarely micropropagated under in vitro conditions (Pierik and Ruibing 1997).

Most of the freshwater aquatic plants are found in environments of Southeast Asia, Malaysia and Indonesia, Vietnam, Laos, and Cambodia as partially, fully submerged or emerged (Kasselmann 1999). Numerous aquatic plants are found in water

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bodies found around us and are ignored as weeds or plants of low economic importance or even no importance. These plants have never been focal points and were left for natural propagation. Increasing world population has increased demands of these plants that is not feasible using propagation through seeds and by letting their natural propagation that need a long time and are most unable to maintain quality.

Although plant biotechnology techniques, tissue culture, and micropropagation are extensively used for culturing terrestrial plants, they have rarely been applied in aquatic plants. The information about in vitro propagation of freshwater aquatic plants is very rare and fragmentary. Aquatic plant biotechnology is fascinating and attractive but largely neglected subject (Kane et al. 1988, 1990; Staritski 1977). This study involves examination of distinctive in vitro micropropagation methods influencing the mode of their propagation, callus morphogenesis, or genetic transformation.

In Vitro Micropropagation of Freshwater Aquatic Medicinal Plants

*Aquatic Job's Tears (Coix aquatica **Roxb.; Poaceae**)*

Katiyar and Chandel (1998) successfully developed somatic embryos and plantlets of two cultivars by indirect somatic embryogenesis. They cultured immature inflorescence and obtained up to 80% calli on N6 medium containing 1–2 mg/L 2,4-D that was followed by induction of somatic embryos and plantlets on MS medium fortified with 0.5 mg/L BA + 0.03 mg/L NAA. They also reported the importance of size, explant age, and cultivar for embryogenesis. Regenerated plantlets were acclimatized in fields where they flowered.

*Centella (Centella asiatica **(L.) Urban; Apiaceae**)*

Centella is one of the major semiaquatic medicinal plants since ancient times. The large number of studies on in vitro propagation has been reported using different explants like leaf or leaf-derived calli for adventitious shoot regeneration. Whereas, stem, shoot tip, and nodal segments have also been used for multiple axillary shoot regeneration. Sivakumar et al. (2006) used shoot tip explants and achieved 88% induction of 16.8 shoots per explant on MS medium fortified with 17.76 μM BA + 1.44 μM GA3. Similarly, Das et al. (2008) have also used shoot tip explant and got 10.2 ± 0.38 shoots per explant on 4.0 mg/L BA + 0.1 mg/L NAA. The micro-shoots were rooted using 1.0 mg/L IBA. The plants had 80% acclimatization. Tiwari et al. (2013) used 2.5–3.0 cm long nodal segments and cultured them on different combinations of BA-NAA or BA-IBA. They noted maximum number of 6.13 ± 0.16 shoots when cultured on medium with 4.0 mg/L BA + 0.4 mg/L NAA. Recently, Roy et al. (2016) compared different growth mediums (MS, B5, and Nitsch) and

PGRs. They used nodes from three different accessions and got maximum number of 5.3 and 6.2 shoots for accession No. 342109 and 347492 using 2.0 mg/L BA + 0.5 mg/L NAA, while accession No. 331514 induced 11.5 shoots on 1 mg/L BA. All accessions produced maximum shoots on MS medium compared to B5 or Nitsch Medium. Mohapatra et al. (2008) used leaf and nodal segment explants and obtained 81.6% regeneration with 8.3 shoots per explant with shoot length of 2.1 cm on leaf explant that cultured on MS medium provided with 3.0 mg/dm^3 BA and 0.05 mg/dm^3 NAA.

Bibi et al. (2011) produced plants from somatic embryogenic calli originated on leaf explants cultured on MS medium containing 4.42 μM BA with 5.37 μM NAA. They achieved maximum of 10 shoots per callus explant followed by rooting. They also checked the antibacterial activities of calli and plant extracts. Similarly, Joshi et al. (2013) optimized callus induction on leaf and stem explants by using 0.5 mg/L BA + 0.3 mg/L NAA achieving maximum of 6.0 shoots from leaf explant and 8.0 shoots from stem explant after re-culturing them on medium supplemented with 0.5 mg/L BA and 0.75 mg/L BA. They induced rooting on medium enriched with 0.5 mg/L IBA followed by acclimatization. Panthalu et al. (2014) noted green, friable, and granular calli on nodal explants cultured on medium containing 2.0 mg/L 2,4-D and 0.5 mg/L BA. They noted maximum of 11.46 shoots when cultured on medium containing 2.0 mg/L BA + 0.5 mg/L NAA. Regenerated shoots were rooted and subjected to adaptation with 80% survival of plants during acclimatization.

Ceylon Hydrolea (Hydrolea zeylanica **Linn. (Vahl): *Hydrophyllaceae)***

Ravi et al. (2012) developed in vitro micropropagation and flowering protocol of *Hydrolea zeylanica*. They used nodal explants and obtained maximum regeneration on MS medium fortified with different BA or KIN in the presence of NAA. Whereas, in vitro flowering was recorded after 8 days of shoot bud regeneration. Regenerated shoots were rooted on MS medium enriched with IBA.

*Chinese Water Chestnut (***Eleocharis dulcis** *Trinius ex Henschel; Cyperaceae)*

Number of studies are available that highlight the importance of different colored lights with different wavelength on regeneration from different explants using different PGRs. Hoque et al. (2001) cultured the embryonal explants awaited on 5 °C for 40 days on ½ × MS medium enriched with 2.7 μM BA + 0.5 μM NAA + 0.5 μM GA3. Thereafter, chilled and no-chilled explants were inoculated on ½ × MS

medium enriched with different cytokinins, auxins, and GA3. A liquid ½ × MS medium provided with 1.1 BA + 0.5 μM NAA proved the best for shoot proliferation. 100% rooting was recorded when the explants were cultured in liquid ½ × MS medium enriched with 1.1 μM BA + 0.5 μM NAA + 1.1 μM IBA. Hoque and Arima (2004) evaluated the effects of different color lights with different wavelength on in vitro shoot regeneration of water chestnut using cotyledonary nodes or nodal explants. Mixed color lights resulted in early and higher shoot proliferation (95.6%), shoots per explant (25.7) using MSMA medium provided with 2.7 μM BA + 0.5 μM NAA + 0.5 μM GA3.

Hoque et al. (2006) used embryonal explants of three water chestnuts varieties for callus induction on ½ × MS medium supplemented with 2.5 mg/L 2,4-D + 1 mg/L BA. For shoot induction, ½ × MS liquid medium containing 1.1 mg/L BA + 0.5 mg/L NAA was found optimum for all three varieties. Whereas, 0.5 mg/L GA3 was found best for shoot elongation. Maximum root induction was recorded on liquid ½ × MS medium enriched with 1.1 mg/L IBA. Jun et al. (2011) used terminal bud as explants and cultured on MS medium using different concentrations of BA and NAA and found the efficacy of BA:NAA ratio on explants proliferation. The optimum medium for shoot induction was 0.20 mg/L 6-BA + 0.02 mg/L NAA which generated 81.40%. Whereas, optimum medium for shoots was 0.50 mg/L 6-BA + 0.10 mg/L NAA.

Recently, Gao et al. (2015) compared the temporary immersion bioreactor system (TIBS) with conventional semisolid medium technique for propagation of Chinese water chestnut using shoot tip explant. TIBS conditions for 15 min of immersion after every 8 h resulted in 36.5-fold more shoot multiplication with shoot-forming capacity (SFC) of 68.9. Maximum shoot multiplication rate (43.7 times) in TIBS was recorded on MS medium supplemented with 4 mg/L BA and 0.5 mg/L NAA. No rooting was observed during multiplication in TIBS, and these were rooted on medium containing 2.0 mg/L NAA with 95% survival during acclimatization.

Coontail or Hornwort (*Ceratophyllum demersum *L.; Ceratophyllaceae)

Coontail is one of the most popular aquatic plants used for phytoremediation and is also considered as medicinal plant due to containing rich bioactive compounds. In recent years, number of protocols have been established for in vitro regeneration using shoot tip and nodal explants. Karataş et al. (2014a) cultured shoot tips, first and second nodal explants on agar solidified or liquid MS medium fortified with 0.05–0.80 mg/L BA. Liquid culture medium was found superior compared to solidified medium, and maximum shoot regeneration frequency from all explants were recorded on liquid medium with 0.05 mg/L BA. Second nodal explants were most responsive and regenerated 16.75 and 204.33 shoots per explant on solid and liquid

culture medium, respectively. They also suggested slightly acidic to alkaline water requirement for best growth of these plants in the aquaria. Later on, Dogan et al. (2015) used the same explants to liquid culture medium containing TDZ. First nodal explant generated maximum of 138.44 shoots per explant when cultured on MS medium containing 0.40 mg/L TDZ. Thereafter, they excised ≈2.0 cm long shoots and transferred them to liquid medium containing GA3 which resulted in shoot length up to 16.08 cm on medium containing 0.80 mg/L GA3. There are other studies on coontail in which researchers propagated shoots under in vitro conditions and used these shoots to control water quality (Karataş et al. 2016a). Similarly, extracts taken from in vitro grown shoots of coontail were used to check antioxidant properties (Karataş et al. 2015) and as insecticide (Emsen et al. 2016) against some stored grain pests. In all these studies, 100% survival rate was recorded when shoots were transferred to aquariums for adaptation with or without aeration.

Creeping Coldenia** (*Coldenia procumbens* **Linn.; Boraginaceae)

The studies about in vitro regeneration of *Coldenia procumbens* revealed the use of nodal segments (Jahirhussain et al. 2016) and shoot tip explants regenerated axillary shoot regeneration on BA and KIN. 100% shoot regeneration was observed on both explants when cultured on medium containing 10 μM BA or 6 μM BA. Whereas, 100% regeneration on nodal segments and shoot tips was observed on medium supplemented with 8 μM KIN or 10 μM KIN, respectively. On the other hand, 15 shoots per explant were noted on nodal segment and 14 shoots per shoot tip when they were cultured on medium with 10 μM BA or 6 μM BA, respectively. Whereas, 10.6 shoots per nodal segment and 10 shoots per shoot tip were obtained, respectively, on medium fortified with 8 μM KIN or 10 μM KIN. They also reported BA as more responsive than KIN in both studies.

Creeping Jenny** (*Lysimachia nummularia* **L.; Primulaceae)

Karataş and Aasim (2015a) reported in vitro whole plant regeneration of creeping jenny, an important aquatic medicinal plant. They used shoot tip and first and second nodal segment explants by culturing them on MS medium supplemented with BA-NAA and achieved improved shoot regeneration frequency of 58.33–83.33%, 33.33–83.33%, and 41.67–91.67%, respectively. MS medium enriched with 1.25 mg/L BA was found optimum for generating maximum number of shoots as 9.30, 8.94, and 8.11 for shoot tip and first and second nodal segment explant, respectively. In vitro regenerated shoots were rooted directly on all treatments irrespective of explant type and were transferred directly to aquariums for adaptation with high rate of survival.

Dwarf Hygro (Hygrophila polysperma Anderson; Acanthaceae)

Dwarf hygro is an important part of Ayurvedic system and also known as bioindicator to detect algae in aquatic system. There are few reports on in vitro propagation of dwarf hygro using leaf, shoot tip, and nodal segment explants. Çinar et al. (2013) cultured shoot tip and first nodal segment explant on liquid MS medium containing BAP for axillary shoot regeneration. They recorded maximum of 25.33 (shoot tip) and 21.67 (first nodal segment) shoots on MS medium containing 0.10 mg/L BA. Shoot tip explants were subcultured which improved number of shoots per explant that ranged 26.02–36.91. In another study, Karataş et al. (2014b) used liquid MS medium containing BAP for adventitious shoot regeneration using leaf explant. Maximum number of shoots (5.11) were noted on MS medium containing 1.0 mg/L BAP. Thereafter, subculture on liquid medium containing GA3 improved shoot regeneration to maximum of 10.92 shoots with shoot length of 1.24 cm on MS medium supplemented with 1.0 mg/L BA + 1.0 mg/L GA3 or 0.5 mg/L BA − 1.0 mg/L GA3, respectively. Whereas, Karataş et al. (2013a) reported in vitro adventitious shoot regeneration by culturing leaf explants on agar solidified MS medium supplemented with either of KIN/TDZ singly or with 0.10 mg/L IBA. They achieved maximum of 16.33 shoots per explant on MS medium containing 0.80 mg/L KIN + 0.10 mg/L IBA and 20.55 shoots when cultured on MS medium enriched with 0.10 mg/L TDZ + 0.10 mg/L IBA. These shoots were rooted using IBA and acclimatized in aquariums with a range of pH levels that showed that the plants could survive at pH level of 6–9 pH in aquariums without any difficulty.

Dwarf Water Clover (Marsilea minuta L.; Marsileaceae)

Shekhawat and Manokari (2015) used rhizome explants cultured on MS medium supplemented with BA. Maximum shoot regeneration frequency (96%), shoots (6.2), and shoot length (2.72 cm) was recorded on medium containing 0.5 mg/L BA. These shoot clusters were subcultured on 0.25 mg/L BA with induction of 79.0 shoots per cluster. These shoots were elongated on ½ × MS medium devoid of PGRs and rooted after fourth subculture.

East Indian Globe Thistle or Kamdaryus (Sphaeranthus indicus Linn.; Asteraceae)

In vitro regeneration studies for *Sphaeranthus indicus* indicated the use of axillary bud and shoots tip explant for axillary shoot regeneration. Ravipaul et al. (2008) reported axillary shoot regeneration using shoot tip and axillary bud explants. They achieved maximum shoot regeneration frequency of 90% and 75%, respectively, for axillary bud and shoot tip explants cultured on medium containing 4.0 mg/L

BA. Thereafter, the shoots were rooted using MS medium containing 2.0 mg/L IBA followed by hardening with 90% survival rate. Recently, two studies revealed the effects of silver nitrate and different carbon sources on in vitro shoot regeneration. Harathi and Naidu (2016) investigated the silver nitrate on shoot regeneration potential using shoot tip explant. Medium containing 1.0 mg/L KIN + 0.1 mg/L KIN + 0.4 mg/L $AgNO_3$ was optimized for maximum shoots that was recorded 34.3. Their results highlighted the positive effects of $AgNO_3$ on shoots length. Regenerated shoots were rooted on 0.4 mg/L $AgNO_3$ + 2.0 mg/L NAA in the media. Another study by Harathi et al. (2016) highlighted the role of different carbon sources in the presence of 0.4 mg/L silver nitrate, 1.0 mg/L KIN, and 0.1 mg/L NAA using nodal explants. Maximum of 29.1 shoots were recorded on MS medium containing 3% fructose + 0.4 mg/L $AgNO_3$ compared to control without silver nitrate. Their results also revealed positive effects of different sucrose concentrations in the following order as 3% fructose > sucrose > maltose > glucose on nodal explants of *S. indicus*. In vitro shoots were rooted on medium containing 1.0–2.0 mg/L NAA, IBA, and 1–0.6 mg/L $AgNO_3$. Besides axillary shoot regeneration, Yarra et al. (2010) reported adventitious shoot regeneration *Sphaeranthus indicus* using leaf segment explants. The maximum number of 12 shoots with the highest shoot length of 3.0 cm were recorded using 4.4 μM BA + 1.71 μM IAA and 2.46 μM IBA containing MS medium for rooting with adaptation to external environment.

Eclipta *(Eclipta prostrata* ***(Linn.) Linn.; Asteraceae)***

Eclipta prostrata is a potent medicinal aquatic plant shoot multiplication or in vitro flowering using nodal segment explants cultured on MS medium supplemented with 4.44 μM BA. Rooting was recorded on MS medium supported with 0.44 μM BA (Gawde and Paratkar 2004). Maximum shoots were noted on 1 mg/L BA using nodal segment explants. Whereas, BA and GA3 were used for further multiplication of shoots (Dhaka and Kothari 2005). Husain and Anis (2006) used 10 M BA for axillary bud proliferation and gained 23 shoots per explants. Whereas, culture of node explants at lower concentration of 2 M BA resulted in 79.0 shoots per explants. Regenerated shoots were rooted on ½ × MS medium enriched with 0.5 mg/L IBA followed by 90% survival rate. Whereas, Singh et al. (2012) used transverse thin cell layer (tTCL) culture explant taken from nodal segment. MS medium fortified with 13.2 μM BA + 4.6 μM KIN resulted in 100% shoot proliferation with 32.6 shoot buds per explant. They rooted the plants and acclimatized them with 90–100% survival rate. Yesmin et al. (2015) cultured nodal segment explants on MS medium fortified with BA/KIN alone or in combination with IAA or NAA. They obtained maximum number of 18.40 shoots per explants. When cultured on medium enriched with 1.0 mg/L BA + 0.1 mg/NAA. ½ × MS medium supplemented with 1 mg/L IBA resulted in 96% rooting that was followed by successful adaptation.

Use of cotyledonary node explants followed by the establishment of plants in soil was reported by Baskaran and Jayabalan (2005). They also obtained maximum shoots on medium provided with 4.4 μM BA + 9.2 μM KIN + 2.4 μM 2iP. Sharan

et al. (2014) used seed as explant for multiple clonal propagation. They cultured the sterilized seed explants on MS medium containing 2 mg/L BA + 1 mg/L NAA followed by subculture of shoots which resulted in multiple whole plant proliferation. These plantlets were separated and acclimatized successfully. Besides axillary shoot regeneration, adventitious shoot regeneration from leaf explant has also been reported by Sharma et al. (2013). They cultured leaf explants and reported MS medium supplemented with 1.0 mg/L 2,4-D + 0.5 mg/L BA for callus induction. Whereas, 1.0 mg/L BA + 0.1 mg/L NAA was found best for shoot proliferation from calli. On the other hand, Dar et al. (2016) induced an average of 4–7 flowers when cultured on medium supported with 4 mg/L KIN. Whereas, they attained maximum of 13 flowers when using 5 mg/L 2iP.

Epaltes *(Epaltes divaricata* ***L. Cass.: Asteraceae***

Barathi and Agastian (2015) developed both direct and indirect regeneration protocols of *Epaltes divaricata* L. using different explants. Maximum callusing was achieved on shoot tip explants on medium with maximum number of 10.77 shoots and mean shoot length. Contrarily, nodal explants generated 1.86 shoots with average length of 3.0 cm when cultured on MS medium containing 1.0 mg/L BA + 0.1 mg/L KIN. Regenerated plantlets were rooted using IBA with survival rate of 73.33% during acclimatization.

Indian Heliotrope *(Heliotropium indicum* ***Linn.; Boraginaceae)***

Indian heliotrope is an important medicinal herb used for curing different diseases. It is rich in different bioactive compounds like alkaloids and steroids. Bagadeka and Jayaraj (2011) achieved in vitro callogenesis and rhizogenesis using stem and leaf explants, cultured on MS medium enriched with 2,4-D, NAA, and IBA (1.0 or 3 mg/L) and KIN or BA (2 mg/L). Their results indicated the strong rhizogenesis compared to callus induction. Extracts taken from the roots showed the presence of bioactive compounds. In another study, Priyadarshini et al. (2014a) developed calli for isolation of bioactive compounds. They used 2,4-D, IBA (0.5–5 mg/L), and BA (0.5–1.0 mg/L) singly or in combination for internodal segments or leaf explants. Higher calli induction (89.8%) on internodal segment was recorded on MS medium supplemented with 1.5 mg/L 2,4-D + 1.0 mg/L BA.

The other studies indicated the greater potential of regeneration using apical or axillary buds or nodes for axillary shoots induction. Kumar and Rao (2007) developed in vitro axillary regeneration protocol using apical or axillary buds. They noted maximum number of 32.6 and 20.2 shoots per explant on apical or axillary buds incubated on MS medium provided with 1.0 mg/L + 0.5 mg/L BA + 0.05 mg/L IAA after 30 days of culture. They also achieved 85.0% rooting by using 0.1 mg/L IBA

followed by successful adaptation. Hassan et al. (2010) cultured apical and axillary buds taken from young sprouted plantlets. The highest regeneration frequency (92%) with an average of 12 shoots per explant was achieved on MS medium fortified with 0.5 mg/L BA + 0.1 mg/L GA3. Subculture to the same medium increased the shoots per explant up to 18. These shoots were rooted on 0.5 mg/L IBA containing ½ × MS rooting medium with 85% survival during acclimatization. Priyadarshini et al. (2014b) inoculated apical shoots and nodal explants on MS medium provided with BA or KIN alone or in combination with IAA, NAA, or GA3. Their results revealed 87% shoot regeneration with average of 5.4 shoots using 1.0 mg/L BA. Whereas, addition of GA3 with 1.0 mg/L KIN resulted in 92% shoot regeneration. Regeneration frequency of 94% was noted with 14.8 shoots on apical shoot explant on the same medium. ½ × MS medium containing 0.2 mg/L IBA was the most successful medium for rooting with 74% survival during acclimatization.

***Job's Tears** (Coix lacryma-jobi **Linn.; Poaceae**)*

Mochida and Tsujimoto (2001) used Job's tears (*C. lacryma-jobi* L.) as pollen parent for wheat crosses to produce haploid wheat plants due to availability of pollen throughout the year. After pollination, they treated the explants with 2,4-D, detached tillers followed by embryo culture.

***Limnophila** (Limnophila aromatica **R.Br.; Plantaginaceae**)*

Karataş and Aasim (2015b) reported first ever in vitro multiplication of *Limnophila aromatica*, an important aquatic or semiaquatic medicinal herb. They used sterilized shoot tip explants and cultured them on medium fortified with 0.25–2.0 mg/L BA + 0 or 0.25 mg/L NAA with 100% shoot regeneration frequency and maximum number of 44.22 shoots per explant on medium containing 1 mg/L BA. Contrarily, longer shoots were achieved after a culture on medium containing 0.25 mg/L BA + 0.25 mg/L NAA. In vitro regenerated shoots were successfully rooted (100%) using NAA (0.25–1.0 mg/L NAA) with 100% survival in aerated aquariums for acclimatization.

***Neeramulli** (Hygrophila schulli **Buch.-Ham.) M.R. Almeida & S.M. Almeida; Acanthaceae**)*

Hygrophila schulli (*Asteracantha longifolia* synonym) is an important medicinal aquatic herb, and efforts have been made to develop in vitro regeneration protocol for caulogenesis and axillary or adventitious shoot regeneration in recent years with numerous reports on direct and indirect shoot regeneration. Pal et al. (2014)

successfully developed calli of *H. schulli* using cotyledonary leaf and cotyledonary node explants cultured on MS medium enriched with 0.2 mg/L 2,4-D + 0.2 mg/L BA. Subculture to MS medium supplemented with 1–2 mg/L BA alone or in combination with 0.1 mg/L NAA resulted in green somatic embryos which turned into plantlets. Whereas, Kumar and Nandi (2015) used internode explants using different cytokinins and auxins and achieved maximum number of shoots per explant on MS medium supplemented with 2.0 mg/L BA + 0.5 mg/L NAA. They rooted the shoots by using 0.5 mg/L IBA with 86.7% survival after transplantation.

The studies related to adventitious shoot regeneration revealed the use of leaf explant cultured on MS medium using different concentrations of cytokinins + auxins or cytokinins singly. Mishra et al. (2006) successfully developed the indirect and direct adventitious shoot regeneration using leaf explant cultured on different concentrations of BA or KIN. They obtained maximum of 6.6 shoots when cultured on medium with 2.0 mg/L BA. Contrarily, maximum number of 3.4 shoots were noted on 2.5 mg/L KIN containing medium. Panigrahi et al. (2006) reported adventitious shoot regeneration using leaf explant. They obtained maximum of 21.8 shoots or buds per explant when cultured on MS medium supplemented with 2.0 mg/L BA + 0.5 mg/L NAA. Shoots with more than 3 cm length were successfully rooted on medium enriched with 0.1 mg/L NAA. A study reported by Behera et al. (2010) highlighted the adventitious shoot regeneration protocol when inoculated leaf explant on MS medium contained TDZ, BA, and NAA. They achieved 158.4 shoots, when they cultured leaf explants on medium enriched with 0.5 mg/L TDZ. They converted shoots into plantlets by applying 0.1 mg/L NAA followed by 80% survival rate.

Roundleaf Toothcup** (Rotala rotundifolia **(Roxb.) Koehne; Lythraceae)

There are only two studies about in vitro whole plant regeneration using nodal segments or shoot tip explants. Micheli et al. (2006) obtained direct plantlet regeneration from uninodal explants and cultured on LS medium enriched with 1.0 or 4.0 mg/L BA and achieved 67.5% shoots regeneration MS medium supplemented with 4.0 mg/L BA. However, shoot length (26.4 mm) and weight of shoot clump (11.22 g) were more on medium supplemented with 1.0 mg/L BA. Recently, Karataş et al. (2014c) reported two steps of adventitious shoot regeneration protocol of *Rotala rotundifolia*. At first, they cultured shoot tip explants on different concentrations of BA (0.25–2.00 mg/L BA). They recorded maximum (53.33%) shoot regeneration frequency and 17.06 shoots per explant on medium enriched with 1.0 mg/L BA. Thereafter, they transferred the explants to medium supplemented with 0.20 mg/L GA3 which resulted in 100% shoot regeneration. However, maximum number of shoots (24.0) were achieved on medium containing 0.25 mg/L BA − 0.20 mg/L GA3. No separate phytohormonal treatments were made for rooting. All plantlets survived in aquariums for acclimatization and survived at pH level 6–9 during acclimatization.

Sessile Joyweed (Alternanthera sessilis; Amaranthaceae)

The studies on *A. sessilis* report adventitious shoot regeneration using leaf (Singh et al. 2009) or internode explants (Das and Borua 2014). Callus induction on leaf explants was achieved using MS medium fortified with 1 mg/L BA and 1 mg/L 2,4-D for 2 weeks followed by subculture to ½ × MS medium containing 1 mg/L IAA and 1 mg/L BA that resulted in achieving 10 shoots per explant. They successfully rooted the shoots on ½ × MS medium containing 1 mg/L IBA (Singh et al. 2009). In another study, Das and Borua (2014) used internode explants for callus induction using MS medium provided with 0.5 mg/L or 1.0 mg/L 2,4-D by regenerating 124 shoots by subculturing each of them on MS medium containing 1.0 mg/L BA and adenine sulfate. They rooted the in vitro regenerated shoots with 100% acclimation under field conditions.

Besides adventitious shoot regeneration, axillary shoot regeneration has also been reported by using shoot tips, nodal segments (Gnanaraj et al. 2011), and nodes (Shekhawat et al. 2017). Gnanaraj et al. (2011) gained 94.3% shoot proliferation with maximum of 23.4 shoots per explant on MS medium containing 2.0 mg/L BA. Whereas, nodal segment explants generated maximum of 90.4% shoot proliferation frequency and 15.2 shoots per explant when cultured on MS medium provided with 1.5 mg/L BA. 97.4% rooting frequency with 6.3 rootlet per shoot was recorded on ½ × MS medium enriched with 3 mg/L IBA. Recently, Shekhawat et al. (2017) successfully developed multiple shoot regeneration system and rooting. They obtained an average of 8.0 shoots per node with an average shoot length of 4.7 cm on nodal segments on medium enriched with 2.0 mg/L BA. They increased the shoot regeneration by subculture of fresh shoots on medium containing 1.0 mg/L each of BA and kinetin, and 0.1 mg/L indole-3-acetic acid (IAA) and additives showed 23.8 shoots per explant. About 85% shoots were rooted on ½ × MS medium containing 1.5 mg/L IBA with an average of 18 roots per shoot. Thereafter, they successfully adapted plants in greenhouse conditions.

Sola Pith Plant (Aeschynomene aspera Linn.; Fabaceae)

Gnanaraj et al. (2011) cultured nodal explants and obtained 93.60% regeneration and 10.6 shoots per explant on medium containing 3.0 or 5.0 mg/L BA. Whereas, longer shoots (5.50 cm) were noted when cultured on medium having 3.0 mg/L KIN. The micro-shoots were rooted on ½ × MS medium supplemented with 1.0 mg/L of IBA followed by acclimatization in greenhouse and open-field conditions. Whereas, Sen et al. (2013) used node explants from field-grown plants that were cultured on full or ½ × MS medium supplemented with different cytokinins singly or with auxins and observed in range of 1–4.33 shoots per explant with 100% rooting and 86.67% survival rate after adaptation.

*Spreading Sneeze weed (Centipeda minima **A. Braun and Ascheron; Asteraceae**)*

Chang et al. (2011) used stem explants for adventitious shoots, rooting, and acclimatization of *C. minima*. They reported MS medium fortified with 0.4 mg/L BA + 0.1 mg/L NAA or 0.4 mg/L BA + 0.1 mg/L NAA + 0.5 mg/L GA3 for optimum adventitious shoot regeneration. They rooted the plants by pulse treated shoots with 40 mg/L NAA for 6 min followed by culture on N6 medium containing 0.2 mg/L NAA. Thereafter, they acclimatized the plants under ambient conditions of temperature and humidity.

*Sweet Flag (Acorus calamus**; Araceae**)*

Studies about in vitro regeneration of *Acorus calamus* revealed the use of explants like apical shoot meristem, rhizome segments, or rhizome buds which resulted in axillary shoot regeneration using either agar solidified or liquid medium. Hettiarchchi et al. (1997) cultured apical shoot meristem explants on MS medium containing different cytokinins and auxins and obtained maximum number of 26 shoots from liquid medium provided with 1.0 or 2.0 mg/L BA. Anu et al. (2001) cultured rhizome buds under 12 h photoperiod on MS medium containing BA (8.87 μM) and NAA (5.37 μM) and gained 8–10 shoots per explant. They rooted plants devoid of growth regulators. Whereas, Verma and Singh (2012) used sterilized rhizome segments cultured on MS medium enriched with different cytokinins and auxins. They obtained maximum number of shoots (4.4) from medium containing 2.0 mg/L BA + 0.5 mg/L NAA. Fifty percent rooting frequency was achieved with 1.0 mg/L IBA. Contrarily to these studies, Dixit et al. (2014) successfully developed adventitious shoot regeneration system by using basal leaf explants. They placed the explants on MS medium supplemented with 2.5 mg/L for 10 days followed by culture on MS medium supplemented with 1.0 mg/L BA + 1.0 mg/L NA and recorded 91.6% shoot regeneration frequency with 4.3 shoots per explant. They elongated the shoots by using 0.25 mg/L BA, 0.25 mg/L GA, and 0.1 mg/L IAA.

*Water Hyssop or Brahmi (Bacopa monnieri **(L.) Pennell**)*

Water hyssop is one of the most popular aquatic or semiaquatic plants used for medicinal purposes in India where it is known as Brahmi. Brahmi-based drugs are available in India for curing disorders and diseases, and plant becomes rarely endangered due to its huge demand. Therefore, the large number of studies about in vitro micropropagation of water hyssop has been reported especially during the last decade. In these studies, researchers developed protocols for its conservation and for secondary metabolites production. These studies reveal the direct organogenesis

(Sharma et al. 2016) or indirect organogenesis (Rout et al. 2011) through calli culture. Moreover, number of explants and plant growth regulators has been employed by researchers in order to enhance plant production.

Studies on water hyssop reveals both axillary and adventitious shoot regeneration depending on the explant type. Results on axillary shoot regeneration highlight the use of shoot tip explant (Pandiyan and Selvaraj 2012; Sharma et al. 2016) or nodal segment explants by cutting stem into small pieces carrying nodes (Vijayakumar et al. 2010; Gurnani et al. 2012; Mehta et al. 2012; Pandiyan and Selvaraj 2012; Ghasolia et al. 2013; Begum and Mathur 2014; Subashri and Koipillai 2013; Jain et al. 2014; Mohanta and Sahoo 2014; Behera et al. 2015; Vijay et al. 2016; Wangdi and Sarethy 2016; Dixit and Thakur 2017). In all these studies, high shoot proliferation frequency was achieved using different PGRs. Contrarily, adventitious shoot regeneration potential of water hyssop is also very high, and the number of studies reveals the higher shoot proliferation using internode explant (Rao et al. 2012; Subashri and Koipillai 2013; Naik et al. 2014). Whereas, Karataş et al. (2013b) compared the potential of first, second, and third segment explant and achieved 21.89, 21.22, and 23.11 shoots per explant, respectively, on MS medium containing 0.25 mg/L BA + 0.25 mg/L NAA. Leaf is another potent explant used for in vitro propagation of water hyssop used as a whole (Joshi et al. 2010; Vijayakumar et al. 2010; Rao et al. 2012; Bhusari et al. 2013; Karataş et al. 2013b; Koul et al. 2014; Naik et al. 2014; Karataş and Aasim 2014) or by cutting leaves into small pieces (Karataş et al. 2016b). All these reports highlight the high shoot proliferation by direct or indirect organogenesis.

Results on water hyssop also reveal the high rooting frequency by using different auxins like IAA, NAA, or IBA. After rooting, these plants were planted in soil with ambient water or directly in the aquariums where high percentage of plants survived (Karataş et al. 2013b, 2016b; Karataş and Aasim 2014). A study conducted by Karataş et al. (2013b) for acclimatization in water with pH range of 4–10 revealed pH 7 or 8 for better growth in aquariums.

Water Lettuce (Pistia stratiotes L.; Araceae)

Water lettuce is a multipurpose plant used for phytoremediation to medicinal and pharmacological studies. However, the limited number of reports is available on in vitro regeneration of water lettuce. Zhang et al. (2008) developed calli from condensed stem using 2.26 μmol/L 2,4-D + 0.88 μmol/L BA. Thereafter, they shifted the calli to medium containing 4.44 μmol/L BA + 0.54 μmol/L NAA which gave shoots within 2 weeks. Regenerated shoots were rooted on medium containing 0.54 μmol/L NAA that was followed by adaptation. Aasim et al. (2013) optimized the sterilization followed by establishing a regeneration protocol. They used surface sterilized explants using 60% H_2O_2 and cultured them on MS medium and MS medium containing various concentrations of BAP-kinetin to regenerate shoots. No shoot regeneration was noted on liquid and solidified MS medium. However, multiplication using sucrose-free liquid MS medium containing 0.05–0.40 mg/L kinetin

or BAP resulted in 2–4 plantlets per explant. In another study, Aasim et al. (2017) successfully developed whole plant regeneration of water lettuce using shoot meristem explants cultured under red:blue (3:1) light emitting diodes (LEDs) at 16 h light photoperiod using TDZ as PGR. Maximum of 39.67 plantlets were induced on explants cultured on agar solidified MS medium enriched with 0.10 mg/L TDZ. All of the regenerated plants survived in aquariums.

Water Pepper *(Persicaria hydropiper* ***(L.) Delarbre; Polygonaceae)***

Studies about *Polygonum hydropiper* revealed only two reports about in vitro regeneration. Marc et al. (1994) initiated callus and cell suspensions culture as well as root and shoot cultures from mature *P. hydropiper* plants. Their results showed detection of polygodial only in shoot cultures. Hasan and Sikdar (2010) reported efficient plant regeneration protocol by using shoot tip explant of *Polygonum hydropiper* (L.) by achieving 96.6% shoot induction frequency with maximum of 9.0 shoots when cultured on MS medium containing 2.0 mg/L KIN. They also achieved 90% rooting with maximum number of 12.0 roots per shoot on MS medium containing 1.0 mg/L IBA. Thereafter, they successfully acclimatized these plants with 100% survival rate.

Water Spinach *(Ipomea aquatica* ***Forssk.; Convolvulaceae)***

Water spinach is an important aquatic plant used both as vegetable and medicinal purposes. There is no report on micropropagation or adventitious shoot regeneration of the plant except some reports on callus culture. Masanori et al. (1997) have reported *Agrobacterium tumefaciens*-mediated introduction of foreign genes by using node explant. Prasad et al. (2006) have reported higher antioxidant activity of in vitro induced calli compared to mother plants. Kirdmanee et al. (2006) have used this plant in phytoremediation and have highlighted the screening of in vitro grown seedlings of water spinach against inorganic salts and temperature. Their results revealed the high salts and temperature tolerance in 10 cultivars.

White Ginger Lilly *(Hedychium coronarium* ***J. Koenig; Zingiberaceae)***

Butterfly ginger is one of the most important medicinal aquatic plants subjected to in vitro propagation with rhizome or rhizome buds used as explant for direct axillary shoot regeneration. Bisht et al. (2012) used shoot tip explant for shoot

regeneration and reported BA with 5.76 shoots per explant compared to KIN with 3.60 shoots per explant. In another experiment, they used BA with NAA, IAA, and IBA and obtained maximum number of 7.90 shoots from medium supported with 1.0 mg/L BA and 0.5 mg/L − 1 NAA. The shoots were rooted on 0.5 mg/L NAA and the plantlets were acclimatized. Mohanty et al. (2013) cultured rhizome explants on MS medium containing BA-NAA. They achieved whole plant regeneration with 3.6 shoots per explant and an average of 4.0 roots per plant on medium containing 2.0 mg/L BA and 0.5 mg/L NAA with 100% acclimatization. Parida et al. (2013) cultured axillary bud explants and recorded maximum of 13.2 shoots per explant when cultured on MS medium fortified with 3 mg/L BA − 3 mg/L KIN − 0.2 mg/L TDZ. They also reported continuous proliferation of shoots when subcultured after every 4 weeks. They rooted the shoot clusters on medium with 3 mg/L KIN + 0.5 mg/L IAA with 6.3 roots per cluster. Verma and Bansal (2013) used different additives like activated charcoal (AC), casein hydrolysate (CH), coconut milk (CM), silver nitrate ($AgNO_3$), and phloroglucinol (PG) with 1 mg/L BA using rhizome explant. They obtained maximum number of 6.56 shoots and 10.86 cm long shoots on medium enriched with 1 mg/L BA + 1 mg/L PG. They rooted the plants on ½ × MS liquid medium containing NAA with 80% acclimatization. Verma and Bansal (2014a) used rhizome bud explants cultured on different concentrations of BA, KIN, and TDZ. They recorded maximum number of 14.21 shoots per explant on medium enriched with 1.0 mg/L TDZ and 12.89 cm shoot length on medium provided with 1.0 mg/L BA. The plants were rooted on liquid ½ × MS medium containing 1.0 mg/L NAA with acclimatization.

Besides of direct axillary shoot regeneration, Verma and Bansal (2014b) also reported multiple shoot regeneration on rhizome explants through indirect somatic embryogenesis. They induced calli on different concentrations of 2,4-D, IAA, and NAA for 4 weeks followed by culture on different concentrations of BA or KIN used singly for somatic embryogenesis and shoot proliferation. They achieved best calli on 0.5 mg/L 2,4-D and achieved maximum shoots.

White Snowflake *(Nymphoides indica* ***(L.) Kuntze; Menyanthaceae)***

A single report is available for in vitro whole plantlet regeneration of *Nymphoides indica* L. Thwaites O. Kuntze from petiole explants by Jenks et al. (2000). They used different combinations of cytokinins (0–25 μM 2-iP, BA, or KIN) and auxins (0–25 μM NAA or IAA). The best combination for shoot proliferation (80%) and shoots per explants (11.5) was 0.8% TC agar solidified MS medium containing 10 μM BA + 20 μM IAA with addition of 0.56 mM myoinositol, 1.2 μM thiamine-HCl, and 116.8 mM sucrose. They also confirmed direct or indirect adventitious shoot induction by histological analysis.

Conclusion

This study explains micropropagation protocols employed in propagation, callus induction, and transformation of some important freshwater aquatic medicinal plants belonging to families Acanthaceae, Araceae, Asteraceae, Apiaceae, Boraginaceae, Convolvulaceae, Cyperaceae, Fabaceae, Hydrophyllaceae, Lythraceae, Marsileaceae, Menyanthaceae, Poaceae, Polygonaceae, and Zingiberaceae. Although there are rare and fragmented reports on these plants, the trend is encouraging and will help in setting a systematic propagation trend of these plants cheaply for larger worldwide availability.

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Arsenic and Heavy Metal (Cadmium, Lead, Mercury and Nickel) Contamination in Plant-Based Foods

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Introduction

Plant-based foods are important commodities that supply key biological elements for sustainability of human lives on Earth. Food plants grown in accordance with the codes of safe and healthy cropping techniques are considered important sources of dietary fibres, carbohydrates, proteins, lipids, vitamins, minerals and bioactive compounds (Raskin et al. 2002). For example, cereals are the most important food in the world that fulfil more than 50% of total human calorie requirements (FAO 2013). Plant-based foods not only provide nutrition for humans but also have therapeutic uses. There is evidence that diets comprising mainly vegetables and fruits may normalize blood pressure and alleviate other health complications (Boeing et al. 2012). Pulses are important source of proteins and have several other nutritional and health benefits (Mudryj et al. 2014).

Plant-based foods contain a number of the essential trace elements for humans (such as iron Fe, copper Cu, zinc Zn, chromium Cr, selenium Se, manganese Mn and molybdenum Mo). Although arsenic (As) and nickel (Ni) are thought to play some beneficial roles in humans at ultra-trace levels, essentiality of these two elements has not been confirmed regarding a biological function in humans. Toxic elements (such as cadmium Cd, lead Pb and mercury Hg), on the other hand, do not

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have any biological importance in the human body. All these trace elements cause health concerns in humans at concentrations above permissible levels (Liu et al. 2013).

Food plants grown in contaminated environments may accumulate heavy metal(loid)s in edible portions at above-permissible levels, which has become a global challenge in healthy food production. Both point and non-point sources of these elements can contaminate soils, waters and atmosphere. Plant roots and leaves absorb these pollutants from the contaminated environment. Ultimately, heavy metal(loid)s end up in the edible plant parts and are thus introduced into the human food chain.

More than 80% of Cd intake in humans was estimated to come from consumption of cereals and vegetables (Khan et al. 2014). In fact, dietary intake of As, Cd, Pb, Hg and Ni is of major health concern for humans (WHO/FAO 2016; EFSA 2017). Exposure to these elements can cause health-impairing effects such as retarded growth, cancer, impaired immunity, endocrine disruption and even death (IARC 2017). Long biological half-lives of these elements and their potential to be retained in human tissues without degradation (thus accumulating over time) make them particularly damaging.

Worldwide, food safety issues have gained researchers' attention in terms of health risks associated with consumption of contaminated foodstuffs. In fact, ingestion of heavy metal(loid)s through polluted foods and food products is the major route of entry of these elements into the human body (the other two routes are inhalation and surface contact). There are reports on intake of contaminated plant-based foods generating health issues in India, China and other countries (Tripathi et al. 1997; Zheng et al. 2007; Singh et al. 2010; Harmanescu et al. 2011; Saha and Zaman 2013). Due to this reason, a comprehensive appraisal of the literature is required regarding levels and sources of heavy metals and As contamination of common plant-based foods. Such information will not only help understand the current situation regarding the metal/metalloid hazards, but it will also help in policy decisions regarding formulation and achievement of the future targets.

Accumulation of metals in edible portions of plants depends on their concentration in the environment, bioavailability in soils, absorption by plant roots or foliage and remobilization within plant tissues (Viehweger 2014). In addition, variability exists among plant species (as well as among genotypes) in the uptake of various heavy metals and their accumulation in edible portions. Due to species differences as well as differential internal transport pathways and mechanisms, vegetables would accumulate heavy metals from contaminated soils, but fruits would not (Sattar et al. 1989, e.g. Cd).

This chapter first provides general description of plant-based foods and toxicity of important trace elements. The focus of the chapter is on summarizing and critically evaluating information on the content of heavy metals in plant-based foods of diverse origin. Factors affecting accumulation of heavy metals in plant-based foods and possible strategies to facilitate safe food production are also discussed.

Plant-Based Foods

Plant-based foods are fresh or minimally processed and comprise grains, fruits, vegetables, pulses, nuts and oils. Plants are the key source of energy, carbohydrates, proteins, lipids, vitamins and minerals for animals and humans (USDA 2018). Global consumption of plant-based foods and the nutritional composition of selected food items are listed in Tables 1 and 2, respectively. Globally, plant-based foods provide on average about 1800 kcal capita^{-1} day^{-1}. Apart from providing energy and nutrition, plants are also well known to produce important secondary metabolites that have therapeutic uses for humans (Poiroux-Gonord et al. 2010). These may include a number of vitamins, provitamins and bioactive compounds. Natural antioxidants of plant origin, including phenolic compounds, vitamins and carotenoids, reduce the oxidative stress-linked diseases (Dykes and Rooney 2007; Okarter and Liu 2010). Selected plant-based foods may also help reduce the risk of cardiovascular diseases, different types of cancers and type 2 diabetes (Holmes 2002; WCRF/AICR 2007; MedlinePlus 2014).

A brief description of global consumption and nutritional importance of each category of plant-based foods is given below.

Cereal grains and their products are consumed more than any other plant- or animal-based food (Table 1). On average, cereals provide nearly 1300 kcal capita^{-1} day^{-1} globally. Wheat, maize and rice collectively contribute about 75% of the total

Table 1 Consumption of plant-based foods and their contribution to energy consumption by humans

Food category	Consumption or energy	World	Asia	Africa	South America	North America	Europe	Oceania
Cereals	g capita^{-1} day^{-1}	403	426	414	318	293	362	249
	kcal capita^{-1} day^{-1}	1292	1422	1284	967	812	1007	764
Vegetables	g capita^{-1} day^{-1}	385	484	185	144	311	315	278
	kcal capita^{-1} day^{-1}	95	119	48	39	70	80	75
Fruits	g capita^{-1} day^{-1}	213	197	181	265	295	260	243
	kcal capita^{-1} day^{-1}	97	87	107	128	120	110	109
Nuts	g capita^{-1} day^{-1}	6	6	5	2	13	11	18
	kcal capita^{-1} day^{-1}	16	15	13	5	35	25	43
Pulses	g capita^{-1} day^{-1}	20	18	32	29	14	7	6
	kcal capita^{-1} day^{-1}	68	62	110	98	47	24	19
Oils	g capita^{-1} day^{-1}	31	25	24	42	81	48	55
	kcal capita^{-1} day^{-1}	271	215	208	369	677	226	471

Source: FAO Food Consumption Database (FAO 2013)

Table 2 Nutritional content of selected plant-based foods on fresh-weight bases

Nutrient	Value per kg	Maize	Tomato	Banana	Cashew nuts	Lentil	Soybean oil
Proximates							
Ash	g	6.2	5.0	8.2	25	27	0.0
Carbohydrates	g	187	39	228	302	634	0.0
Dietary fibre	g	20	12	26	30	101	0.0
Energy	kcal	860	180	890	5530	3520	8840
Lipids	g	14	2.0	3.3	438	11	1000
Proteins	g	33	8.8	11	182	246	0.0
Water	g	760	945	749	52	83	0.0
Minerals							
Calcium	mg	20	100	50	370	350	0.0
Copper	mg	0.5	0.6	0.8	22	7.5	0.0
Iron	mg	5.2	2.7	2.6	67	65	0.5
Magnesium	mg	370	110	270	2920	470	0.0
Manganese	mg	1.6	1.1	2.7	17	14	0.0
Phosphorus	mg	890	240	220	5930	2810	0.0
Potassium	mg	2700	2370	3580	6600	6770	0.0
Selenium	µg	6.0	0.0	10	199	1.0	0.0
Sodium	mg	150	50	10	120	60	0.0
Zinc	mg	4.6	1.7	1.5	58	33	0.1
Vitamins							
Folate (vitamin B9)	µg	420	150	200	250	4790	0.0
Lutein + zeaxanthin	µg	6440	1230	220	220	0.0	0.0
Niacin (vitamin B3)	mg	18	5.9	6.7	11	26	0.0
Pantothenic acid (vitamin B5)	mg	7.2	0.9	3.3	8.6	21	0.0
Phylloquinone (vitamin K)	µg	3.0	79	5.0	341	50	1839
Riboflavin (vitamin B2)	mg	0.6	0.2	0.7	0.5	2.1	0.0
Thiamine (vitamin B1)	mg	1.6	0.4	0.3	4.3	8.7	0.0
Total ascorbic acid	mg	68	137	87	5.0	45	0.0
Vitamin A	IU	1870	8330	640	0.0	390	0.0
Vitamin B6	mg	0.9	0.8	3.7	4.2	5.4	0.0
α-Carotene	µg	160	1010	250	0.0	0.0	0.0
α-Tocopherol (vitamin E)	mg	0.7	5.4	1.0	9.2	4.9	82
β-Carotene	µg	470	4490	260	0.0	230	0.0

Source: Food composition and nutrition database of the US Department of Agriculture (USDA 2018)

cereal production in the world. As compared to continents of North America and Europe, people from Asia and Africa are fulfilling their energy requirements mostly from cereals. In general, rural populations consume more cereals than urban populations.

Cereals are substantial sources of carbohydrates and dietary fibre, water-soluble vitamins (e.g. thiamine, riboflavin, niacin and vitamin B6), minerals (e.g. Zn, Fe, calcium Ca, potassium K, sodium Na, phosphorus P and magnesium Mg) and amino acids (e.g. arginine and lysine) (Table 2). Wheat bran contains about 45% of dietary fibre (Fardet 2010) that has an important role in the colonic faecal transit time (Payler et al. 1975). Cereal brans are also important in reducing cholesterol level in humans.

Vegetables are mainly consumed in Asian countries, about 484 g capita^{-1} day^{-1}, followed by countries in Europe and North America (Table 1). Vegetables are an important source of dietary fibre, vitamins, minerals, phenolics and flavonoids (Table 2). Along with high nutritional quality, vegetables also provide several vital substances for humans, such as bioactive molecules in cruciferous vegetables that have anti-inflammatory properties (Manchali et al. 2012). Red onions are rich in flavonoid with average contents (based on gallic acid equivalents in fresh weight) of about 3.1 g kg^{-1} (Lin and Tang 2007). Green leafy vegetables are rich sources of Fe, Ca, β-carotene, ascorbic acid and several other minerals and vitamins (Gupta et al. 2005). Due to the presence of important phytochemicals, leafy vegetables may reduce the risk of coronary heart diseases and the ischemic stroke risk (Joshipura et al. 2003). Intake of vegetables with low-fat dairy products can lower the hypertension as effectively as prescription medicines (Sacks et al. 1999).

Fruits consumption, unlike that of cereals and vegetables, is greater in developed countries than developing or underdeveloped nations (Table 1). On average, fruit consumption in North America and Europe is 260–295 g capita^{-1} day^{-1}. Fruits are good source of dietary fibre, water-soluble vitamins (ascorbic acid, thiamine, riboflavin and niacin), important minerals (e.g. P, K, Mg, Fe), phenolic compounds, flavonoids and anthocyanins (Table 2; Beattie et al. 2005). Fruits are also rich in carbohydrates and polyunsaturated fatty acids and low in total fat (Barros et al. 2010). Phytochemicals in fruits may be helpful in combating several diseases. For example, anthocyanins and hydroxycinnamic acid in blueberries and cranberries were found to protect endothelial cells against H_2O_2 that would otherwise result in oxidative and inflammatory stresses (Youdim et al. 2002).

Nuts consumption is low in Asia, Africa and South America (Table 1). However, people living in North America, Europe and Oceania have appreciable consumption rates of 11–18 g capita^{-1} day^{-1}. Nuts, such as cashews, have a high-energy value of about 5.5 kcal g^{-1} (Table 2). Nuts are high in proteins, carbohydrates, minerals, niacin, pantothenic acid, folates and tocopherols (Blomhoff et al. 2006). Frequent consumption of nuts is related to lowered prevalence of diabetes mellitus and coronary heart diseases (Sabaté and Ang 2009).

Pulses are edible legume seeds (family Fabaceae) mainly consumed in Africa (32 g capita^{-1} day^{-1}) and South America (29 g capita^{-1} day^{-1}) (Table 1). They are important sources of minerals, folate and proteins (Table 2). Because of high protein content, pulses may be substitutes for meat products for vegetarians and are also cheap protein sources for poor people. Pulses are low in fat and glycaemic index. In addition to nutritional aspects, pulses have important therapeutic roles in humans (Iqbal et al. 2006). Insoluble fibre in pulses is beneficial in reducing the risk of colon cancer. Phytonutrients and antioxidants in pulses also have anticancer properties (Campos

et al. 2013). Frequent consumption of pulses (four to five servings per week) reduced the risk of cardiovascular diseases by more than 10% (Flight and Clifton 2006).

Plant oils are derived from plant sources and are mainly used in cooking. There are appreciable contents of oils in different oil seed crops (about 20% in soybean and over 40% in sunflower and canola) (Sarwar 2013; USDA 2018). Compared to other continents, North America has higher consumption of plant oils (81 g capita^{-1} day^{-1}) (Table 1). Oils contain lipids, fat-soluble vitamins (E and K), essential fatty acids and antioxidants (e.g. soybean oil; Table 2). Olive oil is prominent source of monounsaturated fatty acids, polyphenolic compounds, squalene and α-tocopherol (Stark and Madar 2002). Olive oil may impart therapeutic benefits and reduce the risk of several diseases.

The quality of a plant-based food is determined not only by the nutritive value but also by levels and types of contaminants in the food. Plant-based foods might be contaminated by pesticides, heavy metals, metalloids and other toxins. Such pollutants may cause health issues in humans.

Effects of As, Cd, Pb, Hg and Ni on Human Health

Toxic elements for humans that have been reported in foods are Cd, Pb and Hg. These elements do not have any known physiological roles in human metabolism and are lethal to human health at trace levels. However, As and Ni are thought to play some beneficial roles in humans at ultra-trace levels and are toxic only above permissible levels. Detailed reports of health effects of these elements on humans can be found elsewhere (Mertz 1986; Järup 2003; Tchounwou et al. 2012; Sigel et al. 2013; Jaishankar et al. 2014); however, a brief description is given here.

Arsenic is not considered an essential element for human, although ultra-trace levels of As were reported to play some beneficial roles in animals. Growth and reproduction were significantly improved in control goats receiving normal As levels than As-deprived goats (Anke et al. 1980; Schmidt et al. 1984). Studies on rats suggested that As has a physiological role related to methionine metabolism (Uthus 1992; Uthus 2003). However, there is no known biological function of As in humans (Wilcox 2013).

Long-term exposure to above permissible levels of As can cause cardiovascular diseases, diabetes, oxidative stresses and various types of cancers (Ng et al. 2003; Naujokas et al. 2013). Arsenic is involved in cardiovascular diseases because it dilates blood vessels and has negative effects on endothelial tissues (Das et al. 2010; Moon et al. 2012). Arsenic-induced inhibition of cellular enzymes such as pyruvate dehydrogenase, suppression of cellular antioxidants such as glutathione (Samikkannu et al. 2003; Ferrario et al. 2008) and generation of several types of reactive oxygen species (ROS) in cells are linked to oxidative damage to lipids, proteins and DNA (Valko et al. 2007; Roy et al. 2009). Arsenic is genotoxic; it also affects cell proliferation and signalling, transcription and repair of DNA, epigenetic regulation and apoptosis (Waalkes et al. 2004; Bailey and Fry 2014).

Cadmium has obvious carcinogenic effects on humans (ATSDR 2017). Cadmium is mostly bound to metallothioneins in the body, and this complex is transported to various tissues and organs (Ohta and Cherian 1991). Due to a lack of excretory mechanism for Cd, this metal resides in tissues, having half-life of about 20–35 years in kidney cortex. Other organs affected by Cd toxicity are the liver, pancreas and lungs (Jomova and Valko 2011). Lung cancer caused by occupational and environmental Cd exposure has been well documented (NTP 2011). The carcinogenic nature of Cd relates to its ability to induce oxidative stress by the generation of ROS (such as superoxide and OH· radicals and hydrogen peroxide) and reactive nitrogen species (RNS) (such as NO) (Satarug et al. 2002; Waisberg et al. 2003).

Noncarcinogenic effects of Cd on human populations consuming Cd-contaminated cereals and vegetables have also been reported (Zheng et al. 2007). Cadmium exposure may cause hazards to kidneys and bones. In the kidney, the initial tubular damage may progress to formation of stones and chronic renal failure that may be related to an increased excretion of Ca from the body (Hellström et al. 2001; Jin et al. 2004; Nogawa et al. 2004). Similarly, Cd may also reduce mineral density in bones leading to skeletal damages (fractures, osteomalacia and osteoporosis) in long-term exposures (Staessen et al. 1999; Alfvén et al. 2000; Nordberg et al. 2002; Jin et al. 2004).

Cadmium exposure is closely related to cardiovascular diseases and hypertension (Menke et al. 2009; Angeli et al. 2013). Elevated Cd levels (an increase of about 50% in blood) was linked to 35% increase in risk of stroke and 48% increase in risk of heart failure (Peters et al. 2010). Blood and urinary Cd are involved in peripheral arterial disease (Jarup and Akesso 2009). Chronic exposure to Cd reduces the functioning of endothelial cells resulting in cell death, vascular inflammation, release of cytokines, aggregation of smooth muscle cells (Messner and Bernhard 2010) and angiogenesis (Woods and Fearon 2009). These cellular disturbances result in hypertension and ischemic heart disease.

Lead can damage cells of animals by a decrease in antioxidants and production of free radicals (Ercal et al. 2001). Up to 40% reduction in the concentration of glutathione in cells was observed due to intake of Pb (Hunaiti and Soud 2000). This may lead to elevated levels of ROS that induce carcinogenesis by damage to existing DNA, delayed replication of DNA and clastogenicity (Silbergeld and Waalkes 2000). Hence, Pb is a well-known carcinogen (IARC 2017). Studies also found that Pb can induce toxicity and apoptosis in human cancer cells that have multiple consequences ranging from oxidative stresses to death of human subjects (Yedjou and Tchounwou 2007). Lead was also involved in renal tumours (Waalkes et al. 1995), and lung and stomach cancers (Steenland and Boffetta 2000).

Noncarcinogenic effects of Pb poisoning were reported in both occupational and dietary exposures. Production of ROS and RNS in humans under Pb exposure led to hypertension, elevated blood pressure, kidney damage and neurological disorders (Apostoli et al. 2005; Valko et al. 2007; Verstraeten et al. 2008). Long-term exposure to Pb may result in memory deterioration, prolonged reaction times and reduced ability to understand. Children exposure to Pb may lead to behavioural disturbances in learning and concentration (Järup 2003).

Mercury and its compounds are involved in neurological disorders, genotoxic effects, oxidative stresses, cardiovascular diseases, different types of cancers and several other diseases in humans (Bonacker et al. 2004; Zahir et al. 2005; WHO 2016). Neurological disorders in children related to Hg exposure have been associated with dysplasia of cerebral and cerebellar cortex, neuronal ectopia and several other neurological disorders (Sakamoto et al. 2002; Johnson 2004). Exposure at high doses is related to impairment of motor functions in all age groups (Dietrich et al. 2005).

Genotoxicity of Hg is due to its negative effects on microtubules, mitosis and DNA repair mechanism (Crespo-López et al. 2009; Tchounwou et al. 2012). In the human body, a methylmercury-induced chromosomal damage in germ line cells gives rise to an abnormal offspring. Chromosomal abnormality is strongly associated with binding of Hg to a microtubular assembly; complete inhibition of the assembly was observed at 10 μM Hg (Bonacker et al. 2004). Exposure of blood cell cultures to mercuric chloride resulted in abnormal mitosis (Rao et al. 2001).

Intake of Hg can induce oxidative stress, increased secretion of β-amyloid 1–40 and 1–42 and cytotoxicity that may lead to Alzheimer's and Parkinson's diseases (Olivieri et al. 2002; Crespo-López et al. 2009; Tchounwou et al. 2012).

Nickel, in ultra-trace levels, is thought to play some essential roles in animals and humans. In rats and goats, Ni deficiency decreased conception and growth rates, and increased abortions (Nielsen et al. 1975; Spears et al. 1986; Yokoi et al. 2003). The Ni essentiality is linked to its possible roles in the production of haemoglobin and red blood cells and in gene expressions for the regulation of enzymes in the liver and kidney (Nielsen et al. 1984; Spears et al. 1986; Stangl and Kirchgessner 1996). However, the positive effects of dietary Ni may only be due to wide presence of Ni-dependent enzymes in beneficial microflora in the animal gut (Tremaroli and Bäckhed 2012).

Nickel exposure to above permissible levels, both occupational and dietary, can cause allergy, lung fibrosis, iatrogenic poisoning, organ system toxicity, dermatitis and cancer (Friberg and Elinder 1993; Salnikow and Zhitkovich 2007). These diseases are mediated by malfunctioning in metabolic pathways that underlie inflammation, stress response, oxidative stress, cell proliferation and cell death (Nielsen et al. 1999; Denkhaus and Salnikow 2002). Nickel ions induce the production of tumours by inhibiting lymphocyte activity. Plausible mechanisms of Ni carcinogenicity include mutagenesis, damage to chromosomes, formation of Z-DNA and inhibition of DNA excision repair (Costa and Klein 1999; Cangul et al. 2002).

Arsenic, Cd, Pb, Hg and Ni in Cereal Grains

Plants have several mechanisms to prefer essential and avoid toxic elements. Thereby, the uptake of toxic heavy metal(loid)s by roots and their transport from roots to above-ground parts and then particularly to grains are minimized (Khan et al. 2014). However, non-essential elements can use non-specific transporters

and soluble metal carriers for their uptake and transport in plants. Under metal(loid)-contaminated environments, therefore, a significant amount of toxic heavy metal(loid)s can be accumulated into cereal grains (Table 3).

Arsenic concentration in cereal grains ranged from 0 to 1.70 mg kg^{-1} (Table 3). In total diet study of the UK and France, As concentration in miscellaneous cereals was lower than the maximum permissible limit of 0.10 mg kg^{-1} (Leblanc et al. 2005; Rose et al. 2010). Concentration of As in wheat grains produced by farmers in Spain (Hernández-Martínez and Navarro-Blasco 2013) and in rice grains collected from different cities of Cambodia (Wang et al. 2013) was also within the permissible levels.

Polluted agricultural soils or irrigation water may contaminate cereal grains with As. For example, in India, As concentration in rice and wheat grains collected from the fields irrigated by As-contaminated basin water was above the safe limit (Bhattacharya et al. 2010). Similarly, concentration of As in rice produced in As-contaminated acidic soil (total soil level of 100 mg As kg^{-1}) in China was also above the maximum permissible limit (Geng et al. 2017). Surveys from maize fields in Bangladesh (Islam et al. 2014) and rice fields in Vietnam (Phuong et al. 1999) reported As contamination in maize grains.

Cadmium in cereal grains collected from local markets in France, Ethiopia, the UK, Australia, Nigeria and India had not-detectable to safe levels of Cd (≤0.10 mg kg^{-1}) (Table 3). However, concentration of Cd in some of the samples of cereal grains collected at the Saudi Arabia markets was above the safe limit (Ali and Al-Qahtani 2012). Oat and millet grains from Finland had Cd levels below the maximum permissible limit (Ekholm et al. 2007); and similar results were reported for wheat flour in Brazil and Spain (Santos et al. 2004; Tejera et al. 2013). Field-grown cereals in various countries also had safe levels of Cd (Phuong et al. 1999; Harcz et al. 2007; Islam et al. 2014).

Cadmium concentration in cereals is related to contaminated environments and inputs (Table 3). For example, toxic levels of Cd in soils resulted in contaminated grains of sorghum (Angelova et al. 2011) and barley (Hymete and Eticha 2015). Due to the application of contaminated biosolids, concentration of Cd in organically grown rice and wheat grains was also above the safe limit (Chandorkar and Vaze 2013).

Lead concentration in a study on Swedish rye was below a detection limit (Jorhem et al. 2001). Concentration of Pb in market surveys of most countries was within safe levels (≤0.20 mg kg^{-1}) (Table 3). However, some of the rice samples purchased from markets in Australia (Rahman et al. 2014) and Saudi Arabia (Ali and Al-Qahtani 2012) had Pb concentration above the maximum permissible limit. Oat and millet grains collected from agricultural fields in Finland (Ekholm et al. 2007) and wheat grains from a field in Belgium (Harcz et al. 2007) had safe levels of Pb. Wheat flour in Brazil and Spain also had safe levels of Pb (Santos et al. 2004; Tejera et al. 2013).

Contaminated organic wastes may result in increased Pb concentration in grains (Cang 2004; Chandorkar and Vaze 2013). Grains of rice, maize and millet collected from contaminated agricultural fields of Nigeria (Orisakwe et al. 2012) and Ethiopia

Table 3 Concentration of arsenic, cadmium, lead, mercury and nickel in cereal grains

Cereal	Country	Study/contamination	As	Cd	Pb	Hg	Ni
			mg kg^{-1}				
Barley	Ethiopia	Market survey (Tegegne 2015)		nd	0.03		nd
Barley	Ethiopia	Farmer field (Hymete and Eticha 2015)		0.22–1.95	0.82–5.64		
Barley	India	Market survey (Singh and Garg 2006)				0.06	<0.01
Maize	Greek	Market survey (Karavoltsos et al. 2002)		<0.01			
Maize	Ethiopia	Market survey (Tegegne 2015)		nd	nd		0.07
Maize	Nigeria	Contaminated soil (Orisakwe et al. 2012)		nd	1.01		nd
Maize	Nigeria	Market survey (Akinyele and Shokunbi 2015)		<0.01	<0.08		0.09
Maize	Bangladesh	Field survey (Islam et al. 2014)	0.08–1.70	0.02–0.53	0.04–1.30		0.24–0.80
Millet	Finland	Market survey (Ekholm et al. 2007)		0.03	0.02		2.20
Millet	Nigeria	Contaminated field (Orisakwe et al. 2012)		nd	3.54		0.44
Millet	India	Market survey (Singh and Garg 2006)				0.10	
Miscellaneous	UK	Market survey (Rose et al. 2010)	0.01	0.02	0.01	0.02	0.16
Miscellaneous	France	Market survey (Leblanc et al. 2005)	<0.01	0.01	0.02	0.01	0.47
Oats	Finland	Market survey (Ekholm et al. 2007)		0.03	0.05		1.90
Rice	Greek	Market survey (Karavoltsos et al. 2002)		0.01			
Rice	China	Contaminated soil (Geng et al. 2017)	0.83				
Rice	Nigeria	Farmer field (Orisakwe et al. 2012)		nd	61		nd
Rice	Saudi Arabia	Market survey (Othman 2010)			0.02–0.03		
Rice	Saudi Arabia	Market survey (Ali and Al-Qahtani 2012)		0.92	6.16	0.02	

(continued)

Table 3 (continued)

Cereal	Country	Study/contamination	As	Cd	Pb	Hg	Ni
			mg kg^{-1}				
Rice	France	Market survey (Leblanc et al. 2005)	0.02	<0.01	0.01	0.01	0.02
Rice	Nigeria	Market survey (Akinyele and Shokunbi 2015)		<0.01	<0.08		0.12
Rice	India	Organic farming (Chandorkar and Vaze 2013)		0.52	0.10		0.91
Rice	Cambodia	Market survey (Wang et al. 2013)	0.01–0.09				
Rice	Australia	Market survey (Rahman et al. 2014)		0.01–0.02	0.02–1.30		0.06–0.40
Rice	India	Peri-urban areas (Tripathi et al. 1997)		0.01	0.02		
Rice	Vietnam	Field survey (Phuong et al. 1999)	0.03–0.78	0.00–0.01			0.13–2.02
Rice	Bangladesh	Field survey (Islam et al. 2014)	0.06–1.60	<0.01–0.07	0.07–1.30		0.03–2.60
Rice	India	Contaminated soils (Bhattacharya et al. 2010)	0.06–0.60				
Rye	Sweden	Market survey (Jorhem et al. 2001)		0.02	nd		0.10
Sorghum	Ethiopia	Market survey (Tegegne 2015)		nd	0.08		0.43
Sorghum	Nigeria	Market survey (Orisakwe et al. 2012)		nd	nd		0.53
Sorghum	Bulgaria	Contaminated area (Angelova et al. 2011)		1.56	10.30		
Wheat	India	Peri-urban area (Tripathi et al. 1997)		0.01	0.02		
Wheat	Ethiopia	Market survey (Tegegne 2015)		nd	0.05		0.27
Wheat	Nigeria	Farmer field (Orisakwe et al. 2012)		nd	nd		0.39
Wheat	Saudi Arabia	Market survey (Ali and Al-Qahtani 2012)		1.90	2.80	0.02	
Wheat	Bangladesh	Field survey (Islam et al. 2014)	0.08–1.50	<0.01–0.66	0.03–1.30		0.10–3.60
Wheat	Nigeria	Market survey (Akinyele and Shokunbi 2015)		<0.01	<0.08		0.06

(continued)

Table 3 (continued)

Cereal	Country	Study/contamination	As	Cd	Pb	Hg	Ni
			mg kg^{-1}				
Wheat	Belgium	Organic and conventional farming (Harcz et al. 2007)		0.10	0.04–0.10		<0.01
Wheat	Spain	Field survey (Hernández-Martínez and Navarro-Blasco 2013)	0.02			<0.01	
Wheat	India	Contaminated soil (Bhattacharya et al. 2010)	0.01–0.19				
Wheat	Brazil	Market survey (Santos et al. 2004)		<0.01–0.02	<0.01–0.02		0.01–0.14
Wheat	Spain	Samples from flour industry (Tejera et al. 2013)		0.03	0.04		0.08
Wheat	India	Organic farming (Chandorkar and Vaze 2013)		0.76	0.12		1.28
Maximum permissible limits			0.10[a]	0.10[b]; 0.20[c]	0.20[b]	0.10[d]	0.65[e]

nd not-detectable
[a]Edible oil
[b]Cereal grains other than wheat grains;
[c]Wheat grains
[d]Food items (WHO/FAO 2016)
[e]Based on consumption of two servings of cereals per day by an adult weighing 50 kg (EFSA 2017; USDA 2018)

(Hymete and Eticha 2015) had Pb levels above the safe limit (Table 3). Contaminated soils in Bangladesh (Angelova et al. 2011) and Bulgaria (Islam et al. 2014) resulted in Pb contamination in cereal grains.

Mercury concentration in cereal grains also varied with source of contamination; however, it was always below the maximum permissible level of 0.10 mg Hg kg^{-1} (Table 3).

Nickel concentration in cereal grains collected from local markets in many countries was within permissible limits (≤0.65 mg kg^{-1}) (Table 3). However, oat and millet grains collected from food stores in Finland had Ni concentration up to 1.90 and 2.20 mg kg^{-1}, respectively (Ekholm et al. 2007). Rice grains collected from different regions of Vietnam had maximum Ni concentration of 2.02 mg kg^{-1} (Phuong et al. 1999). Similarly, several grain samples of rice, wheat and maize collected from agricultural fields in Bangladesh had Ni concentration above the safe limit (Islam et al. 2014). Biosolids used in organic farming resulted in contamination of soils and the production of contaminated cereal grains (Chandorkar and Vaze 2013).

Arsenic, Cd, Pb, Hg and Ni in Vegetables

Vegetables are perishable foods mostly grown near the areas of high demand, such as near large cities and towns. Population pressure and large industries pollute the environment around the cities. Therefore, heavy metal(loid) contamination of vegetables is more probable than the food crops grown in remote areas. Moreover, roots/tubers or leaves have higher contaminant concentrations compared with grains or fruits. This is due to additional barriers for toxic elements in transport towards and accumulation into seeds (Khan et al. 2014).

Arsenic concentration in vegetables ranged from 0 to 2.90 mg kg^{-1} (Table 4). Concentration of As in cauliflower, carrot and potato collected from local markets in Spain and in potato and cabbage collected from markets in Croatia and Cambodia ranged from not-detectable to safe levels ($\leq$0.10 mg kg^{-1}) (Martorell et al. 2011; Wang et al. 2013; Stančić et al. 2016). Similarly, field surveys in Bangladesh also reported As concentration in vegetables <0.10 mg kg^{-1} (Alam et al. 2003). However, vegetable samples collected from As-contaminated fields had As concentration above the permissible limit (Rahman and Hasan 2007; George and Gqaza 2015). A field survey from Botswana reported As concentration of 2.90 mg kg^{-1} (Bati et al. 2016).

Cadmium concentration in vegetables grown with Cd-free wastewater or in non-contaminated fields and from most market surveys in various countries was within safe limits ($\leq$0.20 mg Cd kg^{-1} for leafy vegetables and $\leq$0.05 mg Cd kg^{-1} for all other vegetables) (Table 4). However, Cd can accumulate in vegetables grown on roadsides, in cities and at industrial and mining sites. Therefore, some vegetable surveys reported above permissible concentrations of Cd in various vegetables (Karavoltsos et al. 2002; Radwan and Salama 2006; Guerra et al. 2012; Nankishore 2014; Derakhshan et al. 2016; Bati et al. 2016). Concentration of Cd in potato, radish and cucumber grown in Huang Gang city in China was also above the safe limit (Hu et al. 2013). Turnip, carrot, potato and spinach collected from different farms of four major industrial cities in Saudi Arabia had 1.24–4.10 mg Cd kg^{-1} (Ali and Al-Qahtani 2012). Vegetables grown in peri-urban areas of India (Sharma et al. 2009; Ramesh and Murthy 2010; Yadav et al. 2013), Libya (Elbagermi et al. 2012) and Turkey (Demirezen and Aksoy 2006) also had Cd in leafy and fruity vegetables above the safe limits. Similarly, application of contaminated wastewater to agricultural fields in Pakistan (Khan et al. 2015) and India (Saini et al. 2014) resulted in toxic levels of Cd in vegetables.

Lead concentration in various vegetables collected from markets in Nigeria, Yemen, Serbia, Saudi Arabia and Tanzania was below the maximum permissible limits (0.30 and 0.10 mg kg^{-1} for leafy and other vegetables, respectively) (Table 4). However, market surveys in many countries reported toxic levels of Pb in various vegetables. Similarly, selected vegetables grown in agricultural fields in Kyrgyzstan had up to 0.18 mg Pb kg^{-1} (Usubalieva et al. 2013). The Pb contamination in vegetables from markets and field surveys indicate the unstudied sources of contamination in the crop environments.

Table 4 Concentration of arsenic, cadmium, lead, mercury and nickel in edible portions of vegetables

Vegetable	Country	Study/contamination	As	Cd	Pb	Hg	Ni
			mg kg^{-1}				
Brinjal	Bangladesh	Contaminated irrigation (Rahman and Hasan 2007)	0.30				
Cabbage	Nigeria	Market survey (Sobukola et al. 2010)		0.06	0.09–0.17		
Cabbage	Cambodia	Market survey (Wang et al. 2013)	≤0.01				
Cabbage	South Africa	Contaminated field (George and Gqaza 2015)	1.50				
Cabbage	Bangladesh	Field survey (Tasrina and Rowshon 2015)	<0.10	<0.10		<0.03	
Cabbage	Pakistan	Industrial area (Farooq et al. 2008)		0.07	1.92		
Cabbage	Yamen	Market survey (Nogaim et al. 2013)		<0.01	0.07		
Cabbage	Guyana	Market survey (Nankishore 2014)		0.19	0.41		nd
Cabbage	Romania	Near the mining area (Harmanescu et al. 2011)		0.01–0.12	0.05–0.90		
Cabbage	Egypt	Farmer field (Dogheim et al. 2004)		<0.01			
Cabbage	Serbia	Market survey (Škrbić et al. 2013)	<0.03	<0.01	0.05		
Cabbage	Kyrgyzstan	Farmer field (Usubalieva et al. 2013)		0.01–0.03	0.05–0.08		
Cabbage	Ghana	Market survey (Bempah et al. 2011)		0.01–0.08	0.22–0.53		
Cabbage	Ghana	Wastewater irrigation (Lente et al. 2014)			10.5		1.77
Cabbage	Morocco	Wastewater irrigation (Al-Jaboobi et al. 2014)		nd	15.3		85
Cabbage	Brazil	Farmer field (Guerra et al. 2012)		0.12	1.66		0.54
Carrot	Saudi Arabia	Farm survey (Ali and Al-Qahtani 2012)		1.20–1.43	1.13–1.42	0.02–0.03	
Carrot	Saudi Arabia	Market survey (Othman 2010)			0.03		
Carrot	Pakistan	Market survey (Bukhari et al. 2013)			1.70		3.00
Carrot	Egypt	Market survey (Radwan and Salama 2006)		0.01	0.18		

(continued)

Table 4 (continued)

Vegetable	Country	Study/contamination	As	Cd	Pb	Hg	Ni
			mg kg^{-1}				
Carrot	Romania	Grown near the mining area (Harmanescu et al. 2011)		0.01–0.03			
Carrot	Greek	Market survey (Karavoltsos et al. 2002)		0.13–0.16			
Carrot	Serbia	Market survey (Škrbić et al. 2013)	<0.03	<0.01	0.06		
Carrot	Spain	Market survey (Martorell et al. 2011)	nd	0.01	0.06	<0.01	
Carrot	Kyrgyzstan	Market survey (Usubalieva et al. 2013)		0.02	0.03		
Carrot	Libya	Grown near the market (Elbagermi et al. 2012)		0.12	0.21		0.21
Carrot	Ghana	Market survey (Bempah et al. 2011)		0.01–0.05	0.12–0.23		
Carrot	Yamen	Market survey (Nogaim et al. 2013)		0.03	0.07		
Cauliflower	India	Peri-urban area (Sharma et al. 2009)		2.57	1.56		
Cauliflower	Greek	Market survey (Karavoltsos et al. 2002)		0.01–0.01			
Cauliflower	Spain	Market survey (Martorell et al. 2011)	nd	0.01	0.14	0.01	
Cauliflower	China	Farmer field (Hu et al. 2013)		0.04–0.06			
Cauliflower	Brazil	Farmer field (Guerra et al. 2012)		0.08	0.36		0.24
Chinese cabbage	Tanzania	Market survey (Mubofu 2012)		0.01–0.03	0.02–0.02		
Cucumber	Yamen	Market survey (Nogaim et al. 2013)		0.05	0.06		
Cucumber	Saudi Arabia	Market survey (Othman 2010)			0.02		
Cucumber	Egypt	Market survey (Radwan and Salama 2006)		0.15	0.19		
Cucumber	China	Peri-urban areas (Hu et al. 2013)		0.11–0.22			
Cucumber	Kyrgyzstan	Farmer field (Usubalieva et al. 2013)		<0.01	0.03–0.18		
Cucumber	Libya	Grown near the market (Elbagermi et al. 2012)		0.20	0.10		0.22
Cucumber	Turkey	Farmer field (Demirezen and Aksoy 2006)		0.64	6.90		13.5

(continued)

Table 4 (continued)

Vegetable	Country	Study/contamination	As	Cd	Pb	Hg	Ni
			mg kg^{-1}				
Fluted pumpkin	Nigeria	Market survey (Sobukola et al. 2010)		0.01–0.09	0.15–0.27		
Foetid Cassia	Nigeria	Market survey (Yahaya 2013)		nd	0.12		
Kenaf	Nigeria	Market survey (Yahaya 2013)		nd	0.1		
Lettuce	Tanzania	Market survey (Mubofu 2012)		0.02–0.06	0.02–0.07		
Okra	India	Peri-urban area (Sharma et al. 2009)		0.50–1.20	0.30–1.20		
Okra	Bangladesh	Farmer field (Alam et al. 2003)	0.09				
Luffa	Pakistan	Wastewater irrigation (Khan et al. 2015)		0.32	2.34		
Okra	Turkey	Farmer field (Demirezen and Aksoy 2006)		0.58	10.7		2.70
Okra	Ghana	Market survey (Bempah et al. 2011)		<0.01–0.06	0.14–0.26		
Okra	Tanzania	Market survey (Mubofu 2012)		<0.01–0.018	0.02–0.08		
Onion	Botswana	Market survey (Bati et al. 2016)	2.90	0.33	1.60		
Parsley	Iran	Available in the market (Derakhshan et al. 2016)		0.03–0.09	0.01–0.20		
Parsley	Brazil	Market survey (Guerra et al. 2012)		0.18	1.02		0.70
Potato	Saudi Arabia	Farmer field (Ali and Al-Qahtani 2012)		0.99–1.18	1.50–6.19	0.01	
Potato	Croatia	Market survey (Stančić et al. 2016)	0.02–0.03	0.13–0.34		0.01–0.02	
Potato	Saudi Arabia	Market survey (Othman 2010)			<0.01		
Potato	Serbia	Market survey (Škrbić et al. 2013)	<0.03	0.01	<0.01		
Potato	Spain	Market survey (Martorell et al. 2011)	nd	0.05	0.06	0.01	
Potato	China	Peri-urban areas (Hu et al. 2013)		0.09–0.16			
Potato	Kyrgyzstan	Farmer field (Usubalieva et al. 2013)		0.01–0.02	0.08–0.15		
Radish	Pakistan	Market survey (Bukhari et al. 2013)			3.50		8.50
Radish	China	Peri-urban areas (Hu et al. 2013)		0.05–0.09			

(continued)

Table 4 (continued)

Vegetable	Country	Study/contamination	As	Cd	Pb	Hg	Ni
			mg kg^{-1}				
Radish	Pakistan	Wastewater irrigation (Khan et al. 2015)		0.67	11.40		
Spinach	Saudi Arabia	Peri-urban areas (Ali and Al-Qahtani 2012)		4.02–4.13	1.26–2.88	0.01–0.02	
Spinach	India	Peri-urban area (Sharma et al. 2009)		0.98	1.00		
Spinach	Egypt	Market survey (Radwan and Salama 2006)		0.11	0.34		
Spinach	Greek	Market survey (Karavoltsos et al. 2002)		0.04–0.08			
Spinach	Egypt	Farmer field (Dogheim et al. 2004)		<0.01–0.05	0.03–1.40		
Spinach	Nigeria	Wastewater irrigation (Mustapha and Adeboye 2014)		<0.01	0.06		
Spinach	India	Industrial wastes (Ramesh and Murthy 2010)		nd–1.50	28.4–149.5		
Spinach	Libya	Grown near the market (Elbagermi et al. 2012)		0.27	0.32		0.26
Spinach	Nigeria	Field survey (Dike and Odunze 2016)		0.01–0.02	0.90–4.90		0.21–1.20
Spinach	Turkey	Market survey (Bagdatlioglu et al. 2010)		nd–0.05	0.21–1.00		
Spinach	India	Industrial effluent irrigation (Saini et al. 2014)		0.30	0.10		
Spinach	India	Wastewater irrigation (Yadav et al. 2013)		11.2–67.6	12.2–38.2		57–506
Turnip	Saudi Arabia	Peri-urban areas (Ali and Al-Qahtani 2012)		1.24–1.34	1.27–4.37	0.03–0.02	
Turnip	Pakistan	Market survey (Bukhari et al. 2013)			1.70		12.0
Turnip	Bangladesh	Market survey (Linkon et al. 2015)		nd	5.20	nd	4.20
Maximum permissible limits			0.10[a]	0.20[b], 0.05[c]	0.30[b], 0.10[c]	0.10[d]	6.67[e]

nd not-detectable
[a]Edible oil
[b]Leafy vegetables
[c]All other vegetables
[d]Food items (WHO/FAO 2016)
[e]Based on consumption of three servings of vegetables per day by an adult weighing 50 kg (EFSA 2017; USDA 2018)

Spinach irrigated by municipal wastewater in Minna city of Nigeria also had Pb concentration within safe limit (Mustapha and Adeboye 2014). Depending on the source and level of pollution, however, vegetables can be contaminated with Pb, for example, different vegetables grown in peri-urban areas (Dogheim et al. 2004; Demirezen and Aksoy 2006; Sharma et al. 2009; Elbagermi et al. 2012; Yadav et al. 2013; Dike and Odunze 2016) and cabbage grown in industrial and mining zones (Farooq et al. 2008; Harmanescu et al. 2011). Similarly, the use of untreated wastewater for agriculture in different cities in Pakistan (Khan et al. 2015), Ghana (Lente et al. 2014) and Morocco (Al-Jaboobi et al. 2014) caused toxic accumulations of Pb in various leafy and fruity vegetables (Al-Jaboobi et al. 2014; Lente et al. 2014; Khan et al. 2015).

Mercury was not accumulated to toxic levels (>0.10 mg Pb kg^{-1}) in any of the vegetables listed in Table 4.

Nickel concentration in most vegetables was within the permissible limit (≤6.67 mg kg^{-1}) (Table 4). However, some studies reported toxic levels. For example, vegetables collected from local markets in Pakistan had 3–12 mg Ni kg^{-1} (Bukhari et al. 2013). Wastewater irrigation of vegetables in Morocco resulted in 85 mg Ni kg^{-1} (Al-Jaboobi et al. 2014). In India, vegetables grown in peri-urban areas and irrigated with untreated wastewaters had Ni concentration as high as 506 mg Ni kg^{-1} (Yadav et al. 2013).

Arsenic, Cd, Pb, Hg and Ni in Fruits

Unlike vegetables, fruits generally had lower concentrations of contaminants in edible parts (Table 5). The one reason behind this could be the lower contamination levels in orchards than vegetable farms. Moreover, this is due to physiological barriers for toxic heavy metal(loid)s in transport in, and loading/unloading of, phloem and accumulation into fruits (Khan et al. 2014). In fact, there are wide differences in physiology of annual vegetable plants versus perennial fruit trees (Peralta-Videa et al. 2009).

Arsenic contamination to above permissible limit (0.10 mg kg^{-1}) is generally not reported in fruits (Table 5). However, some fruits in market surveys in Botswana (Bati et al. 2016) and Bangladesh (Saha and Zaman 2013) had unsafe levels of As.

Cadmium concentration in fruit samples collected from markets and production areas in most countries was within the safe limits (≤0.05 mg kg^{-1}) (Table 5). Mulberry grown on roadsides in Turkey had Cd concentration within the acceptable level; however, Cd concentration in apples (Hamurcu et al. 2010) and plums (Pehluvan et al. 2012) was above the safe limit.

Yellow plum, fig and black grapes from Turkish markets had a maximum of 0.63 mg Cd kg^{-1} (Duran et al. 2008). Similarly, market surveys in Bangladesh (Saha and Zaman 2013), Botswana (Bati et al. 2016), Brazil (Guerra et al. 2012; Pereira et al. 2014), Poland (Krejpcio et al. 2005) and Serbia (Micić et al. 2013) also reported Cd contamination in some of the fruits. Summer fruits in Pakistan had

0.05–1.00 mg Cd kg^{-1} (Zahoor et al. 2003). Fruits grown near the market in a city in Libya had Cd above the safe limit (Elbagermi et al. 2012). Similarly, concentration of Cd in some fruits collected from farms in polluted areas in Finland (Elbagermi et al. 2012), Nigeria (Orisakwe et al. 2012; Ihesinachi and Eresiya 2014), Kenya (Mausi et al. 2014), the Slovak Republic (Vollmannova et al. 2015) and Pakistan (Akhtar et al. 2010) also ranged above the permissible limit.

Lead concentration in tropical fruits available at markets in Bangladesh, Brazil, Nigeria, Turkey, Saudi Arabia, Spain and India was within the permissible limit (Table 5). In many field and market surveys, however, concentration of Pb in fruits was also reported to be above the safe limit.

In a study conducted in Nigeria, concentration of Pb in avocado was below a detection limit, but its concentration in guava, banana and apple exceeded the safe limit (Orisakwe et al. 2012). Apples and plums grown along the roadsides in Konya city in Turkey also had Pb concentration above the maximum acceptable level (Hamurcu et al. 2010; Pehluvan et al. 2012). Mango and grapes grown near city markets in Libya had Pb concentration above the maximum acceptable concentration (Elbagermi et al. 2012). Lead concentration in tomato, banana and papaya grown in buffer zone around the mining area in India was also above the maximum permissible limit (Barros et al. 2010).

Mercury concentration in fruits listed in Table 5 was below the maximum permissible limit of 0.10 mg kg^{-1}.

Nickel concentration in majority of fruits available in local markets and farms in Turkey, Nigeria, Brazil, the USA, Pakistan, Finland, the Slovak Republic and India was within the safe levels ($\leq$2.92 mg kg^{-1}) (Table 5). Like most of the other heavy metals, however, concentration of Ni in some fruits exceeded the limit. For example, mulberry fruits grown in the south-eastern region of Serbia had 3.60 mg Ni kg^{-1} (Micić et al. 2013). Similarly, some fruits were found to be contaminated with Ni in market surveys in Brazil (Pereira et al. 2014), Libya (Elbagermi et al. 2012), Turkey (Duran et al. 2008) and Pakistan (Zahoor et al. 2003). Avocado, orange and pineapple collected from Nigerian fruit farm had 1.16 to 3.34 mg Ni kg^{-1} (Ihesinachi and Eresiya 2014). Mangoes in some orchards in Pakistan also had unsafe levels of Ni (Akhtar et al. 2010).

Arsenic, Cd, Pb, Hg and Ni in Nuts

Nuts are mostly grown in hilly areas with low human population density and environmental contamination. Therefore, only few studies have reported heavy metal(loid) contamination in nuts (Table 6).

Arsenic concentration in various nuts collected at Swedish and South African markets was within the safe limit ($\leq$0.10 mg kg^{-1}) (Table 6).

Cadmium concentration in hazelnuts, walnuts and almonds from markets in Sweden, Spain and Germany was within the permissible limit ($\leq$0.05 mg kg^{-1}) (Table 6). However, Sattar et al. (1989) reported above-permissible levels of Cd in

Table 5 Concentration of arsenic, cadmium, lead, mercury and nickel in fruits

Fruit	Country	Study/contamination	As	Cd	Pb	Hg	Ni
			mg kg^{-1}				
Apple	Finland	Farmer field (Ekholm et al. 2007)		0.01	0.13		0.07
Apple	Libya	Grown near the markets (Elbagermi et al. 2012)		0.06	0.20		1.00
Apple	Nigeria	Fruit farm (Orisakwe et al. 2012)		nd	0.22		nd
Apple	Pakistan	Market survey (Zahoor et al. 2003)		1.00	3.08		8.90
Apple	Poland	Market survey (Krejpcio et al. 2005)		0.00–0.05	0.01–0.32		
Apple	Saudi Arabia	Market survey (Othman 2010)			0.02		
Apple	Spain	Market survey (Martorell et al. 2011)	≤0.01	≤0.01	0.03–0.04	<0.01	
Apple	Turkey	Grown along the roadside (Hamurcu et al. 2010)		0.16	2.21		0.42
Apricot	Pakistan	Market survey (Zahoor et al. 2003)			1.14		1.00
Avocado	Nigeria	Fruit farm (Orisakwe et al. 2012)		nd	nd		0.72
Avocado	Nigeria	Fruit farm (Ihesinachi and Eresiya 2014)					3.34
Banana	Bangladesh	Market survey (Saha and Zaman 2013)	0.13	0.58	0.42		
Banana	Ghana	Market survey (Bempah et al. 2011)		nd	0.01–0.03		
Banana	India	Grown near the mining area (Barros et al. 2010)		0.01	0.80		0.40
Banana	Nigeria	Market survey (Sobukola et al. 2010)		≤0.01	0.11–0.18		
Banana	Nigeria	Fruit farm (Orisakwe et al. 2012)		nd	0.46		nd
Banana	Pakistan	Market survey (Ismail et al. 2011)					0.06
Banana	Saudi Arabia	Market survey (Othman 2010)			0.01		
Black berry	Bangladesh	Market survey (Saha and Zaman 2013)	0.13	0.62	0.90		
Black grapes	Turkey	Market survey (Duran et al. 2008)		0.63	7.80		2.12

(continued)

Table 5 (continued)

Fruit	Country	Study/contamination	As	Cd	Pb	Hg	Ni
			mg kg^{-1}				
Blackberry	Slovak Republic	Polluted air (Vollmannova et al. 2015)		0.05–0.08	0.03	<0.01	0.12–0.78
Cherry	Poland	Market survey (Krejpcio et al. 2005)		<0.01–0.04	0.01–0.14		
Cherry	Turkey	Farmer field (Bagdatlioglu et al. 2010)		nd	0.01		
Fig	Turkey	Market survey (Duran et al. 2008)		0.28	10.10		4.31
Grape	Libya	Grown near the market (Elbagermi et al. 2012)		0.05	0.40		0.63
Grape	Nigeria	Fruit farm (Orisakwe et al. 2012)		0.14	0.33		0.08
Grape	Turkey	Farmer field (Bagdatlioglu et al. 2010)		nd	<0.01–0.02		
Grapes	Nigeria	Market survey (Sobukola et al. 2010)		≤0.01	0.09–0.1		
Guava	Bangladesh	Fruit farm (Sajib et al. 2014)	nd	nd	nd		
Guava	Bangladesh	Market survey (Saha and Zaman 2013)	0.13	1.50	0.88		
Guava	Nigeria	Fruit farm (Orisakwe et al. 2012)		nd	0.58		nd
Guava	Pakistan	Market survey (Ismail et al. 2011)					0.04
Litchi	Bangladesh	Farmer field (Sajib et al. 2014)	nd	nd	nd		
Litchi	Bangladesh	Market survey (Saha and Zaman 2013)	0.13	2.14	1.20		
Mango	Bangladesh	Farmer field (Sajib et al. 2014)	nd	nd	0.02		
Mango	Bangladesh	Market survey (Saha and Zaman 2013)	0.13	3.02	1.45		
Mango	Brazil	Market survey (Guerra et al. 2012)		0.12	0.14		0.08
Mango	Ghana	Market survey (Bempah et al. 2011)		0.01–0.06	0.02–0.12		
Mango	India	Mango orchard (Ravi et al. 2012)	<0.01	<0.01	<0.01		<0.01

(continued)

Table 5 (continued)

Fruit	Country	Study/contamination	As	Cd	Pb	Hg	Ni
			mg kg^{-1}				
Mango	Kenya	Mano orchard (Mausi et al. 2014)		0.07–0.09	0.31–0.61		
Mango	Libya	Grown near the market (Elbagermi et al. 2012)		0.36	1.82		5.14
Mango	Nigeria	From street vendor (Ogunkunle et al. 2014)	nd	0.09	1.62	nd	0.05
Mango	Pakistan	Mango orchard (Akhtar et al. 2010)		0.22	1.30		6.28
Mango	Pakistan	Market survey (Ismail et al. 2011)					0.06
Mango	Pakistan	Market survey (Zahoor et al. 2003)		0.41	1.08		6.20
Miscellaneous	America	Market survey (Mehari et al. 2015)		<0.10	10.00		<0.10
Miscellaneous	Chile	Market survey (Muñoz et al. 2005)	0.01	<0.01		<0.01	
Mulberry	Serbia	Farmer field (Micić et al. 2013)		24.6	0.90		3.60
Mulberry	Turkey	Grown along the roadside (Pehluvan et al. 2012)		0.05	3.89		0.66
Orange	Kenya	Farmer field (Mausi et al. 2014)		0.05–0.06	0.44–0.65		
Orange	Libya	Grown near the market (Elbagermi et al. 2012)		0.03	0.20		1.10
Orange	Nigeria	Farmer field (Ihesinachi and Eresiya 2014)		0.10	5.80		2.99
Orange	Nigeria	Market survey (Sobukola et al. 2010)		<0.01	0.11		0.12–0.13
Orange	Saudi Arabia	Market survey (Othman 2010)			0.01		
Orange	Spain	Market survey (Martorell et al. 2011)	≤0.01	0.01	0.07–0.08	<0.01	
Papaya	Brazil	Market survey (Guerra et al. 2012)		0.23	0.15		0.06
Papaya	Ghana	Market survey (Bempah et al. 2011)		<0.01–0.04	0.01–0.09		
Papaya	India	Grown near the mining area (Barros et al. 2010)		0.02	0.60		0.90

(continued)

Table 5 (continued)

Fruit	Country	Study/contamination	As	Cd	Pb	Hg	Ni
			mg kg^{-1}				
Peach	Pakistan	Market survey (Zahoor et al. 2003)		0.05	0.94		2.60
Peach,	Finland	Farmer field (Ekholm et al. 2007)		<0.01	0.12		0.75
Pear	Ghana	Market survey (Bempah et al. 2011)		0.01–0.03	0.09–0.14		
Pear	Poland	Market survey (Krejpcio et al. 2005)		<0.01–0.06	0.01–0.09		
Pear	Spain	Market survey (Martorell et al. 2011)	≤0.01	≤0.01	0.02–0.03	≤0.01	
Pears	Saudi Arabia	Market survey (Othman 2010)			<0.01		
Pindo Palm	Brazil	Market survey (Pereira et al. 2014)		0.06	nd		4.99
Pineapple	Ghana	Market survey (Bempah et al. 2011)		0.00–0.05	0.03–0.11		
Pineapple	Nigeria	Fruit farm (Ihesinachi and Eresiya 2014)		0.08	5.01		1.16
Pineapple	Nigeria	Market survey (Sobukola et al. 2010)		≤0.01	0.02–0.12		
Plum	Turkey	Grown along the roadside (Pehluvan et al. 2012)		0.14	2.82		0.50
Raspberry	Finland	Farmer field (Ekholm et al. 2007)		0.03	0.14		0.88
Raspberry	Slovak Republic	Polluted atmosphere (Vollmannova et al. 2015)		0.02	nd	0.01	0.14
Strawberry	Brazil	Market survey (Guerra et al. 2012)		0.04	0.05		0.07
strawberry	Finland	Farmer field (Ekholm et al. 2007)		0.04	0.05		0.24
Strawberry	Poland	Market survey (Krejpcio et al. 2005)		0.00–0.13	0.02–0.34		
Strawberry	Saudi Arabia	Market survey (Othman 2010)			0.02		
Strawberry	Spain	Market survey (Martorell et al. 2011)	≤0.01	<0.01	0.10	<0.01	
Strawberry	Turkey	Farmer field (Bagdatlioglu et al. 2010)		nd	<0.01–0.10		
Tangerine	Nigeria	Market survey (Sobukola et al. 2010)		≤0.01	0.09–0.10		

(continued)

Table 5 (continued)

Fruit	Country	Study/contamination	As	Cd	Pb	Hg	Ni
			mg kg^{-1}				
Tomato	Botswana	Market survey (Bati et al. 2016)	1.20	0.38	1.42		
Tomato	India	Grown in the mining area (Barros et al. 2010)		0.01	0.30		0.20
Tomato	Turkey	Farmer field (Bagdatlioglu et al. 2010)		nd	0.01		
tomatoes	Saudi Arabia	Market survey (Othman 2010)			0.03		
Water melon	Ghana	Market survey (Bempah et al. 2011)		nd	nd		
Watermelon	Brazil	Market survey (Guerra et al. 2012)		0.02	0.17		0.05
Watermelon	Nigeria	From street vendor (Ogunkunle et al. 2014)	nd	<0.01	1.76	nd	0.14
Watermelon	Nigeria	Market survey (Sobukola et al. 2010)		≤0.01	0.01–0.12		
Yellow guava	Brazil	Market survey (Pereira et al. 2014)		0.03	nd		3.40
Yellow plum	Turkey	Market survey (Duran et al. 2008)		0.63	7.80		3.58
Maximum permissible limit			0.10[a]	0.05[b]	0.10[c], 0.20[d]	0.10[a]	2.92[e]

nd not-detectable
[a]Edible oils or any food item
[b]Fruiting vegetables
[c]Citrus fruits, pome fruits or stone fruits
[d]Berries or any other small fruit (WHO/FAO 2016)
[e]Based on three servings of fruits per day by an adult weighing 50 kg (EFSA 2017; USDA 2018)

almonds, walnuts, pine nuts and peanuts collected from local markets in Peshawar, Pakistan.

Lead concentration in various nuts from the UK, Germany and Sweden was within the acceptable level (≤0.10 mg kg^{-1}) (Table 6). However, some studies reported Pb concentration in nuts above the safe limit, for example, in almonds, pine nuts and peanuts from Pakistan and Spain (Sattar et al. 1989; Cabrera et al. 2003).

Mercury concentration is reported only in a few studies on nuts (Table 6). Concentration of Hg in miscellaneous nuts from different markets in the UK and Sweden was within the permissible limit of 0.10 mg Hg kg^{-1} (Rodushkin et al. 2008; Rose et al. 2010).

Nickel concentration in all nuts listed in Table 6 was in the permissible levels, except cashew nuts from a Swedish market.

Table 6 Concentration of arsenic, cadmium, lead, mercury and nickel in nuts

Edible nuts	Countries	Study	As	Cd	Pb	Hg	Ni
			mg kg^{-1}				
Almond	South Africa	Market survey (Moodley et al. 2007)	0.01				
Almond	Spain	Market survey (Cabrera et al. 2003)		nd–0.01	0.26–0.37		0.19–0.15
Almond	Pakistan	Market survey (Sattar et al. 1989)		0.24	1.02		
Almonds	Sweden	Market survey (Rodushkin et al. 2008)	<0.01	<0.01	<0.01	<0.01	1.10
Brazil nut	South Africa	Market survey (Moodley et al. 2007)	0.02				
Brazil nut	Sweden	Market survey (Rodushkin et al. 2008)	<0.01	<0.01	<0.01	<0.01	4.60
Cashew nut	Sweden	Market survey (Rodushkin et al. 2008)	<0.01	<0.01	<0.01	<0.01	6.70
Cashew nut	Spain	Market survey (Cabrera et al. 2003)		nd	0.20–0.29		0.16–0.17
Hazelnut	Germany	Peri-urban area (von Hoffen and Säumel 2014)		0.01	0.02		
Hazelnut	Spain	Market survey (Cabrera et al. 2003)		≤0.01	0.30–0.39		0.26–0.46
Hazelnuts	Sweden	Market survey (Rodushkin et al. 2008)	0.01	0.01	<0.01	<0.01	1.30
Miscellaneous	UK	Market survey (Rose et al. 2010)			<0.01	<0.01	3.02
Peanut	Pakistan	Market survey (Sattar et al. 1989)		0.09	0.12		
Peanut	Spain	Market survey (Cabrera et al. 2003)		≤0.01	0.22–0.36		0.20–0.36
Pine nut	Pakistan	Purchased from retailer agency (Sattar et al. 1989)		0.12	0.49		
Pistachio	Spain	Market survey (Cabrera et al. 2003)		nd	0.15–0.25		0.13–0.34
Walnut	Germany	Peri-urban area (von Hoffen and Säumel 2014)		<0.01	0.01		
Walnut	South Africa	Market survey (Moodley et al. 2007)	0.02				
Walnut	Pakistan	Market survey (Sattar et al. 1989)		0.11	0.19		
Walnuts	Sweden	Market survey (Rodushkin et al. 2008)	<0.01	<0.01	<0.01	<0.01	1.60
Maximum permissible limit			0.10[a]	0.05[b]	0.10[c]	0.10[a]	5.00[d]

nd not-detectable
[a]Edible oils or any food item
[b]Fruiting vegetables
[c]Citrus fruits, pome fruits or stone fruits (WHO/FAO 2016)
[d]Based on one serving of nuts per day by an adult weighing 50 kg (EFSA 2017; USDA 2018)

Arsenic, Cd, Pb, Hg and Ni in Pulses

Similar to nuts, pulses are not generally contaminated with heavy metal(loid)s. However, contamination in the environment resulted in toxic levels of various heavy metal(loid)s in pulses (Table 7).

Arsenic concentration in different pulses grown in India was in the safe limit of ≤0.10 mg kg^{-1} (Table 7). The concentration of As in pulses collected from markets in Pakistan, Bangladesh and India ranged from 0.01 to 1.80 mg As kg^{-1} (Bhattacharya et al. 2010; Rehman et al. 2011; Ahmed et al. 2016; Krohn et al. 2016). Chickpea and lentil grown in contaminated fields had As contamination to unsafe levels (Bhattacharya et al. 2010).

Table 7 Concentration of arsenic, cadmium, lead, mercury and nickel in pulses

Pulses	Country	Study/contamination	As	Cd	Pb	Hg	Ni
			mg kg^{-1}				
Broadbean	Mexico	Field experiment (Garrido et al. 2005)					3.12
Broadbean	Turkey	Market survey (Bagdatlioglu et al. 2010)		nd	0.05		
Chickpea	Bangladesh	Market survey (Ahmed et al. 2016)	0.07–0.21				
Chickpea	India	Contaminated field (Bhattacharya et al. 2010)	0.02–0.90				
Chickpea	India	Field survey (Samal and Garg 2012)	0.06–0.08	0.07–0.08	0.17–0.19		
Chickpea	India	Field survey (Samal and Garg 2012)	0.06–0.08	0.07	0.16–0.18		
Chickpea	Spain	Market survey (Martorell et al. 2011)	0.01	<0.01	0.03	0.01	
Cowpea	India	Field survey (Samal and Garg 2012)	0.05–0.06	0.01	0.13–0.14		
Cowpea	China	Peri-urban area (Hu et al. 2013)		0.06–0.11			
Cowpea, brown	Iraq	Field survey (Ismael et al. 2016)		0.09	0.20		3.50
Cowpea, red	Iraq	Field survey (Ismael et al. 2016)		0.09	0.20		3.80
Cowpea, white	Iraq	Field survey (Ismael et al. 2016)		0.09	0.18		2.04
Green bean	Roam	Industrial area (Beccaloni et al. 2013)	0.13	0.05	0.01		

(continued)

Table 7 (continued)

Pulses	Country	Study/contamination	As	Cd	Pb	Hg	Ni
			mg kg^{-1}				
Green bean	Egypt	Market survey (Radwan and Salama 2006)		0.01	0.08		
Haricot bean	Spain	Market survey (Martorell et al. 2011)	0.01	0.01	0.05	0.01	
Horse gram	India	Field survey (Samal and Garg 2012)	0.05–0.06	0.08–0.09	0.15–0.17		
Kidney bean	China	Peri-urban areas (Hu et al. 2013)		0.06–0.13			
Lentil	Ethiopia	Field survey (Leshe and Tessema 2014)		0.09–0.30	1.40–1.80		1.40–1.80
Lentil	Bangladesh	Market survey (Ahmed et al. 2016)	0.02–0.13				
Lentil	Bangladesh	Contaminated field (Krohn et al. 2016)	<0.01				
Lentil	India	Contaminated field (Bhattacharya et al. 2010)	0.00–0.14				
Lentil	Pakistan	Market surveys (Rehman et al. 2011)	0.10–1.80	0.02–0.13	0.14–1.60		0.04–4.00
Lentil	Spain	Market survey (Martorell et al. 2011)	0.01	0.01	0.04	0.01	
Lentil, green	India	Field survey (Samal and Garg 2012)	0.06–0.07	0.02	0.12–0.14		
Lentil, black	India	Field survey (Samal and Garg 2012)	0.05–0.06	0.02	0.15–0.17		
Lentil, red	India	Field survey (Samal and Garg 2012)	0.05–0.06	0.08–0.09	0.17–0.18		
Mung bean	India	Field survey (Samal and Garg 2012)	0.05–0.07	0.05–0.06	0.14		
Mung bean	Nigeria	Market survey (Akinyele and Shokunbi 2015)		0.08	<0.08		0.08
Mungo beans	India	Farmer field (Samal and Garg 2012)	0.06–0.07	0.16–0.17	0.35–0.39		
Mungo beans	Pakistan	Market surveys (Rehman et al. 2011)	0.06–1.60	0.00–0.13	0.08–1.60		0.05–4.00
Pigeon pea	India	Field survey (Samal and Garg 2012)	0.06–0.08	0.19–0.21	0.15–0.17		
Red kidney bean	India	Field survey (Samal and Garg 2012)	0.06–0.08	0.06–0.08	0.17–0.20		
Maximum permissible limits			0.10[a]	0.10[a]	0.20[b]	0.10[a]	0.38[c]

nd not-detectable
[a]Edible oil or food items
[b]Pulses (WHO/FAO 2016)
[c]Based on consumption of two servings of pulses per day by an adult weighing 50 kg (EFSA 2017; USDA 2018)

Cadmium concentration in pulses of market surveys from Egypt, Spain and Nigeria and a field survey from Kurdistan was within the acceptable limit (≤0.10 mg kg^{-1}) (Table 7). However, market and field surveys from Pakistan (Rehman et al. 2011) and India (Samal and Garg 2012) reported Cd levels in some pulses above the permissible limit. Pulses grown in peri-urban areas in China and contaminated areas in Ethiopia had Cd concentration above the safe limit (Hu et al. 2013; Leshe and Tessema 2014).

Lead concentration in nearly all pulses from market surveys in India, Nigeria, Turkey, Egypt and Spain was within safe limits (≤0.20 mg kg^{-1}) (Table 7). However, a market survey in Pakistan reported safe to toxic levels of Pb in pulses (Rehman et al. 2011). A field survey of polluted area in Ethiopia also reported Pb concentration in pulses above the safe limit (Leshe and Tessema 2014).

Mercury concentration in pulses was within the safe limit of ≤0.10 mg kg^{-1} (Table 7).

Nickel concentration in pulses available in selected markets in Nigeria was within the safe limit (≤0.38 mg kg^{-1}) (Akinyele and Shokunbi 2015; Table 7). However, pulses grown on contaminated and peri-urban areas of different countries had above-permissible levels of Ni (Garrido et al. 2005; Leshe and Tessema 2014; Ismael et al. 2016). Concentration of Ni in some of samples of pulses collected at different markets in Pakistan was also above the safe limit (Rehman et al. 2011).

Arsenic, Cd, Pb, Hg and Ni in Plant Oils

All plant oils had safe levels of As, Cd, Pb, Hg and Ni (Table 8). This might partly relate with selective extraction of oils from oil seeds at industries by sophisticated processing and purification techniques. Due to the same reasons, mineral contents of plant oils are very low (Table 2).

Sources and Remedies

Plant-based foods might have toxic levels of heavy metals especially if produced in contaminated environments (Tables 3–8). The negative health effects of excessive dietary intakes of these metals are already well known (Zheng et al. 2007). Contaminated soils, irrigation waters, agricultural inputs and atmosphere are major factors responsible for accumulation of toxic levels of elements in plant-based foods (Okoronkwo et al. 2005). Soils, waters and atmosphere are contaminated due to effluents and emissions from mining areas, industries and cities. Toxic levels of heavy metal(loid)s should be removed from wastes before spreading on land. If the environment is already contaminated, proper remediation strategies must be adopted for safe food production.

Table 8 Concentration of arsenic, cadmium, lead, mercury and nickel in plant oils

Oils	Countries	Study	As	Cd	Pb	Hg	Ni
			mg kg^{-1}				
Almond	Iran	Market survey (Mohammadpourfard et al. 2015)	0.01	0.01	0.02	nd	
Apricot	Iran	Market survey (Mohammadpourfard et al. 2015)	0.01	0.02	0.02		
Corn	China	Market survey (Zhu et al. 2011)	0.01–0.02	<0.01	0.01		0.03–0.04
Miscellaneous	France	Market survey (Leblanc et al. 2005)	0.05	<0.01	0.01	<0.01	0.02
Miscellaneous	UK	Market survey (Rose et al. 2010)	<0.01	<0.01	<0.01	<0.01	<0.04
Olive	Greek	Market survey (Karavoltsos et al. 2002)		<0.01			
Olive	Spain	Market survey (Martorell et al. 2011)	≤0.01	<0.01	0.02–0.03	<0.01	
Olive	China	Market survey (Zhu et al. 2011)	0.01–0.02	<0.01	0.01–0.02		0.05–0.06
Olive	Spain	Market survey (Llorent-Martínez et al. 2011)	<0.01	<0.01	<0.01	<0.01	<0.01
Olive	Italy	Market survey (Benincasa et al. 2007)	0.01	<0.01			0.05
Olive	Austria	Market survey (Cindric et al. 2007)			<0.01		1.10
Peanut	China	Market survey (Zhu et al. 2011)	0.01–0.02	<0.01	0.01–0.02		0.02–0.03
Soybean	Spain	Market survey (Llorent-Martínez et al. 2011)			<0.01		0.21–0.25
Soybean	China	Market survey (Zhu et al. 2011)	0.01–0.02	<0.01	0.01–0.02		0.04–0.06
Soybean	Austria	Market survey (Cindric et al. 2007)			<0.01		0.21
Sunflower	Spain	Market survey (Martorell et al. 2011)	≤0.01	≤0.01	0.02–0.03	<0.01	
Sunflower	China	Market survey (Zhu et al. 2011)	0.01	<0.01	0.01–0.02		0.03–0.04
Sunflower	Spain	Market survey (Llorent-Martínez et al. 2011)	<0.01	nd	<0.01	<0.01	nd
Sunflower	Austria	Market survey (Cindric et al. 2007)			<0.01		0.09–0.11
Maximum permissible limit			0.10[a]	0.05[b]	0.10[a]	0.10[a]	3.43[c]

nd not-detectable
[a]Edible oils or any food item
[b]Fruiting vegetables (WHO/FAO 2016)
[c]Based on three servings of oil per day by an adult weighing 50 kg (EFSA 2017; USDA 2018)

Soil contamination by heavy metal(loid)s is a result of industrial activities, mining (Almås et al. 2006; Almås et al. 2007; Degryse et al. 2007; Oorts et al. 2008; Chen et al. 2009) or long-term applications of contaminated wastes (Horswell et al. 2006; Kim et al. 2007; Antoniadis 2008). Heavy metal(loid)s in soils occur as soluble, exchangeable, organically bounded, calcium carbonate-bound, metal oxides-bound and structural parts of primary and secondary minerals (Alloway 1995). Various properties and constituents of soils influence total quantity and plant-available fraction of elements in soils.

Manipulations of soil pH, redox potential and organic matter can influence the availability of toxic elements to plants. Soil pH controls exchange, precipitation, dissolution, redox and complexation reactions of heavy metals in soils (Chuan et al. 1996). Therefore, the pH of soil solution governs the equilibria among labile and non-labile pools of elements in soils. Soil pH influences the binding of cations on soil constituents, firstly by the competition between protons and metal/metalloid cations for binding sites and secondly by ionization of surface functional groups (Tipping et al. 2003; Bradl 2004).

Soil redox potential can significantly influence pH and speciation of elements in soils (Chuan et al. 1996). Under reduced conditions, As toxicity is more prevalent because As is mobile in the reduced form (Horswell and Speir 2006; Oorts et al. 2008). Heavy metals such as Cd and Ni are mostly present as divalent forms, and these metals do not take part in redox reactions.

Soil organic matter is involved in retention and release of cations. Stable organic complexes of heavy metal(loid)s are not easily available to plants (Kalis et al. 2006; Amery et al. 2008); hence, application of stable organic matter can immobilize the labile fractions of toxic elements in soils.

Soil remediation is often the only option available to restore the soils for safe production of food crops. However, remediation of soil to safe levels of pollutants requires huge investment of money and time. Every soil remediation strategy has its pros and cons (Mulligan et al. 2001). Thermal remediation and air aspiration may remove elements from soils that can form volatile compounds. However, the atmosphere is contaminated by the volatilized pollutants. In acidic soils, liming is helpful to precipitate labile species of toxic elements by increasing soil pH (Hale et al. 2012). Soil pollution can also be reduced by stabilized forms of organic matter that immobilize heavy metal(loid)s (Ondrasek et al. 2009; Zhang et al. 2013; Elouear et al. 2016). However, decomposition of organic matter and dissolution of precipitated forms reintroduce the inorganic pollutants in the soil solution. Microbes can also immobilize these elements in soils. Phytoremediation is another well-known technique used to reduce contamination levels in soils and waters by the use of hyperaccumulator plant species (Bothe 2011; Tangahu et al. 2011). Plants belonging to Brassicaceae family grown alone or in combination with specific microorganisms significantly decreased the level of heavy metals in soils (Bothe 2011). Restoration of contaminated soils with deep ploughing is also considered a good measure to leach toxic elements from the root zone (Zhou et al. 2000). However, leaching to aquifers may contaminate underground water reservoirs. Based on the above discussion, it is clear that soil remediation strategy must be carefully selected considering the nature of the contaminant (Mulligan et al. 2001).

Water contamination by heavy metal(loid)s from different sources has become a global issue (Dike et al. 2004). Such pollutants in irrigation water may be introduced by natural processes, such as weathering of rocks and minerals, and by anthropogenic activities, such as industrial, domestic and agricultural wastes (Bilos et al. 2001). River waters passing through urban areas are continuously contaminated by drainage of untreated or inadequately treated city wastes and industrial effluents (Bilos et al. 2001; Othman 2001; ul Islam et al. 2007; Ismail et al. 2014). Industrial activities like metal workshops, garages, tanneries, pharmaceuticals and various factories produce huge amounts of effluents that often contain toxic elements. Such contaminated river water is used to irrigate agricultural fields that lead to contamination of soils and plant-based foods (Chary et al. 2008).

Treatment of wastewater is imperative to produce contamination-free food crops (Mulligan et al. 2001). Construction of wetlands to clean effluents and sewage is one of the strategies to improve the quality of irrigation water (Williams 2002). Macrophytes may take up substantial amounts of metal(loid)s from contaminated water (Sood et al. 2012). Filters and adsorbents can separate pollutants from wastewaters. Adsorbents like papaya wood, potato peel, bentonite, anaerobic sludge and nanosized metal oxides have good removal efficiencies for heavy metals from wastewaters (Saeed et al. 2005; Ali 2012). Photocatalysts, such as TiO_2 and SiO_2, can be used to remove Pb from aqueous solutions (Harraz et al. 2013; Rashed 2015).

Agricultural inputs, other than contaminated irrigation water, can also be a source of inorganic pollutants for food crops. Concentration of Cd in soils differed with application rates of P fertilizers (Lambert et al. 2007). This is due to the impurities of Cd in rock phosphate. Biosolids may contaminate soils with As, Cd, Pb, Hg and Ni (Beshah et al. 2015). Continuous application of contaminated biosolids may result in leaching of pollutants to the aquifer (McLaughlin et al. 2000). Therefore, soil amendments and fertilizers must be carefully screened for contaminants before their applications to fields.

Air can be a source of toxic elements for plants (Haiyan and Stuanes 2003). In urban and industrial areas, heavy metal(loid)s emitted from industries and automobiles may be absorbed by surfaces of leaves and edible portions of plants during their growth (Zhou et al. 2000). Therefore, emissions should be treated, and environmentally friendly fuels should be used to avoid contamination of the environment. Moreover, food plants should be grown at some distance from highways and industrial areas.

Choice of plants for a particular area can be a safe strategy to produce contamination-free food crops. Due to perishability and high market demand, vegetables are generally grown near populated areas that tend to be more polluted than the remote areas. Moreover, plant species differ in the uptake of elements from soils and their distribution in various plant tissues (Peralta et al. 2001). With the help of selective uptake at root surfaces and root-induced changes in the rhizosphere, plant roots favour the uptake of nutrients (Gilroy and Jones 2000; Dakora and Phillips 2002). However, there are non-specific cation channels on root surfaces and non-essential heavy metal(loid)s can be absorbed by plants (Sharma et al. 2007). Moreover, root exudates can also increase bioavailability of heavy metal(loid)s to

plants (Courchesne et al. 2008; Maqsood et al. 2011). Therefore, growing crops in heavy metal(loid)-contaminated areas should consider food safety issues. If the environment cannot be remediated, food plants should not be grown in the contaminated areas or near the sources of contamination.

Conclusions

Plant-based foods are important in providing energy and nutrition to human populations around the world. Along with other intake routes into the human body, dietary intakes of heavy metal(loid)s via consumption of plant-based foods is becoming a serious threat for human health.

Contamination of agricultural soils, irrigation waters and air by industrial effluents, city wastes, biosolids and toxic emissions especially in densely populated developing countries may result in heavy metal(loid) contamination of plant-based foods. Food crops grown in cities, peri-urban areas, industrial zones, roadsides and mining sites often contain high concentrations of toxic elements in edible portions. Vegetables and fruits had relatively higher contamination levels than nuts, pulses and cereals. This may be partly due to preferable cropping of vegetables and fruits in peri-urban and industrial areas of high food demand. Plant oils had permissible levels of all heavy metals in all the listed studies, and Hg was reported to be within safe limits in all the studies.

Accumulation of toxic levels of heavy metal(loid)s in edible plant parts clearly relates with levels of contamination in the environment. Therefore, the situation requires urgent actions on careful remediation of the contaminated environments. However, remediation of the contaminated environments to safe levels requires substantial investment of money and time. Nevertheless, foods plants should not be grown in the contaminated areas or near the sources of contamination. Additionally, heavy metal(loid)s in wastes and agricultural inputs must be monitored by strict law enforcement to ensure healthy food production.

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Ganoderma lucidum: A Macro Fungus with Phytochemicals and Their Pharmacological Properties

Md Faruque Ahmad

Introduction

Mushrooms are the reproductive structures (fruiting body or sporocarp) of certain fungi (Jonathan et al. 2009). It is estimated that there are around 70,000 species which are described among approximately 1.5 million species of mushrooms globally (Prasad and Wesely 2008). *Ganoderma lucidum* (Fr.) Karst. (Ganodermataceae), basidiomycetous fungi, has been employing as a health remedy in China, Japan, and Korea for centuries (Kim and Kim 1990). More than 250 Ganoderma species have been described globally (Wasser 2011). However, in aspects of therapeutic applications and literature citations, Ganoderma generally refers to the species of *G. lucidum* (Fig. 1). The fungus has been used as a traditional Chinese medicine (TCM) to treat different ailments for more than 4000 years, and regular use of mushroom extracts is supposed to preserve human vitality and to promote longevity (Wachtel-Galor et al. 2003; Chang and Buswell 1999).

Common Names

China	Lingzhi, ling zhi cao, ling chih, hong ling zhi, chi zhi
Japan	Reishi, mannentake, rokkaku-reishi
United States	Reishi mushroom (Herbs of Commerce)
Korea	Young ji
Vietnam	Ling chi

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Fig. 1 *Ganoderma lucidum*: (**a**) Basidiocarp and (**b**) Powder

Taxonomical Classification

Kingdom	Fungi
Phylum	Basidiomycota
Class	Agaricomycetes
Order	Polyporales
Family	Ganodermataceae
Genus	Ganoderma species
Species	*G. lucidum*

Various biological active constituents of *Ganoderma lucidum* that have been revealed include triterpenoids, carbohydrate, proteins, proteopolysaccharides, sterols, alkaloids, nucleotides, lactones and fatty acids, vitamins, and minerals (Chang and Buswell 2003; Ahmad et al. 2013a; Wachtel-Galor et al. 2003; Paterson 2006; Akihisa et al. 2007). Various medicinal properties have been reported from the these pharmacologically active compounds, such as immunomodulatory (Ishimoto et al. 2017), anticancer (Jin et al. 2016; Lakshmi et al. 2006), antioxidant (Krishna et al. 2016; Gill et al. 2016a), antiarthritic (Pan et al. 2017), hypoglycemic (Seto et al. 2009; Berger et al. 2004), carcinostatic (Sliva, 2003), cardioprotective ((Rajasekaran and Kalaimagal 2012), anti-inflammatory (Cai et al. 2016), proapoptotic (Gill et al. 2016b), anti-allergic (Ji et al. 2007; Tasaka et al. 1988a, b), anti-angiogenic (Song et al. 2004), antiosteoporotic (Elhassaneen et al. 2016), antinociceptive (Sheena et al. 2003), antiviral (Li and Wang, 2006), anti-HIV (Akbar and Yam 2011), antifungal (Nayak et al. 2010), antibacterial (Quereshi et al. 2010), antiandrogenic (Fujita et al. 2005), and hepatoprotective properties (Powell 2015).

Table 1 Types of Reishi and their use

Color	Taste	Use
Red	Bitter	Aids internal organs and improves memory
Blue	Sour	Improves eyesight and liver function
White	Hot	Protects kidney
Yellow	Sweet	Strengthen spleen function
Black	Salty	Improves lung function
Purple	Sweet	Enhances function of eyes joints, helps complexion

Diversity of Reishi

Although different species of Reishi are available, only six types have been considered in superior aspect to reveal potential biomedical applications. These are red, black, blue, yellow, white, and purple (Table 1). Among these, black and red Reishi have demonstrated the most significant health-enhancing effects, and both are therefore widely applied in the global health supplement market today. Black reishi is unevenly shaped and can measure up to 10 inches in diameter, although most mature specimens are about 6 inches in diameter. The majority of reishi products declare to be using black Reishi (Babu and Subhasree 2008). High content of polysaccharides in red Reishi makes it particularly potent (Komoda et al. 1989).

Cultivation

The basidiocarp (fruiting body) of *Ganoderma* is cultivated worldwide by various methods including wood log, pot or bottle, sawdust bag, and tank cultivation.

Over the past three decades, numerous studies have been demonstrated to find out the best method to produce high quality of products. Most of the commercial *G. lucidum* products derive from its basidiocarp (Fig. 2).

It has become recent interest in producing biologically active substances by fermentation techniques which is creating a center of attention. Advantage of fermentation over traditional basidiocarp cultivation is the reduction in the time spent to obtain the product of interest. The production of basidiocarp takes at least 3–5 months, and these traditional techniques do not guarantee a standardized product since the composition of the substrate, which affects basidiocarp composition, varies from batch to batch (Huang and Liu 2008). While the appreciable amounts of ganoderic acid and polysaccharides can be obtained by submerged fermentation after only 2–3 weeks (Wagner et al. 2003; Ahmad et al. 2013c). Some nutritional components of fermented and non-fermented *G. lucidum* can be seen in Table 2.

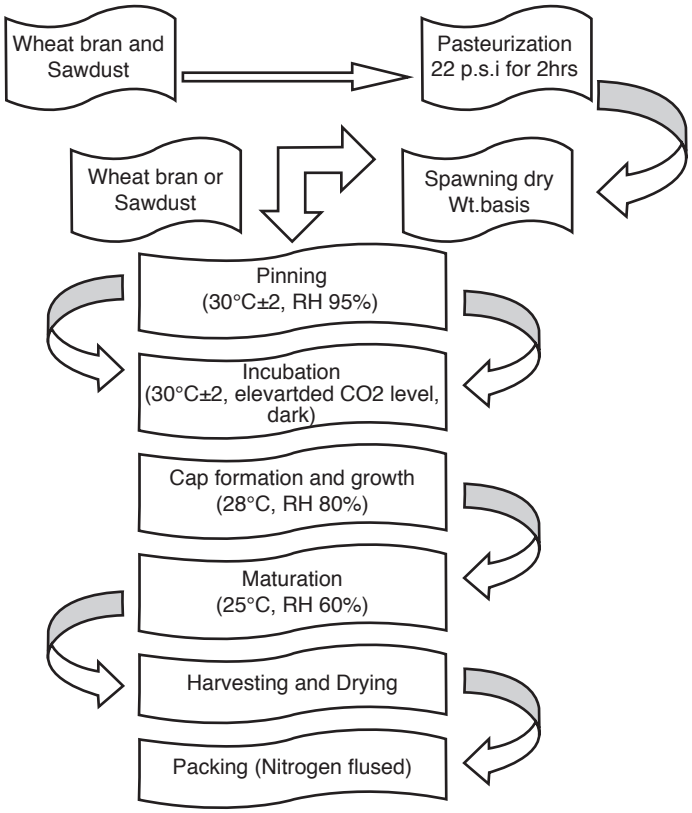


Fig. 2 General scheme of mushrooms production

Table 2 Main nutritional component of fermented products of *G. lucidum* and a non-fermented control on a dry basis

Component (%)	Fermented	Non-fermented
Protein	16.5 ± 0.7	11.0 ± 0.5
Reducing sugar	20.6 ± 0.8	4.2 ± 0.2
Starch	25.3 ± 0.8	64.5 ± 1.5
Crude fat	8.5 ± 0.3	10.3 ± 0.6

Marketed Formulation Other Than Medicinal

Physician and nutritionist have the same opinion that a person with a healthy diet intake does not need any supplementation but the modern lifestyle exposes to several diseases; various reasons such as lacking of exercise, pollutants, or unhealthy diet intakes are causes of high health risk. However, circumstances and situations make food supplements play important role in healthier life. The use of such supplements is a challenge to accomplish pleasing therapeutical outcomes with reduced side effects, as compared to other therapeutic agents. About two third of American population takes at

least one type of nutraceutical health product (Ahmad et al. 2011, Ahmad et al. 2013b). Among all the health products, *G. lucidum* is being widely used as nutritional supplements. That is why *G. lucidum* is available in various forms like capsule, powder, tea, tablet, coffee, jelly, and beverages in the market (Table 3). Now the intense interest of the public has become toward worth full effect of *G. lucidum* not only as a medicinal but also as a nutraceuticals and food supplements.

Table 3 Various formulations other than medicinal form of *G. lucidum* in the market

Different form	Application	Preparation/compound	References
Food	Food and drug	Extract	Boh et al. (2000)
	Food containing dietary fiber	Extract powder	Mayuzami and Fujiwara (1993)
	Fermented food	Mycelium	Tamura et al. (1987)
	Food or beverage for improving saccharide metabolism	Extract	Fujiwara and Sawai (1993)
	Yoghurt	Mycelial powder	Yoshii and Kai (1984)
	Healthy candy	Fruiting body powder or extract	Yamashita and Sato (1994)
	Jelly	Culture medium containing high viscous polysaccharides	Tsujikura and Higuchi (1991)
	Thickeners, emulsifiers, humectants. In food, chemical and pharmaceutical industries	Viscous polysaccharides	Murata et al. (1989)
Drinks	Coffee	Extract	Terada and Murata (1985)
	Health tea	Extract and finely cut fruiting body	Feng (1995)
	Health tonic	Protein-polysaccharide	Liang (1993)
	Alcoholic beverage		Yu (1994)
	Beer	Extract	Gao (1996)
		Aroma (instead of hops)	Naoi (1990)
	Wine	Fruiting body	Lin et al. (1998)
	Health drink vinegar	Fruiting body	Numata (1988)
Nutraceuticals	Organic capsules	*G. lucidum* with varying amounts of the different compounds	ganogoldreishi.coffee.com/products/nutraceuticals
	Organic sliced		
	Additional Supplements		
	Organic powder		
	Grape Seed capsule		
	Ganoderma tea bag		
Miscellaneous	Taiwan healthy pure toothpaste	*G. lucidum* with varying amounts of the different compounds	
	SOD facial mask		
	Elite lotion		

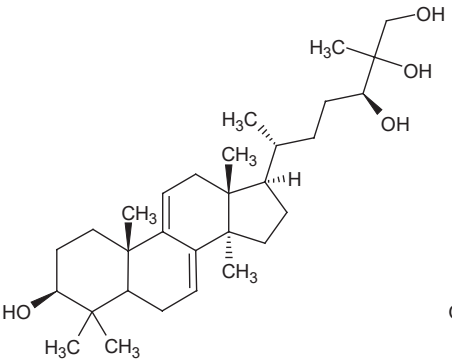
Methodology

The survey of the literature has been done by using PubMed and goggle scholar search engines to collect the phytochemical and pharmacological aspect of *G. lucidum* for the period of 1983–2017. The search was completed with keywords such as *G. lucidum* phytochemistry, *G. lucidum* and immunomodulatory, *G. lucidum* and antihistaminic, *G. lucidum* and hepatoprotective, *G. lucidum* and anticancer, *G. lucidum* and cardiovascular disease, *G. lucidum* and polysaccharide, *G. lucidum* and triterpenes, etc. Effort was made to document only the relevant literature in support of *G. lucidum*.

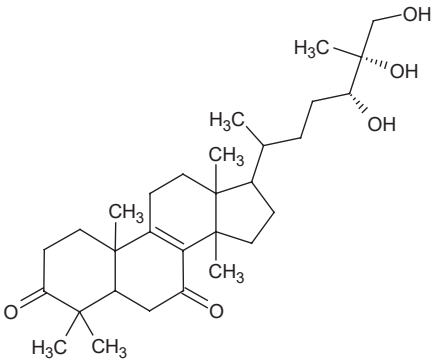
Major Bioactive Constituents and Their Pharmacological Properties

Triterpenes

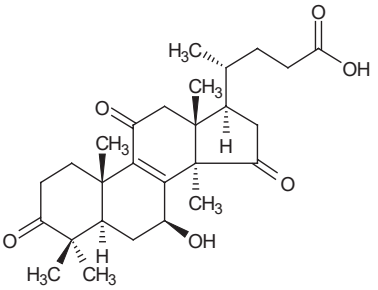
Terpenoids of *G. lucidum* have drawn considerable attention owing to their well-known pharmacological activities. Several highly oxygenated and pharmacologically active triterpenes have been isolated from *G. lucidum* (Cao et al. 2017; Huie and Di 2004) (Fig. 3). Various constituents of the triterpenes fraction consisted of ganoderic acids A, B, C, and D, lucidenic acid B, and ganodermanotriol (Table 4). More than 130 isoforms of ganoderic acids have been searched from fruiting bodies, spores, and mycelia of *G. lucidum* (Chen and Yu 1990). Some of them isolated more than 20 years ago and originally named ganoderic acids U, V, W, X, and Y that exhibited cytotoxicity against hepatoma cells in vitro (Toth et al. 1983). Ganoderic acids (GA) target different adaptor proteins contributing in cellular signaling pathways leading to the arrest of cell adhesion, proliferation, survival, invasion, metastasis, and other mitogenic processes (Lin et al. 2003; Jiang et al. 2004; Lin 2005). Cisplatin is a chemotherapy medication used to treat different types of cancers. Through suppressing JAK-1 and JAK-2 accessory proteins of JAK-STAT-3 signaling, ganoderic acid A augments chemosensitivity and aggravates the cytotoxicity of cisplatin in liver cancer cell lines (HepG2) (Yao et al. 2012). GA-C inhibiting the biosynthesis of farnesyl pyrophosphate (FPP) and the subsequent posttranslational modification via competitively inhibition of protein prenyltransferase (PPP). These modifications are important for cell membrane association and transforming activities (Lokody 2014; Komoda et al. 1989). Against hepatic carcinoma (HepG2, HepG2.2.15) and leukemia (P-388), similar cytotoxicity was also observed with methyl lucidenic acid; lucidenic acids A, C, and N; and ganoderic acid E. Through enhancing immune function in terms of IL-2 and IFN-γ expression and NK cell activity, lanostane triterpenoid ganoderic acid Me inhibited tumor growth and metastasis of Lewis lung carcinoma in T helper 1 responder C57BL/6 mice by employing cytokine-induced killer (CIK) cells to monitor the interaction between



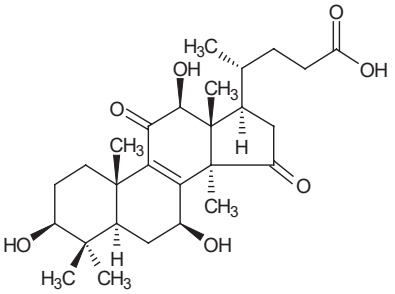
Ganoderiol A



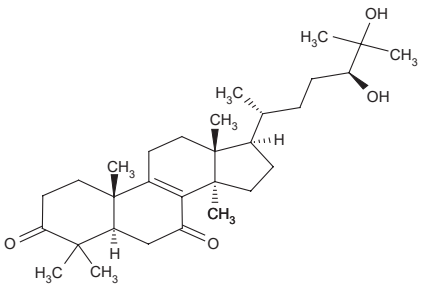
Ganoderiol D



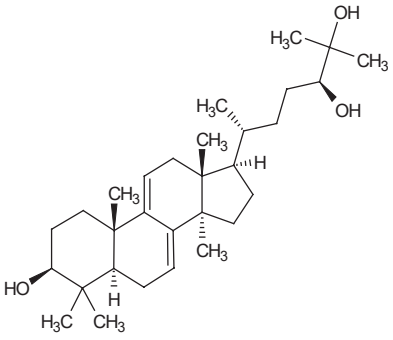
Lucidenic Acid A



Lucidenic Acid C

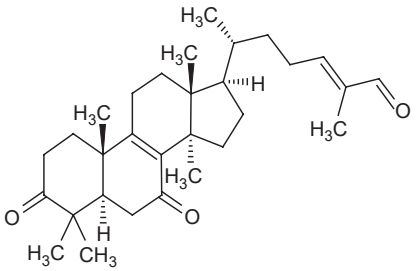


Lucidumol A

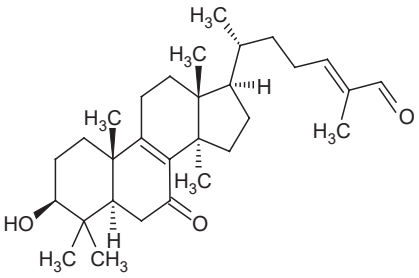


Lucidumol B

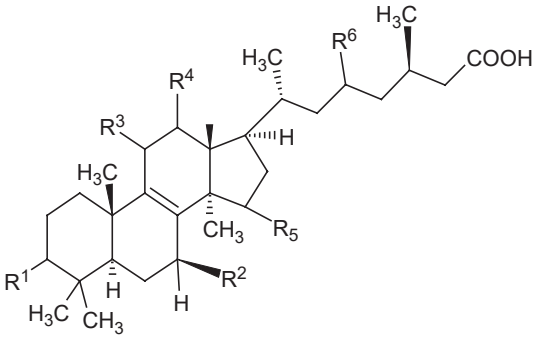
Fig. 3 Pharmacologically active triterpenoids



Lucialdehyde B



Lucialdehyde C



Ganoderic acid A: $R^1=R^3=R^6=O, R^2= R^5= \beta\text{-OH}, R^4=H$

Ganoderic acid B: $R^1=R^3=R^5=R^6=O, R^2=\beta\text{-OH}, R^4=H$

Ganoderic acid C: $R^1=R^3=R^5=R^6=O, R^2= \beta\text{-OH}, R^4=H$

Ganoderic acid D: $R^1=R^3=R^5=R^6=O, R^2= R^4=\beta\text{-OH}$

Ganoderic acid F: $R^1=R^2=R^3=R^5=R^6=O, R^4=\beta\text{-OH}$

Ganoderic acid G: $R^1=R^2= R^4=\beta\text{-OH}, R^3=R^5=R^6=O$

Ganoderic acid H: $R^1=\beta\text{-OH}, R^2=R^3=R^5=R^6=O, R^4=\beta\text{-OAc}$

Ganoderic acid Z: $R^1=\beta\text{-OH}, R^2=R^3= R^4= R^5=R^6=H$

Family of isolated ganoderic acid compounds

Fig. 3 (continued)

Table 4 Biologically active triterpenes and their function

Triterpenes	Biological function	References
Ganoderic acid A, B, C, D	Antioxidative activity	Thyagarajan et al. (2010)
Ganoderic acid F, K, B, D, A, DM	Antitumor activity	Yue et al. (2010); Liu et al. (2009); Yao et al. (2012); Gill and Kumar (2015)
Ganoderic acid α, β, C1, H, G, B	Anti-HIV activity	Min et al. (1998); Akbar and Yam (2011); El-Mekkawy et al. (1998)
Ganoderic acid G, A, F, DM	Anti-inflammatory activity	Wu et al. (2012); Sheena et al. (2003); Joseph et al. (2009)
Ganoderic acid U, V, W, X, Y	Cytotoxic for hepatoma cells	Shiao et al. (1994)
Ganoderic acid Y, F, H, B, D, K, S	Antihypertensive activity	Morigiwa et al. (1986)
Ganoderic acid Me, Mf, Y	Hypercholesterolemic activity	Xu et al. (2010); Hajjaj et al. (2005)
Ganoderic acid Mf, S, A, DM, Me	Apoptosis	Wu et al. (2012); Liu and Zhong (2011)
Ganoderic acid R, S	Antihepatotoxic activity	Hirotani and Furuya (1986)
Ganoderic acid R, T	Cytotoxicity	Ouyang et al. (2014); Liu et al. (2012)
Ganoderic acid E	Neuroprotective effect	Liu et al. (2013a)
Ganoderic acid A, C	Inhibition of farnesyl protein transferase	Toth et al. (1983)
Ganoderic acid F	Inhibition of angiogenesis	Kimura et al. (2002)
Lucidimol (A, B), ganodermanondiol, ganodermanontriol, genoderiol, ganoderiol F	Cytotoxic for sarcoma and lung carcinoma cells	El-Mekkawy et al. (1998); Min et al. (1998, 2000)
Ganoderol B	Inhibits α-Glucosidase	Fatmawati et al. (2011)

G. lucidum polysaccharides (GL-PSs) and cytokines, which mediated antitumor activity and cell proliferation (Wang et al. 2007; Zhu and Lin 2006).

It was found that ganoderiol F and ganodermanontriol demonstrated anti-HIV-1 activity. While, ganoderic acid F plays the role of Inhibition of angiogenesis. In addition, lucidimol (A and B), ganodermanondiol, ganoderiol F, and ganodermanontriol are found to be cytotoxic for sarcoma and lung carcinoma cells (Min et al. 1998, 2000).

Some compounds from *G. lucidum* like ganoderic acids C and D have pharmacologically potential effect to inhibit the histamine release from rat mast cells (Kohda et al. 1985; Tasaka et al. 1988a, b). On the other hand, potential outcome of triterpenoid components on the central nervous system has also been proved in cases of insomnia as well as for mental stabilization (Jia et al. 2005; Chu et al. 2007).

Triterpenoids inhibit farnesyl protein transferase (FPT)-catalyzed posttranslational farnesylation of Ras protein (Lee et al. 1998). Farnesylation is necessary for the membrane translocation, signal transduction, and cell transforming activities of Ras. FPT inhibitors have been verified to block Ras-dependent cell transformation and therefore represent a potential therapeutic strategy for the treatment of human cancer. A few FPT inhibitors have been isolated from *G. lucidum*, namely, ganoderic acid A and ganoderic acid C. The inhibitors are competitive with farnesyl pyrophosphate (FPP) (Shiao 2003; Lee et al. 1998).

A lot of *G. lucidum* triterpenoids inhibit biosynthesis of cholesterol at a postmevalonate step. A few oxygenated triterpenoids from *G. lucidum* and their derivatives inhibit cholesterol biosynthesis from 24,25-dihydrolanosterol (Berger et al. 2004). Various other triterpenes have been given a position in the treatment of liver diseases and said to possess wide hepatoprotective properties (Yang et al. 2006; Lakshmi et al. 2006; Wu et al. 2010). They block platelet-derived growth factor beta receptor (PDGFbetaR), inhibiting the activation and proliferation of hepatic stellate cells, a key event in hepatic fibrosis (Wang et al. 2009).

Hypertension is one of the most important risk factors in several diseases, most notably in the development of atherosclerosis, a highly significant disease process since atheroma of the coronary arteries leads to myocardial infarction. There are eight lanostane triterpenes found those have ACE inhibitory effects among them ganoderic acid F has exhibited greatest effect (Kim et al. 2004; Wachtel-Galor et al. 2004).

Carbohydrates

G. lucidum produces several metabolites with biological activity; polysaccharides are one of them which have medicinal properties. Polysaccharide fractions containing (1→3)β-D-glucans, branched mainly at the C-6 position, exhibit high antitumor activity (Gao et al. 2003a). Beta-D-glucan is a huge sugar molecule made up of many little sugar molecules chained together bound to amino acids. It has been reported in several studies that polysaccharides exhibit their anticancer effect by enhancing the host immune system rather than via a direct cytotoxic effect (Liu et al. 2013b; Lindequist et al. 2005; Chen et al. 1995; Lieu et al. 1992; Wang et al. 1997). It leads to stimulation of various cells such as macrophages, cytotoxic T lymphocytes (CTL), and natural killer (NK) cells. Production of IL-1β, IL-6, interferon-γ (IFN-γ), and TNF-α can be encouraged by *G. lucidum* fruiting bodies in human monocyte-macrophages and T lymphocytes (Wang et al. 1997). These intricate sugars are notable for their ability to stimulate or modulate the immune system by activating immune cells response to foreign invaders, including bacteria, viruses, or tumor cells (Gao et al. 2005). *G. lucidum* polysaccharide extract confirms its immune enhancing ability in cancer patients with increases in NK cell activity and Th1 cytokine levels and decrease in Th2 cytokine levels in advanced lung cancer patients (Gao et al. 2005; Ho et al. 2007). Marked increase in their

phagocytosis activity and production of IL-1β and nitric oxide have been demonstrated by proteoglycan immune-modulating substance of *G. lucidum* (Zhang et al. 2010a) as well as inhibit the production of rheumatoid arthritis more over synovial fibroblasts in vitro, in part through inhibition of NF-κB transcription pathway (Gao et al. 2004b).

Inhibition of reactive oxygen species have been reported by aminopolysaccharide fraction (G009) of *Ganoderma lucidum*. Reactive oxygen species have been implicated in the pathophysiology of cancer (Pincemail 1995). Two cerebrosides from fruiting body of *Ganoderma lucidum* which isolated glycosphingolipids consisting of D-glucose, sphingosine and 2-hydroxypalmitoyl, or 2-hydroxystearoyl fatty acid moiety, respectively, inhibited DNA polymerases, suggesting their probable use for cancer therapy by inhibiting DNA replication (Mizushina et al. 1998). G009 also inhibited iron-induced lipid peroxidation and inactivated hydroxyl radicals and superoxide anions as well as reduced oxidative DNA damage (Lee et al. 2001).

A proteoglycan of *Ganoderma lucidum* (GLIS) fruiting body is mainly composed of D-glucose, D-galactose, and D-mannose with carbohydrate/protein ratio 11.5:1.22. It encourages the proliferation and activation of B lymphocytes, by virtue of which increase production of IL(interleukin)-2, whereas the secretion of IL does not change. In addition, GLIS also improves the expression of PKCα and PKCγ in B cells (Zhang and Tang 2002b).

Polysaccharides of *Ganoderma lucidum* have shown hypotensive, antithrombotic, and hypolipidemic effects, while improvement in ECG and lowered chest pain, palpitation, and shortness of breath has been observed by polysaccharide preparation (Ganopoly) in a double-blind, randomized, multicentered study (Gao et al. 2004b). *G. lucidum* treatment considerably elevates glutathione (GSH) which is the cofactor for antioxidant synthesis and the antioxidant enzymes, glutathione S transferase (GST), glutathione peroxidase (GPx), superoxide dismutase (SOD), and catalase (CAT) indicating antioxidant defense offered by the mushroom that is a major contributor in cardioprotection (Wachtel-Galor et al. 2004).

G. lucidum possesses anti-angiogenic activity (Song et al. 2004). Inhibition of the proliferation of human umbilical cord vascular endothelial cells (HUVEC) in a dose-dependent manner has been observed by *G. lucidum* polysaccharide peptide (*Gl*-PP) isolated from *G. lucidum* (Cao and Lin 2006). High dose of *Gl*-PP for 18 h under hypoxic conditions led to a decline in the amount of secreted vascular endothelial growth factor (VEGF) in human lung carcinoma cells (Kao et al. 2013).

Diabetes mellitus is one of the most common chronic diseases affecting millions of people worldwide. Ganoderans A, ganoderans B and glucans obtained from *G. lucidum* fruiting bodies, exhibit hypoglycemic effects in several test systems and ameliorated the symptoms of diabetes (Zhou et al. 2007; Hikino et al. 1985). Thus many patients with confirmed type-2 diabetes have been treated with polysaccharide fractions from *G. lucidum* (Zhang and Lin 2004). It has been also revealed that *G. lucidum* polysaccharides injection could decrease the serum glucose level and the prevalence of diabetes in the multiple low-dose streptozotocin-induced autoimmune diabetes (Lindequist et al. 2005; Tanaka et al. 1989).

Proteins, Peptides, and Amino Acids

Various bioactive proteins have been isolated from lingzhi. Namely, LZP-1, LZP-2, and LZP-3 from fruiting body and spores of Ganoderma show the mitogenic activity (Huie and Di 2004). Ling Zhi-8 (LZ-8) is a polypeptide consisting of 110 amino acid residues with an acetylated amino terminus (Lin et al. 2011; van der Hem et al. 1995). It is very similar to variable region of the immunoglobulin heavy chain in its sequence and in its predicted secondary structure. It is the first immunomodulatory protein which was isolated from the mycelial extract by using chromatographic and electrophoretic techniques (Kino et al. 1989).

LZ-8 has a chain of biological behaviors which include immunomodulatory effect (Huang et al. 2009; Miyasaka et al. 1992), alleviation of transplant rejection, and antitumor activity (van der Hem et al. 1996). Main biological activities of LZ-8 are similar to lectins, with mitogenic capacity toward mouse spleen cells and human peripheral lymphocytes and agglutination of sheep red blood cells in vitro (Wasser 2005; Haak-Frendscho et al. 1993; Murasugi et al. 1991). It has mitogenic activity and illustrates the immunosuppressive activity in vivo. Intraperitoneal administration of LZ-8, twice weekly into the mice (8 and 12 mg/kg), prevents the production of antibody to the hepatitis B surface antigen (HBs Ag) with the inhibition rates of 83.3% and 96.8%, respectively (Zhou et al. 2002; Gao et al. 2003b; Sun et al. 2004).

Microspherule protein (MSP58) acts a significant function in a variety of cellular processes including transcriptional regulation, cell proliferation, and oncogenic transformation. MSP58 isolated from Ganoderma represses human telomerase reverse transcriptase (hTERT) gene expression and cell proliferation via cooperating with telomerase transcriptional element-interacting factor (TEIF) (Hsu et al. 2014).

Different enzymes from *G. lucidum* have also been isolated like carboxy proteinase, amylase, cellulases, laccase isozymes, and lignin-modifying enzymes α-galactosidase. *G. lucidum* was believed to be involved in the production of D-Galactose in the bioactive polysaccharide such as ganoderan C. (Huie and Di 2004; Smith and Sivasithamparam 2000).

Oxidative stress has been involved with the pathogenesis of many human diseases including cancer, aging, and atherosclerosis (Paterson 2006). Polysaccharides and polysaccharide-peptide complex have been anticipated to be responsible for this antioxidant effect. Antitumor activity has also been reported in a fucose-containing glycoprotein fraction (Rubel et al. 2011; Kawagishi et al. 1993).

Nucleosides, Nucleotides, and RNAs

Yu and Zhai (1979) were the first to report the isolation of adenine, adenosine, uracil, and uridine from the mycelia of a Ganoderma species, *G. capense*. From the mycelium and fruiting bodies of *G. lucidum*, Kim and Nam explored the

distribution of RNA contents and mononucleotides. It was also demonstrated that the level of RNAs from the young basidiocarp mycelium were much higher than those of mature basidiocarp (Kim and Nam 1984). Among all these, nucleosides, uridine, and uracil were searched to be capable of lowering the serum aldolase level of mice suffering from experimental myotonia (Huie and Di 2004).

G. lucidum adenosine has platelet aggregation inhibiting activity. By virtue of which it plays an important role in various disorders of CVS and CNS associated by clots like atherosclerosis, angina pectoris, and stroke (Wang et al. 2009; Kawagishi et al. 1993). In contrast it has been also reported that the administration of crude Ganoderma extracts, known to have a high content of adenosine, had no effect on platelet aggregation in hemophiliac patients who were HIV positive (Gau et al. 1990).

Organic Germanium, Alkaloids, Vitamins, Essential Minerals

Organic germanium is precious compound that gives *G. lucidum* great medicinal values. The presence of organic germanium expresses its effectiveness in fighting cancer. It has been reported that the organic germanium providing antimutagenic or anticarcinogenic properties was beneficial in the treatment of cancer. Organic germanium has been applied as a dietary supplement, and its therapeutic attributes include immune enhancement, oxygen enrichment, free radical scavenging, analgesia, and heavy metal detoxification (Chiu et al. 2000).

Ganoderma has various vitamin contents including vitamins C, B1, B2, B6, E, and β-carotene (Huie and Di 2004 ; McKenna et al. 2002). Those play the great role in maintaining normal physiological functions of the body. Alkaloids are a group of naturally occurring chemical compounds which mostly contain basic nitrogen atoms. Among the different types of alkaloids particularly choline and betaine were isolated from the spores of *G. lucidum* (Paterson 2006). It has been studied that *G. lucidum* fungus may be used as a potential raw material for chitin production (Alvarez et al. 2014).

Dietary Fiber

Dietary fiber is the indigestible portion of plant foods that are not digestible by human or other mammalian digestive enzymes produce the action by the change the nature of the contents of the gastrointestinal tract. Indigestible fibers such as β-glucans (1→3), present in *G. lucidum*, apparently decrease cholesterol absorption in the small intestine or bind to bile acids and as a result accelerate the enteric degradation of cholesterol (Rubel et al. 2011; Fukushima et al. 2000). Fibrous components could affect cholesterol absorption and bile acid recycling (Berger et al. 2004).

Fatty Acids and Sterols

Monounsaturated fatty acids of *G. lucidum* have a propensity to inhibit the production of TNF, one of the proinflammatory cytokines. Study provides scientific supporting evidence for the spores extract as an anti-inflammatory agent (Fukuzawa et al. 2008; Ziegenbein et al. 2006).

The compound oleic acid isolated from the culture broth of *G. lucidum* was found to inhibit the ability of histamine release. This is important for treatment of inflammation, allergies, and anaphylactic shock (Tasaka 1988a).

Ergosterol and ergosteryl esters have been isolated from the fruiting bodies of *G. lucidum* (Yuan et al. 2006) and applied as a suitable marker for evaluating the quality of Ganoderma spore lipid (GSL) that is extracted from the spores of *G. lucidum*. Different pharmacological applications on the basis of different body systems can be seen in Table 5.

Historically mushrooms have had a long and victorious medicinal use especially in traditional medicine for many forms of disorders. Several of these mushroom-derived medicinal products are being used worldwide by historically oriented physicians. Despite such various varieties of mushrooms, *G. lucidum* acts as a king of herbs because of wide pharmacological range in many diseases. Comparative study gives a clue about its versatile function that can be observed in Table 6.

Safety Issues

Reishi is usually regarded as safe. It has been seen in a minute study when taken at a dose of 2 g daily for 10 days failed to find any evidence of ill effects. On the other hand, in another study it has been reported that reishi impairs blood clotting. This study concluded that individuals with bleeding disorders should avoid it. There should have pre- and postsurgery precaution as well as at the time of labor and delivery. Moreover, reishi should be used under a doctor's supervision for individuals taking medications that impair blood clotting, like aspirin, heparin, warfarin, clopidogrel, pentoxifylline, and ticlopidine (Wicks et al. 2007; Su et al. 2000).

Future Prospect

Various pharmacological properties and their health benefits fascinate the researchers and scientist toward finding out several health aspects values. This requires further clinical trials to confirm the efficacy and safety. It is expected that in the nearest future studies on this medicinal mushroom will be conducted on broad scale due to both the uniqueness of their chemical makeup and the number of credible peer-reviewed publications over the past 10 years.

Table 5 Biomedical applications of *G. lucidum*

Application	References
A. Cardiovascular disorders	
1. Increase heart contraction frequency and amplitude	Chu et al. (2012); Kim et al. (2004); Gao et al. (2004a); Wasser (2011); Ahmad (2013a)
2. Coronary dilation and increased coronary circulation	Khatun et al. (2012)
3. Blood pressure regulation with other medication	Ahmad (2018)
4. Relief of oxygen deprivation	Srivastava et al. (2005)
B. Immunomodulatory effects	
1. Anticancer	Ishimoto et al. (2017); Gill et al. (2016b); Srivastava et al. (2005); Deepalakshmi and Mirunalini (2011)
2. Anti-inflammatory	Barbieri et al. (2017); Cai et al. (2016)
3. Antiviral (anti HIV)	Timo et al. (2005)
4. Improve autoimmune disorder	Rubel et al. (2010)
5. Inhibits histamine release in allergy and prevention of anaphylactic shock	Srivastava et al. (2005); Zhang et al. (2010b)
C. Cancer	Jin et al. (2016); Deepalakshmi and Mirunalini (2011); Sun et al. (2014)
1. Enhance the immune system in advanced-stage cancer	Gao et al. (2005)
2. Cytotoxic activity against different cancer cell lines	Thyagarajan (2010); Chen et al. (2010)
3. Maintain leukocyte counts	Zhang et al. (2008)
4. Reduction of toxicity by chemotherapy and elimination of induced leucopoenia (low blood leukocytes) by chemotherapy and radiation	Chen et al. (1995)
5. Accelerate postsurgery recovery	Ogbe et al. (2011)
6. Sedation; pain relief and reduce dependence on morphine in terminal cancer patients	Akihisa et al. (2007)
7. Use during remission to prevent relapse	Khatun et al. (2012)
D. Nerve Disorders	
1. Insomnia/anxiety 2. Psychiatric and neurological afflictions 3. Environmental stress 4. Alzheimer's disease	Babu and Subhasree (2008); Chu et al. (2007); Moreno et al. (2011)
E. Miscellaneous	
1. Hepatoprotective a. Hepatic fibrosis b. Hepatitis B	Li and Wang (2006); Wu et al. (2010); Moreno et al. (2011); Zhang et al. (2002a, b)
2. Hypoglycemic	Zhang and Lin (2004)
3. Hypolipidemic	Rubel et al. (2011)
4. Improve working capacity and rapid recovery to normal physiology	Yang et al. (2001)

(continued)

Table 5 (continued)

Application	References
5. Antioxidant	Gill et al. (2016a); Nahata (2013); Sun et al. (2004); Rubel et al. (2011); Kao et al. (2013)
6. Antiaging activity	Lai et al. (2008)
7. Topical application	Kurtipek et al. (2016)
8. Antimicrobial	Heleno et al. (2013)
9. Anti-arthritic	Pan et al. (2017); Ho et al. (2007)

Table 6 Comparative medicinal values of *G. lucidum* and other medicinal mushrooms

Mushrooms	*Ganoderma lucidum*	*Lentinula edodes*	*Grifola frondosa*	*Tremella fuciformis*	*Poria cocos*
Immune enhancer	+	+	+	+	+
Antitumor	+	+	+	+	+
Antiviral	+	+	–	–	+
Cardiovascular	+	+	–	+	–
Asthma	+	–	–	+	–
Lower cholesterol	+	+	–	+	–
Antidepressant	+	–	–	–	–
References	Ishimoto et al. (2017); Jin et al. (2016); Cheuk et al. (2007); Babu Subhasree (2008)	Bisen et al. (2010); Israilides et al. (2008); Nieminen et al. (2009)	Masuda et al. (2013); Chang (2002); Kodama et al. (2004)	Ooi and Liu (2000); De Baets and Vandamme (2001); Cheng et al. (2002)	Ukiya et al. (2002); Wang et al. (2004)

+Indicates favorable effect of mushrooms
–Indicates not known effect of mushrooms

Conclusion

The review demonstrates that *G. lucidum* is having a great potential for the production of useful bioactive metabolites and it is a prolific resource for drugs. The responsible bioactive compounds belong to several chemical groups, very often they are polysaccharides or triterpenes. Its apparent medical efficacy and the absence of unfavorable side effects and toxins resulting more clinical trials are now in order. Wide application of *G. lucidum* intensifies the interest toward the various techniques besides the traditional one like tank cultivation, solid, and submerged fermentation to get higher production of pharmacological active compounds in short of duration.

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Functional Attributes of Seeds of Two Coastal Germplasms of *Sesbania*

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Introduction

Plant-derived proteins are popular worldwide in food industries owing to their functional attributes in formulations compared to expensive and scarce animal-derived proteins (Chel-Guerrero et al. 2002; Bernardino et al. 2005). Functional properties of foods are dependent on the composition, structure, conformation and physicochemical properties of components, which influence the behaviour of proteins during processing, storage, preparation and consumption (Kinsella 1982; Kohnhorst et al. 1990; Guéguen 1998; Alobo et al. 2009). The most important attributes in food processing include protein solubility, gelation, water-holding capacity, fat-holding capacity, flavour-binding capacity, emulsification, foaming and thickening (Boye et al. 2010). Such functional potential is dependent on the amino acid composition, protein structure/conformation and interactions between proteins and other components like salts, fats, carbohydrates and phenolics. As there is a demand to develop inexpensive protein-rich supplementary foods, there is a shift in the emphasis to utilize lesser-known or wild legumes in the place of popular or well-known legumes (Alobo 1999; Eneche 2005; Seena and Sridhar 2005; Tosh and Yada 2010; Sridhar et al. 2016a). Food industries are in need of appropriate functional properties in legume seed flours to develop cost-effective, affordable and novel food products (Boye et al. 2010).

About 60 species of *Sesbania* (consisting of annuals, perennials, herbs, shrubs and trees) are widely distributed in tropical and subtropical regions (Evans 1990; Veasey et al. 1999). Agricultural, nutritional, pharmaceutical and industrial applications of *Sesbania* have been documented by various researchers (Siddhuraju et al. 1995; Hossain and

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Becker 2001; Bhat and Karim 2009; Vadivel and Biesalski 2010; Pollard et al. 2011: Shreelalitha and Sridhar 2016; Sridhar et al. 2016a). According to Siddhuraju et al. (1995), tribals (Kharis and Ghondas) of Southeast India utilize cooked mature seeds of *Sesbania* as food source. *Sesbania bispinosa* is highly adapted to the coastal region of Southwest coast of India as they tolerate wide soil, geographic and climatic conditions (e.g. water logging; temperature, 36–44 °C; pH, 10) (Prasad 1993; Ipor and Oyen 1997; Anita 2010). Average seed yield of *S. bispinosa* ranges between 600 and 1000 kg/h (India, 600 kg/h; Peru, 900 kg/h; California, 1000 kg/h), while the fibre yield ranges between 100 and 1000 kg/h (http://www.hort.purdue.edu/newcrop/duke_energy/Sesbania_bispinosa.html). Seed yield in *S. bispinosa* in coastal region of Southwest coast of India is about 150–200 g per plant (Shreelalitha 2011). *Sesbania bispinosa* is one of the germplasms conserved in Plant Genetic Resources Conservation Unit (PGRCU), University of Georgia, as it is industrially versatile (useful as fibre, pulp, cover crop, fodder, green manure, ornamental and galactomannan) (Morris 1999; Hossain et al. 2003; Pollard et al. 2011). It also serves as an ornamental plant, fodder and green manure and possesses medicinal and industrial applications (Bhagya and Sridhar 2009). Flowers of *S. bispinosa* are used to treat skin diseases, seed power is used to treat cough and headache and seed oil is used to treat body ache (Morris 1999; Bhagya and Sridhar 2009). The seed gum galactomannan derived from *Sesbania* seeds possesses properties like smooth, transparent, coherent and elastic film useful in sizing textiles and paper industries and also to stabilize mud during oil drilling (Vietmeyer 1986). As galactomannan is a food-grade polysaccharide, it will be utilized as stabilizer and thickener in several food products like ice cream, bakery mixes and salad dressings. Recent studies revealed that the galactomannan of *Sesbania* seeds could be comparable or equivalent to guar gum (or guaran) derived from cluster bean (*Cyamopsis tetragonoloba*) (Pollard et al. 2011).

The novelty of legume-based food/feed and pharmaceutically/industrially useful product could be evaluated precisely by physical, cooking and functional properties (Boye et al. 2010; Karaj and Müller 2010; Tosh and Yada 2010; Pollard et al. 2011; Niveditha et al. 2013; Niveditha and Sridhar 2017). Evaluation of nutritional and functional properties of seeds of wild legumes helps in optimum utilization and possibilities to produce the desired food/feed/pharmaceutical products. As an extension of earlier evaluation of nutritional and bioactive potential of *Sesbania bispinosa* (Shreelalitha 2011; Shreelalitha and Sridhar 2016), the current study envisaged to compare the functional properties of seeds of two *Sesbania* landraces (*S. bispinosa* and *S. speciosa*) adapted to mangrove habitats of Southwest mangroves of India.

Seeds and Processing

Dry pods of *Sesbania bispinosa* (Jacq.) W.F. Wight and *Sesbania speciosa* Taub. were harvested from the five sampling stations of about 100 m distance in Nethravathi mangroves (12°50′ N, 74°49′ E), Mangalore, southwest coast of India during post-monsoon season (January–February) (Fig. 1). Seeds were separated from dry pods, and damaged, sunken, aborted and malformed seeds were eliminated and sun-dried

Fig. 1 Mature plant of *Sesbania bispinosa* with dry pods and dry seeds (left panel); mature plant of *Sesbania speciosa* with dry pods and dry seeds (right panel) grown on the Nethravathi mangroves, Southwest India

until the moisture attained below 10%. Each sample was divided into two sets, the first set was uncooked and the second set was pressure-cooked (6.5 L, Deluxe stainless steel; *TTK* Prestige™, Prestige Ltd., India) with freshwater (1:3 v/v) followed by sun-drying. Uncooked and cooked seeds were milled (Wiley mill, mesh # 30), and the flour was stored in airtight containers in a refrigerator.

Assessment of Functional Properties

Uncooked and cooked seed flours of *S. bispinosa* and *S. speciosa* were analysed for protein solubility, gelation property, water and oil absorption capacities, emulsion properties and foam properties.

Protein Solubility

Protein solubility of the seed flours was determined according to the method outlined by Were et al. (1997). For uncooked and cooked seed flours, each (125 mg) ($n = 5$) was blended with distilled water (25 mL) (Philips HL1643, Philips India Ltd.,

Kolkata, India). The solution was mixed using a magnetic stirrer (1 h, 20 °C) and centrifuged (12,000 *g*, 20 min, 4 °C). The supernatant was filtered through glass wool, and nitrogen was estimated by micro-Kjeldahl method (Humphries 1956). The soluble protein (%) profile was determined (nitrogen solubility × 6.25). The effect of ionic strength (0.2–1.0 M NaCl) and pH (2–10) on protein solubility was also determined:

$$\text{Protein solubility (\%)} = (\text{Quantity of nitrogen in the supernatant} / \text{Quantity of nitrogen in the flour}) \times 100$$

Gelation

The gelation properties were determined by the method outlined by Coffman and Garcia (1977). Uncooked and cooked seed flour suspension in distilled water (2–20%; in 10 mL) was transferred to test tubes and heated in a boiling water bath (1 h) and cooled to room temperature. The samples in tubes were further cooled (4 °C, 2 h), and the least gelation concentration (LGC) was detected when the sample from the inverted test tube did not slip. The effect of ionic strength (0.2–1 M NaCl) and pH (2–10) on LGC was also determined.

Water and Oil Absorption

Water absorption capacity (WAC) and oil absorption capacity (OAC) were determined according to the method outlined by Beuchat (1977). Uncooked and cooked seed flour (1 g) ($n = 5$) was mixed with distilled water (10 mL) or edible oil (Fortune Sunlite Refined Sunflower Oil, Adani Wilmar Ltd., Gujarat, India) in centrifuge tube (30 s). The solution was allowed to stand at room temperature (27 ± 2 °C; 30 min) and centrifuged (5000 *g*; 30 min), and the volume of supernatant was noted in a 10 mL graduated cylinder. The effect of ionic strength (0.2–1.0 M NaCl) on water absorption capacity was also determined.

Emulsion

Emulsion activity and stability of seed flours were determined according to the method outlined by Neto et al. (2001). Seed flour was suspended in distilled water (10 mg/mL), aliquot (5 mL) ($n = 5$) was homogenized (1 min) with edible oil (5 mL) and centrifuged (1100 *g*; 5 min) and the height of emulsified layer and the total contents in the tube were determined to calculate the emulsion activity:

$$\text{Emulsion activity } (\%) = (\text{Height of the emulsified layer} / \text{Height of the total content}) \times 100$$

Emulsion stability was determined by heating the emulsion (80 °C, 30 min) before centrifugation (1100 *g*; 5 min). The effect of flour concentration (2–10%), ionic strength (0.1–1.0 M NaCl) and pH (2–10) on emulsion activity and stability were also determined.

Foam

Foam properties (foam capacity and foam stability) of the seed flour were determined according to Coffman and Garcia (1977). Seed flour sample (2 g) (n = 5) was dispersed in distilled water (100 mL) and whipped vigorously (2 min) in a blender at speed 1. To determine the foam capacity, the volume before and after whipping was recorded, and the volume increase was calculated in percentage:

$$\text{Foam capacity } (\%) = (\text{Volume after whipping} - \text{Volume before whipping} / \text{Volume before whipping}) \times 100$$

Foam stability was determined as the volume of foam that remained after 8 h at room temperature (27 ± 2 °C) and expressed as the percentage of initial foam volume. The increase in foam volume (%) was also assessed at different time intervals (0–480 min). The effect of flour concentration (2–10%), ionic strength (0.1–1 M NaCl) and pH (2–10) on foam capacity and stability were also determined.

Data Analysis

Variations in physical properties between uncooked and boiled seeds, cooking properties between seeds of two species and functional properties between uncooked and cooked seed flours were evaluated by *t*-test using Statistica version 8.0 (StatSoft Inc. 2008).

Functional Properties

Protein Solubility

Protein solubility of seed flours in distilled water and protein solubility with different ionic strengths and pH are given in Fig. 2. Protein solubility of uncooked seed flour was significantly higher than cooked seed flours ($p < 0.05$). The overall protein

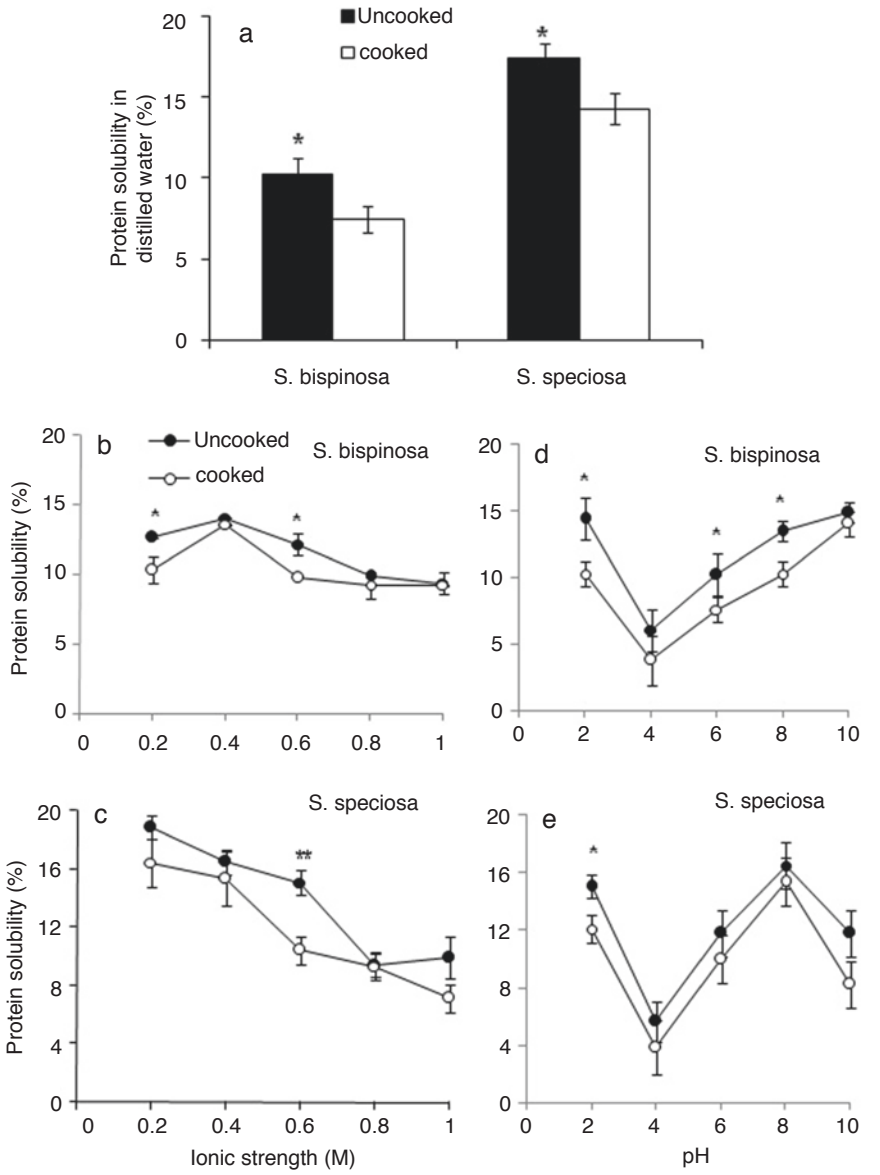

Fig. 2 Protein solubility profile of uncooked and cooked seed flours of *Sesbania bispinosa* and *S. speciosa* in distilled water and different ionic strengths and pH (*t*-test, $*p < 0.05$; $**p < 0.01$)

solubility of both uncooked and cooked seed flours of *S. speciosa* was substantially higher than *S. bispinosa*. Alteration of ionic strength resulted in narrow changes in protein solubility of uncooked and cooked seed flours of *S. bispinosa*, which significantly differed only in 0.2 and 0.6 M. But in *S. speciosa*, the protein solubility gradually decreased with significant difference between uncooked and cooked seed flours at 0.6 M. Protein solubility curves in both uncooked and cooked seeds of *S. bispinosa* on changing pH attained typical '$\surd$' shape, while it was almost similar in *S. speciosa* except for the decrease at pH 10.

Gelation

The least gelation concentration (LGC) of uncooked seed flours was higher than cooked seed flours in both seeds (Fig. 3). The LGC of cooked seed flours of *S. speciosa* was higher than *S. bispinosa* (12% vs. 10%). On the alteration of ionic strength, gelation capacity of cooked seed flours of *S. bispinosa* and uncooked seed flours of *S. speciosa* did not drastically alter. The uncooked seed flour of *S. bispinosa* at 0.6 M and cooked seed flours of *S. speciosa* at 0.4 and 0.6 M showed gelation at least concentration (6% vs. 4%). At both acidic (pH 4) and alkaline (pH 10) conditions, the gelling capacity of uncooked seed flours of *S. bispinosa* was least (12%), while it was at pH 6 in *S. speciosa* (12%). The gelling capacity of cooked seed flours of *S. bispinosa* was least at pH 4 (10%) while at pH 4, pH 8 and pH 10 in *S. speciosa* (12%).

Water and Oil Absorption

The water absorption capacity of uncooked seed flours was significantly lesser than cooked seed flours in both species (Fig. 4). The water absorption capacity of both uncooked and cooked seed flours was higher in *S. bispinosa* than *S. speciosa*. On the increase of ionic strength (0.2–1 M), the water absorption capacity of uncooked and cooked seeds gradually decreased in both species. The water absorption capacity in all ionic strength was significantly higher in cooked seed flours than uncooked seed flours. The oil absorption capacity was higher in uncooked and cooked seed flours of *S. speciosa* than *S. bispinosa* (Fig. 5). However, between uncooked and cooked seed flours, the oil absorption capacity does not significantly differ ($p > 0.05$).

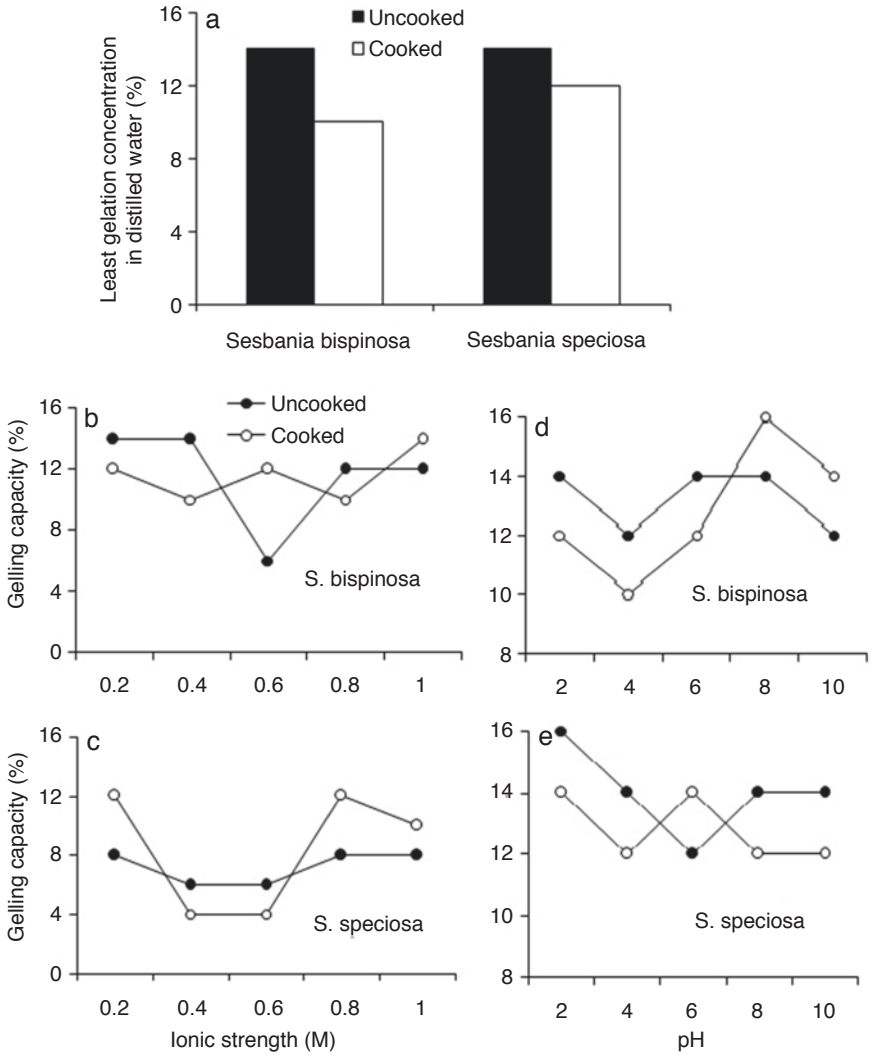

Fig. 3 Gelation profile of uncooked and cooked seed flours of *Sesbania bispinosa* and *S. speciosa* in distilled water and different ionic strengths and pH

Emulsion

Emulsion activity of uncooked seed flours was higher than cooked seed flours in both species (Fig. 6); it showed a gradual decrease on increasing flour concentration. The emulsion stability also followed a similar pattern with that of emulsion activity with an exception of a sudden decrease at 8% flour concentration in *S. speciosa*. The overall emulsion activity and stability were relatively higher in *S. bispinosa* than *S. speciosa* with increased flour concentration. The emulsion activity and stability with increasing

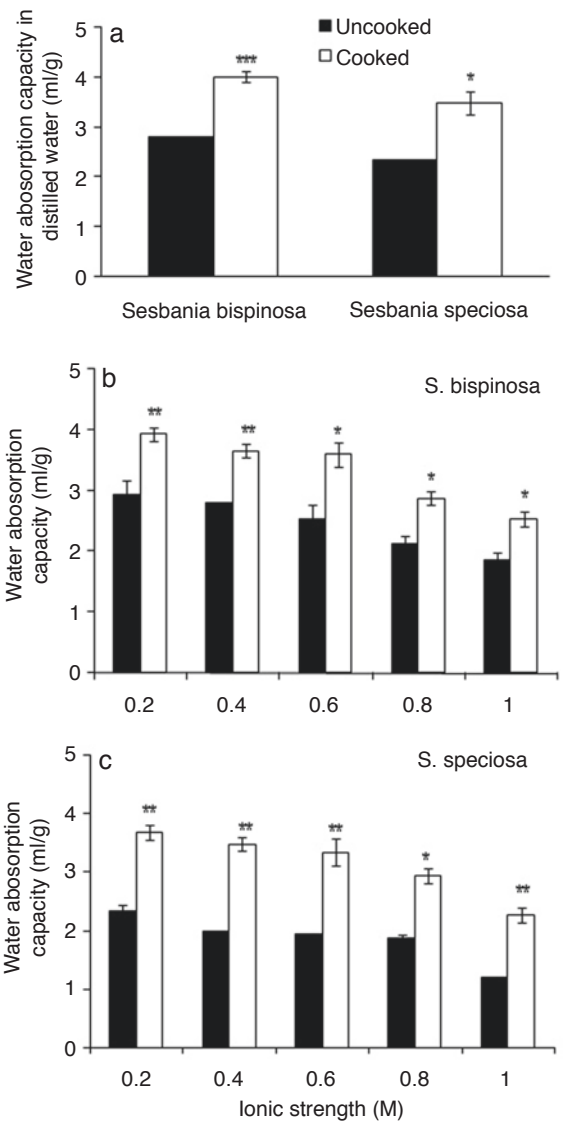

Fig. 4 Water absorption capacity of uncooked and cooked seed flours of *Sesbania bispinosa* and *S. speciosa* in distilled water and different ionic strengths (*t*-test, $*p < 0.05$; $**p < 0.01$; $***p < 0.001$)

ionic strength showed initial elevation followed by gradual decline (Fig. 7). The overall emulsion activity and stability were higher in *S. speciosa* than *S. bispinosa* on changing ionic strength. The emulsion activity was higher in uncooked than cooked seed flours of both species, while it was reverse in emulsion stability with the changing ionic strength. Changing pH showed initial decrease followed by gradual elevation in emulsion activity and stability (Fig. 8). However, a drastic decline in emulsion activity was evident between pH 2 and pH 4 in *S. speciosa*. Emulsion activity was higher in uncooked seed flours than cooked seed flours, while it was reverse in emulsion stability on changing pH.

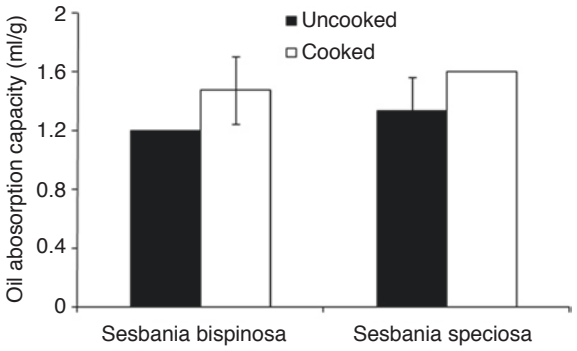

Fig. 5 Oil absorption capacity of uncooked and cooked seed flours of *Sesbania bispinosa* and *S. speciosa*

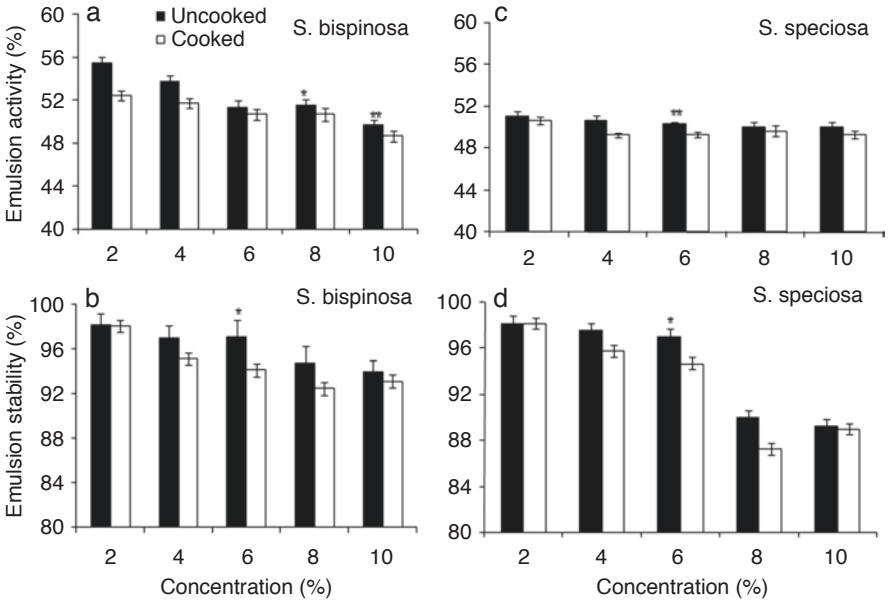

Fig. 6 Emulsion activity and stability of uncooked and cooked seed flours of *Sesbania bispinosa* and *S. speciosa* at different concentrations (*t*-test, $*p < 0.05$; $**p < 0.001$)

Foam

Foam capacity and stability of uncooked seed flour of *S. bispinosa* were higher than cooked seed flour (Fig. 9). Foam capacity of uncooked seed flour at 6% concentration showed highest foam capacity (0 h) and stability (8 h). The foam capacity and stability of *S. speciosa* are also similar to *S. bispinosa* and showed highest foam stability at 10% concentration in uncooked seed flours (Fig. 10). The foam capacity and stability of both seeds were higher in uncooked than cooked seed flours at different ionic strengths, and 0.4 M showed highest foam capacity and stability

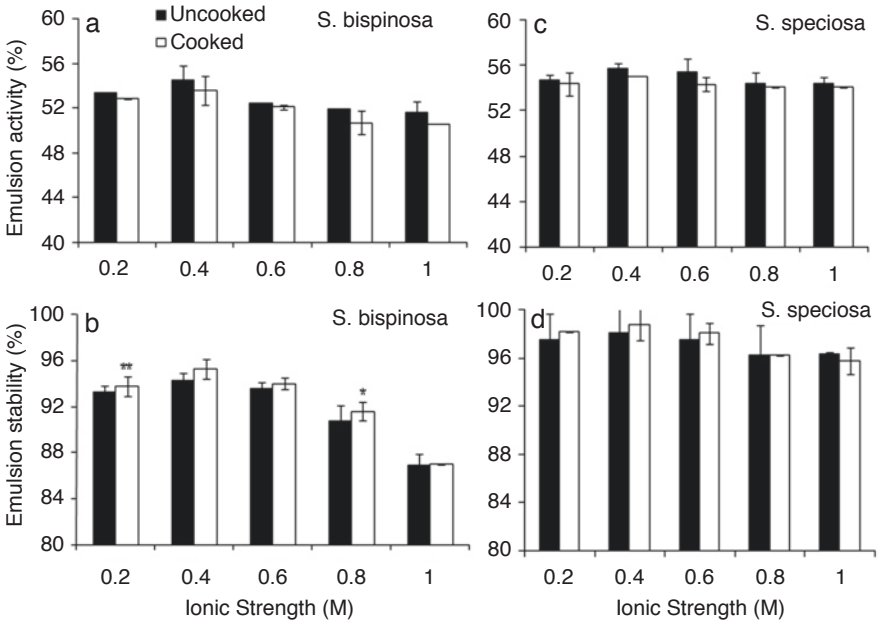

Fig. 7 Emulsion activity and stability of uncooked and cooked seed flours of *Sesbania bispinosa* and *S. speciosa* at different ionic strengths (*t*-test, $*p < 0.05$; $**p < 0.001$)

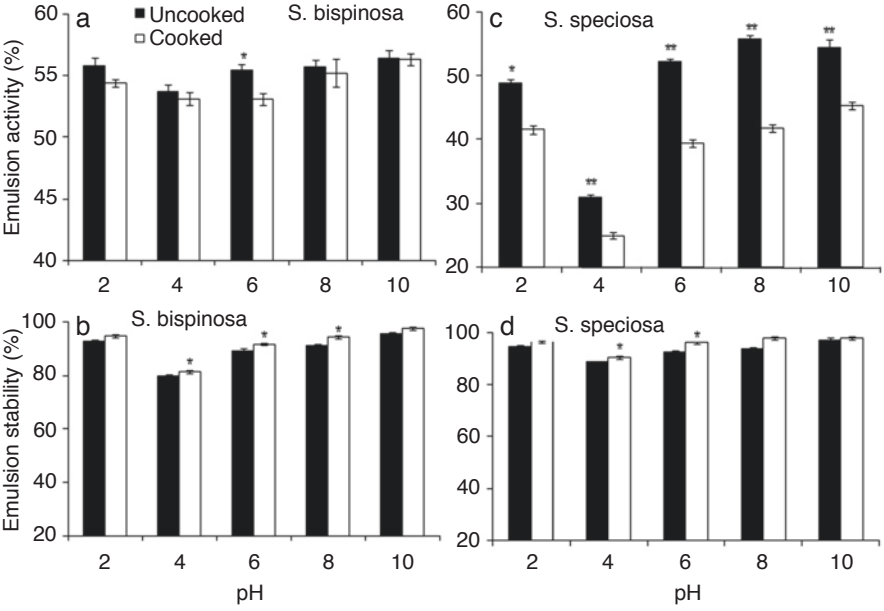

Fig. 8 Emulsion activity and stability of uncooked and cooked seed flours of *Sesbania bispinosa* and *S. speciosa* at different pH (*t*-test, $*p < 0.05$; $**p < 0.001$)

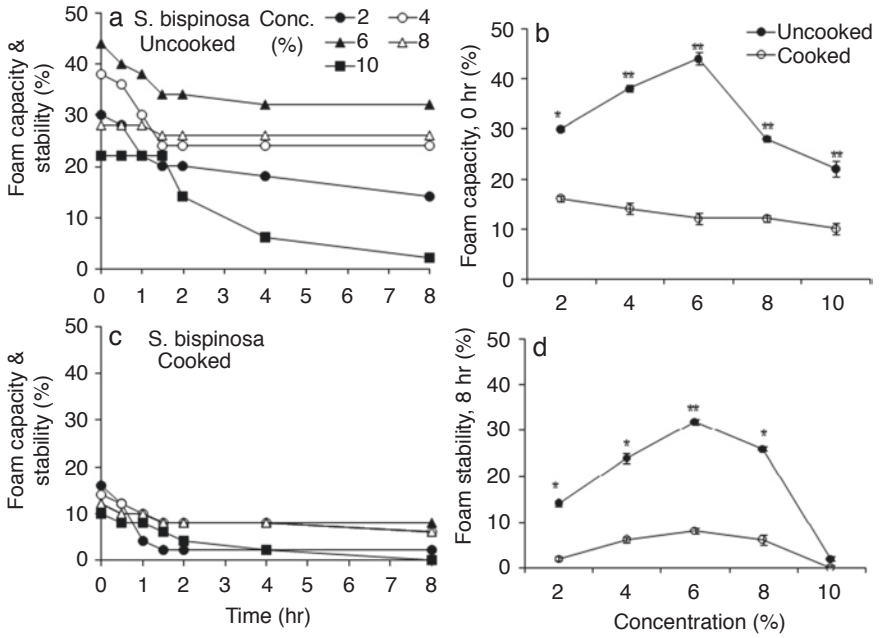

Fig. 9 Foam capacity and stability of uncooked and cooked seed flours of *Sesbania bispinosa* at different concentrations (*t*-test, $*p < 0.01$; $**p < 0.001$)

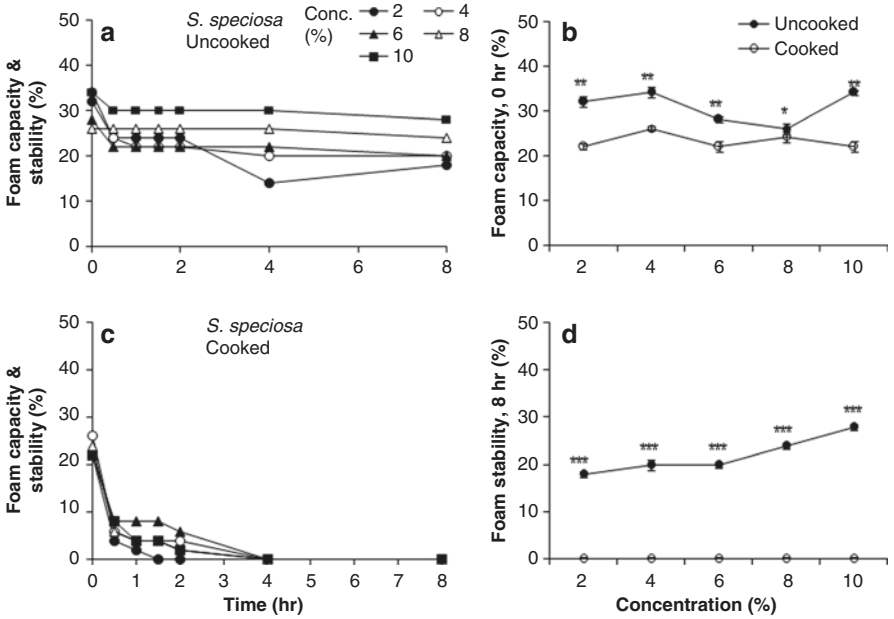

Fig. 10 Foam capacity and stability of uncooked and cooked seed flours of *Sesbania speciosa* at different concentrations (*t*-test, $*p < 0.05$; $**p < 0.01$; $***p < 0.001$)

(Figs. 11 and 12). The foam capacity and stability of both seeds were higher in uncooked than cooked seed flours at different pH; at pH 10 foam capacity was highest, while the stability was highest at pH 8 (Figs. 13 and 14).

Discussion

Suitability of any legume flour for the preparation of food product mainly depends on the nutritional and functional properties (Pour-El 1981). The following sections compare the functional properties of uncooked and cooked seed flours of *Sesbania* seeds in comparison with other legumes.

Protein Solubility

Protein solubility serves as an index of functionality for application in preparation of food products (e.g. soups, beverages and food cakes) requiring gelation, emulsification and foaming properties. Physicochemical changes in seed proteins influence the functional properties especially protein solubility, water absorption capacity, oil absorption capacity and foam properties (Kerr et al. 2000). Proteins along with starch also influence the functional properties of seed flours (Kinsella 1979). The protein solubility profile of uncooked and cooked *Sesbania* seed flours in distilled water and changed ionic strength and pH showed a wide variation. Overall the uncooked flours possessed higher protein solubility in distilled water and different ionic strengths and pH than cooked flours. Protein solubility of seed flours is usually affected by heat treatment (boiling) resulting in protein denaturation and solubility reduction (del Rosario and Flores 1981). The decrease in protein solubility as a result of heat treatment has been reported in other legumes: peanut and cowpea (Singh and Singh 1991; Giami 1993). On the contrary, in *Sesbania* seeds, although bulk density and protein solubility reduced on heat treatment, water and oil absorption capacities significantly improved. Low salt concentrations are known to enhance the protein solubility in seed flours than higher salt concentrations. *S. bispinosa* seed flours at 0.4 M ionic strength showed maximum protein solubility without significant difference between uncooked and cooked seed flours, while in *S. speciosa* maximum protein solubility was seen at 0.2 M ionic strength without significant difference in treatments. Least protein solubility was seen at pH 4 in both seeds, which corroborates with higher protein solubility in the range of pH 4–5 in other legumes such as *Canavalia cathartica*, *C. ensiformis*, *C. maritima* and *Phaseolus lunatus* (Chel-Guerrero et al. 2002; Seena and Sridhar 2005; Lawal and Adebowale 2006). Protein solubility in *Sesbania* uncooked and cooked seed flours ranged between 3.8 and 6.1 at pH 4, which corroborates with other legume seed flours: *Dolichos lablab*, *Glycine max*,

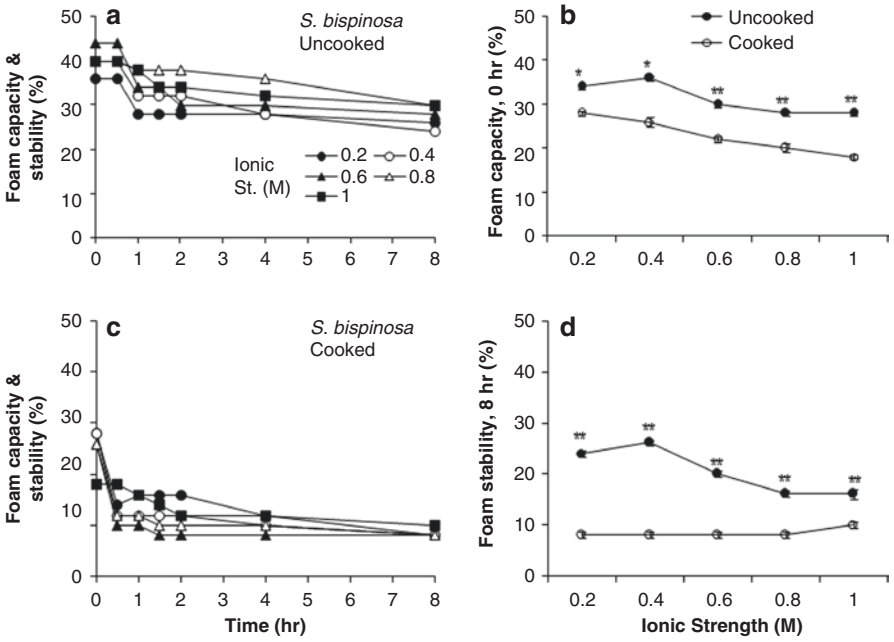

Fig. 11 Foam capacity and stability of uncooked and cooked seed flours of *Sesbania bispinosa* at different ionic strengths (*t*-test, $*p < 0.01$; $**p < 0.001$)

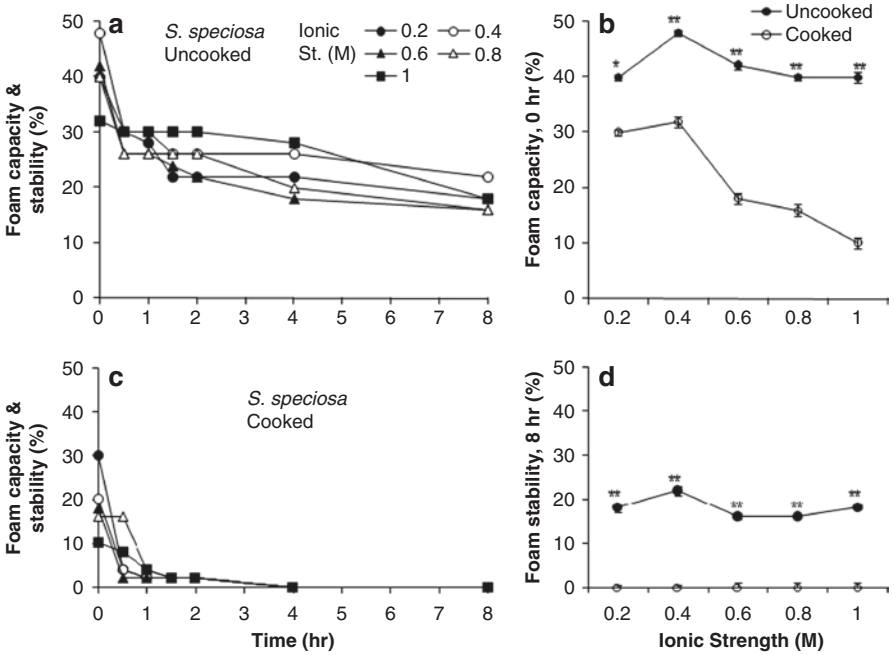

Fig. 12 Foam capacity and stability of uncooked and cooked seed flours of *Sesbania speciosa* at different ionic strengths (*t*-test, $*p < 0.01$; $**p < 0.001$)

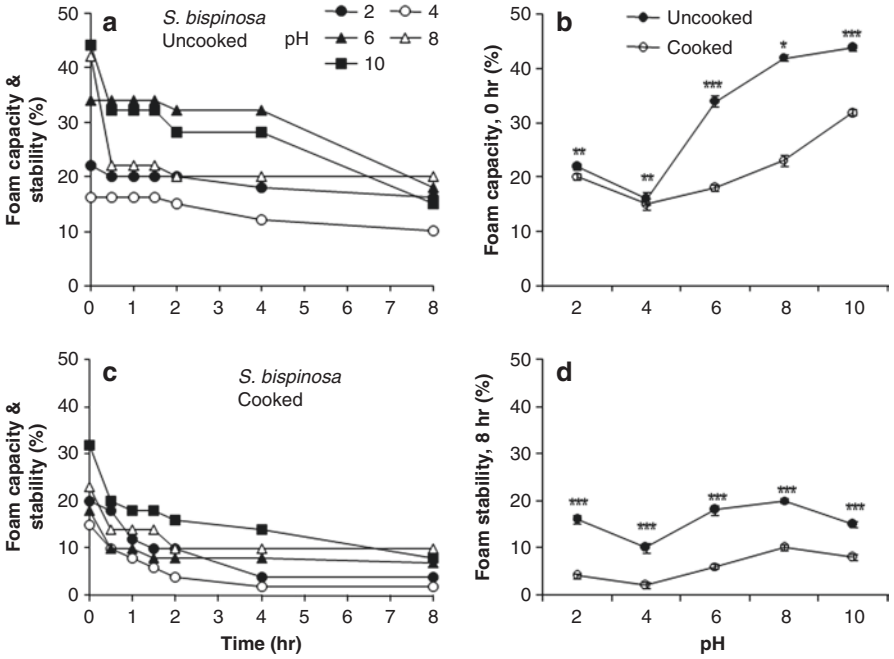

Fig. 13 Foam capacity and stability of uncooked and cooked seed flours of *Sesbania bispinosa* at different pH (*t*-test, $*p < 0.05$; $**p < 0.01$; $***p < 0.001$)

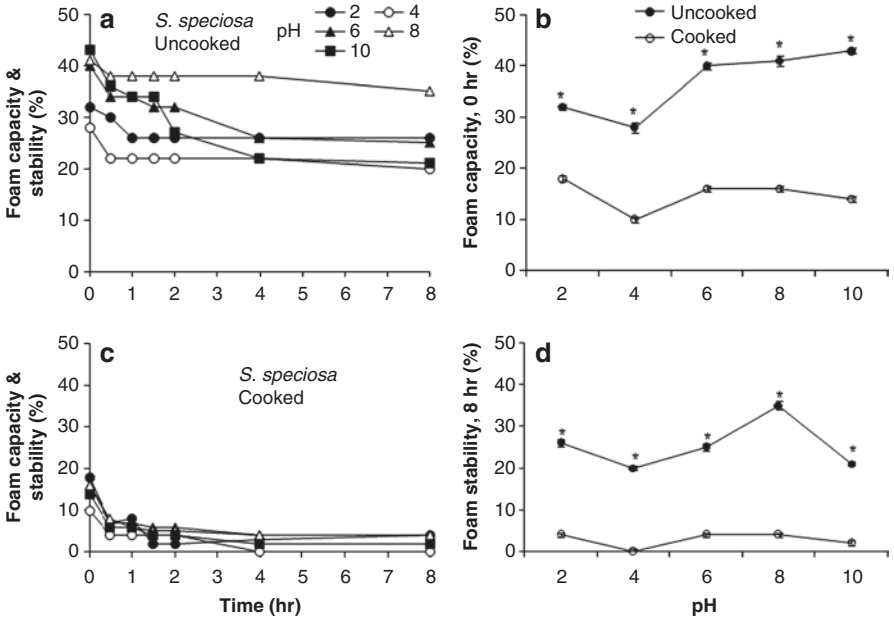

Fig. 14 Foam capacity and stability of uncooked and cooked seed flours of *Sesbania speciosa* with different pH (*t*-test, $*p < 0.001$)

Phaseolus calcaratus and *P. lunatus* (5–5.3%) (Chau et al. 1997; Chel-Guerrero et al. 2002). Proteins with high solubility profiles are required to impart certain characteristics in food formulations (Idouraine et al. 1997). *Sesbania* seed flours in the present study showed maximum protein solubility between pH 7 and 10. Between pH 2 and 10, *S. speciosa* seed flour showed maximum protein solubility, which is similar to the protein solubility pattern seen in the flour of cowpea (Olalekan and Bosede 2010). High protein solubility at alkaline pH reveals that *Sesbania* seed flours could be useful in formulating protein-rich carbonated beverages.

Gelation

There is a wide variation in gelation properties of legume seed flours (Moure et al. 2006), and such variation depends on the relative ratios and interactions of proteins, carbohydrates and lipids in flours (Sathe et al. 1982). The LGC of uncooked and cooked *Sesbania* seed flours in distilled water ranged between 10% and 14%, which is higher than seed flours of cowpea (4%), jack bean (4%) and pigeon pea (6%); comparable to seed flours *Cassia fistula* (10%), great northern bean (10%), mung bean (10%), cowpea (10–12%), soybean (10%), lima bean (12%) and pigeon pea (12%) (Coffman and Garcia 1977; Sathe and Salunkhe 1981; Oshodi and Ekperigin 1989; Oshodi and Adeladun 1993; Padilla et al. 1996; Horax et al. 2004; Akinyede and Amoo 2009; Olalekan and Bosede 2010) are lower than chick pea (14–18%) (Kaur and Singh 2007). Gelation in legume flours involves the formation of a protein-polysaccharide complex (Schmidt 1981), and some legume starches showed remarkable swelling power within the range of 60–90 °C (Wankhede and Ramteke 1982; Hoover and Sosulki 1991). Cooking *Sesbania* seeds decreased the LGC from 14% to 10–12%. Addition of salt up to 5% (w/v) generally improves gelation property by lowering the LGC. Ionic strength at 0.6 M resulted in drastic improvement in LGC of uncooked seed flours (14% vs. 6%) of *S. bispinosa* and uncooked (8% vs. 6%) and cooked (12% vs. 4%) seed flours of *S. speciosa*. Decreases in LGC by the addition of salt have been reported for winged beans and *Mucuna* beans (Sathe et al. 1982; Adebowale et al. 2005). Unlike ionic strength, change in pH did not result in drastic decrease in LGC in both *Sesbania* seed flours. High temperature leads to dissociation of proteins in flours, allows carbohydrates to gelatinize and permits fibre to swell (Mulvihihill and Kinsella 1987; Alobo et al. 2009). Such changes in carbohydrates and fibres in seed flours improve to hold water, flavours and sugars in favour of desired organoleptic properties in food products. Improvement in the gelation property of seed flours is useful in the production of food products especially jams, sauces, custards, ice creams, sausages, puddings and bakery products.

Water and Oil Absorption

Water retention in seed flours depicts the capacity of proteins to absorb water in the presence of dietary fibre in seed flours. The increase in water absorption capacity is due to the depolymerization of starch into short-chain dextrins, which have high affinity for water (Whistler and Daniel 1985). Carbohydrate content of seed flours is considered as one of the significant factors that influences the water-holding capacity (Paredes et al. 1991). Water absorption capacity of uncooked *Sesbania* seeds significantly increased on cooking in distilled water and at different ionic strengths. In both uncooked and cooked seed flours, increased ionic strength resulted in gradual decrease in water-holding capacity. Water absorption in distilled water in uncooked and cooked seed flours (2.3–4 mL/g) is higher than the seed flours of *Canavalia cathartica* and *C. maritima* (1.8–2.4 mL/g), *Mucuna pruriens* (2.17–2.67 mL/g), mung bean (2.1 mL/g) and undefatted soybean flours (1.3–1.75 mL/g) (Oshodi and Ekperigin 1989; Chau et al. 1997; Bhat et al. 2008) compared to *C. ensiformis* (3.5 mL/g) and common beans (red, black and white) (2.9–3 mL/g) (Dzudie and Hardy 1996; Chel-Guerrero et al. 2002). However, the WAC of *Sesbania* seed flours (2.3–4 mL/g) is lower than protein isolates of *Dolichos lablab* (5.1 mL/g), *Mucuna* bean (6 mL/g), *Phaseolus calcaratus* (5.28 mL/g), *P. angularis* (5.05 mL/g), soybean (4.10 mL/g) and winged bean (5.00 mL/g) (Chau et al. 1997; Okezie and Bello 1988; Udensi and Okonkwo 2006) compared to protein isolates of *Phaseolus aureus* (2.26 mL/g) (Mesallam and Hamza 1987). Elevated WAC is advantageous in the preparation of food stuffs such as bread and sausages, which help to maintain the freshness in food. High water absorption capacity is important especially for viscous liquid (e.g. soups and gravies) and baked (e.g. dough and bakery products) food products (Oshodi and Adeladun 1993; Igene et al. 2005). Narayana and Rao (1982) reported that heat treatment enhanced the water absorption capacity in winged beans and cowpea seed flours (Giami 1993). This enhancement of WAC by NaCl would be an added advantage in considering the flour for use in the preparation of meat analogues, sausages, breads and cakes (Altschul and Wilcks 1985).

The oil absorption capacity is an important property, which determines the texture and mouthfeel of the protein material especially in baked foods (Okezie and Bello 1988; Igene et al. 2005). It is valuable in flavour retention and to increase the palatability (Kinsella 1976). Oil-holding capacity of *S. bispinosa* (1.2 mL/g) increased on cooking (1.5 mL/g), which was lower than uncooked (1.3 mL/g) and cooked (1.6 mL/g) seed flours of *S. speciosa*. These values are higher than cowpea (0.69–0.93 mL/g) (Prinyawiwatkul et al. 1997); compared to *Canavalia cathartica* (1.5 mL/g), *C. ensiformis* (1.7 mL/g), *Phaseolus calcaratus* (1.4 mL/g) and winged bean (1.2–1.4 mL/g) (Narayana and Rao 1982; Chau and Cheung 1998; Adebowale and Lawal 2004; Seena and Sridhar 2005) are lower than mung bean (2.2 mL/g), soybean (1.9 mL/g) and *C. ensiformis* seed flours (2.7 g/g) (Dzudie and Hardy 1996; Chau and Cheung 1998; Chel-Guerrero et al. 2002). High

oil-holding capacity values make these flours potentially useful in structural interactions in food, especially in flavour retention, improvement of palatability and extension of shelf life in meat products through reduction of moisture and fat loss.

The WAC and OAC are crucial factors in protein functionality as they influence emulsion and other functional properties. These properties are an important index to absorb and retain water/oil, which influences the mouthfeel of food products like ground meat formulations, doughnuts, pancakes, baked goods and soups.

Emulsion

The emulsion properties of seed flours are dependent on the characteristics of proteins, which vary among seed flours, their quantity and solubility (Kinsella 1976). Thermal processing is known to reduce the emulsion capacity at different concentrations, various molarities and different pH. The present study on seed flours of *Sesbania* is in agreement with the reduction of emulsification capacity of hydrothermally processed winged bean flour at all pH range (Narayana and Rao 1982); heat treatment of soy flour also reduced the emulsion capacity (McWatters and Holmes 1979). The emulsion activity as well as stability was highest in *S. bispinosa* seed flours at 2% concentration, but at 0.4 M ionic strength, emulsion activity and stability were highest in *S. speciosa*. At alkaline pH (pH 10), emulsion activity and stability attained highest in *S. bispinosa*. The emulsion capacity of uncooked and cooked *S. bispinosa* and *S. speciosa* seed flours at different concentrations was lower than jack bean (71.7%) and higher than in pigeon pea (28.73%), while it was comparable with that of cowpea (46.4%) (Olalekan and Bosede 2010). The emulsion activity of seed flours of *S. bispinosa* at 0.2 M ionic strength is comparable to *Canavalia cathartica*, *C. maritima* and *Mucuna pruriens*, while it is lower than *C. ensiformis* (Chau et al. 1997; Seena and Sridhar 2005; Bhat et al. 2008). At 0.4 M, the increase in emulsion activity and stability of uncooked and cooked seed flours of *S. bispinosa* and *S. speciosa* was seen. However, above 0.4 M resulted in the decrease of emulsion activity and stability as seen in sunflower seed flour (Lin et al. 1974). The uncooked *S. bispinosa* (53.7–56.4%) and *S. speciosa* (31–56%) generally exhibited good emulsion activity at different pH levels, with values comparable to those of soybean protein isolate (54–58%), *P. lunatus*, *C. ensiformis* (41.8–56.5%) and *Cassia fistula* (40%) (Akinyede and Amoo 2009). There was a decrease of emulsion activity at pH 4 in *S. bispinosa* (53.7%) and *S. speciosa* (31%), respectively. The emulsion activity of *S. bispinosa* at pH 4 was higher than seed flours of *C. ensiformis* (48.5%), but the emulsion activity of *S. speciosa* was lower than *S. bispinosa* as well as *C. ensiformis*. In both *Sesbania* seed flours, the emulsion activity was almost constant at pH higher than 4. At pH 4 the emulsion activities were about 53.7% and 31% in uncooked seed samples of *S. bispinosa* and *S. speciosa*. With increasing pH, emulsion activity in uncooked

seed flours elevated to a maximum 56.4% in *S. bispinosa* and 54.6% in *S. speciosa*. Similar relationship was reported in emulsion activity against pH in groundnut flours (Ramanathan et al. 1978). High emulsion activity and stability of *Sesbania* seed flours are suitable blends for many products (e.g. sausages, cake buffers and salad dressing).

Foam

Good foam properties of seed flours have been correlated with the amount and solubility of proteins (Aluko and Yada 1993). The percent foam capacity of uncooked seed flours of both *Sesbania* (22–44%) is higher than values reported for flours of quinoa (9%), pearl millet (11.3%), cowpea (16.3%) and jack bean (20.7%) (Oshodi et al. 1999; Olalekan and Bosede 2010) compared to wheat (40%) (Akubor and Badifu 2004) and lower than the flour of pigeon pea (68%), soybean (60%) and sunflower (66%) (Lin et al. 1974; Oshodi and Ekperigin 1989). The foam stability values in uncooked *Sesbania* seed flours were less than the wheat flour (60%) (Akubor and Badifu 2004).

The foam capacity of *Sesbania* seeds in different salt concentrations is higher than flours of soybean (14.6%), sunflower (9.0%) and pigeon pea (2.0%) (Lin et al. 1974; Oshodi and Ekperigin 1989), comparable to values reported for a variety of dehulled African yam bean (21.3–48.4%) (Adeyeye and Aye 1998) and oil seeds (40–50%) (Olaofe et al. 1994). High concentration of salt depressed the foam capacity substantially in *Sesbania* seed flours. There was a concomitant decrease in foam capacity from the concentration above 0.2 M. Low concentration of salt is beneficial as it enhances protein solubility in soybean flour (Narayana and Rao 1984; Akintayo et al. 1999). The foam stability of *Sesbania* seed flours is lower than wheat flour (Akubor and Badifu 2004) and higher than flours of soybean (14.6%) and pigeon pea (20%) (Oshodi and Ekperigin 1989). Uncooked and cooked seed flours of both the species of *Sesbania* exhibited a similar pattern of decrease at pH 4. The low foam stability was concomitant with the low solubility of protein at pH 4.

Vegetable proteins (e.g. defatted and detoxified flour concentrates, isolates and hydrolysates) will be immensely valuable in the production of a variety of food products (Kinsella 1976). However, such preparation is dependent on the functional properties of seed flours to form solution and also behaviour at air-water and oil-water interfaces (Padilla et al. 1996; Adebowale and Lawal 2004). As *Sesbania* seeds possess desired functional properties and adequate quantity of protein, fatty acids, fibre and carbohydrates (Siddhuraju et al. 1995; Shreelalitha and Sridhar 2016; Sridhar et al. 2016b), further studies on functional properties of full-fat and defatted flours are necessary at different concentrations, ionic strengths and pH.

Conclusions

Seeds of *Sesbania* are known for nutritional, medicinal and industrial applications. These seeds are also commercially valuable as an excellent source of fibre and nontoxic food-grade polysaccharide galactomannan. The present study provided additional information on the functional properties of seeds of two landraces of *Sesbania* growing in the mangrove habitats of Southwest India in view of potential food for humans as well as livestock. *Sesbania* seeds studied possess high protein solubility at alkaline conditions and facilitate production of carbonated beverages. Desired least gelation concentration with enhanced water and oil absorption capacities is helpful in the production of many bakery products. Increased emulsion activity and stability are useful in the production of sausages, cake buffers and salad dressers. Owing to nutritional, medicinal and industrial applications, future emphasis on bioactive potential, physical and mechanical properties of seeds of different landraces of *Sesbania* would be of immense value in agricultural and industrial benefits.

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Multiple Uses of Some Important Aquatic and Semiaquatic Medicinal Plants

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Introduction

Traditional medicines or traditional medicinal system refers to the use of traditional knowledge, skill and practices for healing and curing diseases and therapies. The traditional medicinal system is still relied and used in almost every part of the world irrespective of the developments in modern medicinal systems. This might be due to a number of complex socio-economic factors, lack of modern facilities, expensive treatments, education, belief, etc. The basics of traditional medicinal systems include the use of different plants/herbs for healing with transfer of knowledge from generation to generation.

The developments in modern medicinal systems originate from these in one way or another. Rapid developments in science and technology related to pharmacognosy, chromatography, plant biotechnology, molecular biology, etc. has tremendously helped in increasing the values of the traditional medicinal systems that characterise active biological compounds and their applications (Kar 2006).

Use of alternative medicines are referred to as practices involving medicinal byproducts and therapies employed for human benefit with knowledge of their beneficial, toxic and harmful effects (Dasgupta et al. 2011). The use of alternative medicines is based on belief, religion, superstitions, social factors and family-based medicinal systems mostly not recognised by the modern scientific and biological studies (Sampson 1995). These medicines are used for healing chronic diseases like

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fever, headache, etc. and fatal diseases like cancer (Peregoy et al. 2014). Complementary and alternative medicines consist of a wide array of health care practices that are not considered to the tradition without integration into the health system of the country (WHO 2000). However, alternative medicinal systems are being used as complementary medicine (CM) or integrative medicine (IM) used with functional medicines (Zeller et al. 2013), CAM (complementary and alternative medicines) (Cassileth and Deng 2004) or TM (traditional medicines) (Ansari and Inamdar 2010).

Plants which have beneficial pharmacological and therapeutic effects on human health are considered as medicinal plants. These plants are rich in bioactive compounds like alkaloids, sterols, saponins, flavonoids, volatile oils, etc. and are in use since time immemorial (Baytop 1999). Records show the use of herbs as medicine by Chinese scriptures and Egyptian papyrus followed by the development of other traditional medicinal systems. The most famous traditional medication system includes Techiman-Bono ethnomedical system, naturopathy, Western Orthodox, reiki, Native American medicine system, Dormaa medicine system, Ayurvedic, Greco-Arab, Tongan, Unani, Chinese, Iranian and African systems, etc. (Warren and Green 1982; Fink 1990; WHO 2001; Toafa et al. 2001; Kamala Shankar et al. 2004; Saad and Said 2011; Isola 2013). All of the above-mentioned healing systems are in use in one way or the other. Traditional Chinese medicinal systems use around 5000 medicinal products (Li 2000).

Wetland ecosystem has played a significant role in human civilization since a long time ago. Generally people preferred to live close to water bodies or wetland systems in ancient times; where they could grow and irrigate crops for food, clothes and abode. A study of all great civilizations like Indus valley, Mesopotamian and Nile civilizations and modern-day Roman, Greek and Inca civilizations also prove that human beings always preferred to live close to rivers, lakes and oceans to get sustenance for food, shelter and abode. Aquatic plants have significant role in human civilization (Gupta 1987) by providing food, manure, fibre and source of medicines. They are considered as primary producers of the water ecosystem by converting light energy into chemical energy. Besides that, aquatic plants provide shelter and food for animals like fish, insects and other aquatic animals or amphibians (Baldassarre and Bolen 1994). Aquatic plants are also used to control phytoplanktons and moss from water (Oyedeji and Abowei 2012). These plants also anchor the sediments of soft-bottom habitats (Madsen et al. 1996). The population of hydrophytes in the water bodies also shows the fitness and diversity of the water ecosystem (Flint and Madsen 1995). The use of aquatic plants for biomonitoring and for phytoremediation of heavy metals of the aquatic ecosystems also increases their importance. The use of aquatic plants in aquarium industry all over the world also increases their demand in the USA (Crosson 2010), Australia (Petroeschevsky and Champion 2008) and Europe (Brunel 2009).These plants are a rich source of bioactive compounds that are commercially used in healthcare systems for curing diseases and disorders. The wetlands have significant impact on climate (Swapna et al. 2011) and ecosystem of the areas with species richness (Kirim et al. 2014) and provide habitat either on the banks or in water bodies (Maya et al. 2003). Aquatic

plants are major constituent of aquatic ecosystem with different ecological niche (Oyedeji and Abowei 2012) and special adaptations. They grow as emergant, submergant or as floater (Dodds 2002). The most common type of adaptation of aquatic plants is aerenchyma. Besides that, amphibian plants have adapted to grow both on soils fully saturated with water and under aquatic conditions and are also considered as wetland or aquatic plants (Keddy 2010).

It is not possible to give an exact figure; however, a rough estimate shows that around 50,000–80,000 flowering plants are used as medicinal plants all over the world (Duke 2009) by continuous domestication or collection from the wild. On the basis of their usage, these medicinal plants can be used as synergic medicine, supportive medicine or preventive medicine (Rasool Hassan 2012). Medicinal plants provide raw material for synthesising drugs like laxatives, biocides, etc. (Rasool Hassan 2012). In recent years, demands of medicinal plants have increased significantly which in turn have resulted in the increase in cultivation area of some selected plant species, screening of new potential medicinal plants or phytochemicals, conversion of phytochemicals into registered drugs and ultimately increase in trade. However, the evaluation process of isolation of phytochemicals and their pharmacological activities needs time and multidisciplinary approaches (Manjulatha 2006).

Comparison of studies on aquatic plants has revealed that these plants are the most neglected or underutilised plants. Many among them have high potential to be used as medicinal plants, but their uses are confined to specific areas of the world (Swapna et al. 2011), primarily due to the ignorance about these plants or likes or dislikes or eating habits of people. More than 50% of people in many parts of Sri Lanka and Indian states close to the water use aquatic plants as medicinal plants followed by their use as food and ornamental in religious offerings (Munasinghe et al. 2010; Swapna et al. 2011).

Scientists all over the world are interested to characterise them on morphological and genetic basis for phytochemicals and micropropagation and their genetic transformation. This study highlights the use of aquatic plants for medicinal purposes. The present study describes major aquatic, semiaquatic medicinal plants and their medicinal uses.

Major Aquatic and Semiaquatic Medicinal Plants

Acorus calamus *L. (Sweet Flag; Araceae)*

Sweet flag (*Acorus calamus*) is an important amphibian medicinal plant of Araceae family showing wide distribution in temperate, subtropical and warm regions of Asia, Europe and North America over marshy areas, shallow lands and edges of ponds (Verma and Singh 2012). People use its rhizomes, roots and leaves that contain medicinally important alkaloids (choline), acorin and calamine A, bitter glycosides, calamol, gum, resin and starch tannins (Ahmed et al. 2007; Verma and Singh 2012). The major constituent of leaves is β-asarone (27.4–45.5%), whereas

rhizomes contain acorenone (20.86%) and isocalamendiol (12.75%) as the main constituents (Divya et al. 2011). The plant is in use since ancient times in Indian Ayurvedic system, traditional Chinese, American and European healthcare systems (Motley 1994).

The plant is used for healing asthma, diarrhoea, epilepsy, hysteria, insanity, insomnia, melancholia, neurasthenia (Hazra et al. 2007; Mukherjee et al. 2007), heart and lung cancer, skin diseases (Harikrishnan and Hariharan 1999) and piles (Jain et al. 2007). A number of studies have revealed the use of sweet flag as analgesic, anti-inflammatory (Vohora et al. 1990), anti-schizophrenia (Singh et al. 1991), antianxiety (Date and Kulkarni 1995), anticonvulsant (Achliya et al. 2005), antispasmodic (Gilani et al. 2006), antibacterial (Aqil and Ahmad 2007), antiulcer, cytoprotective (Mukherjee et al. 2007) and tranquilliser with CNS depressant activities (Pandi et al. 2009)

Alternanthera philoxeroides *Geiseb. (Alligator Weed; Amaranthaceae)*

The South American *Alternanthera philoxeroides* is a perennial aquatic stoloniferous herb showing a wide distribution in Indo-Malaysia and Australia regions (Swapna et al. 2011) and grows as emergent plant with hollow stems reaching 10 cm in length with a maximum of 20 cm above the water body at flowering stage. It has white inflorescence with seed set of around 6.5% (Liu-qing et al. 2007). The plant is amphibian in nature and grows over a wide range of habitats including freshwater, coastal areas, cultivated/agricultural lands, riverbanks and wetlands (Anonymous 2016). A number of studies conducted described the use of alligator weed for curing various diseases like dengue virus (Jiang et al. 2005), diarrhoea, fistula ani, hazy vision, malaria, night blindness, postnatal complaints, prolapsus ani and puerperal fever (Rahman and Gulshana 2014), dysentery (Jain et al. 2007), blood vomiting and eye troubles (Shankar and Mishra 2012). Furthermore, sulphated polysaccharide compounds have significant in vitro anti-HIV activity (Lewis and Lewis 2003), acute cough and intestinal worms (Panda and Misra 2011).

Alternanthera sessilis *(L.) R.Br.(Sessile Joyweed; Amaranthaceae)*

The tropical American annual *Alternanthera sessilis* is a semiaquatic plant found mainly at the banks of rivers, canals and ponds. It is an erect (approximately 1 m long), creeping well-branched herb that produces small white coloured self pollinated flowers and fruits throughout the year. It is believed that these fruits and seeds have dispersed through water and wind (Jansen 2004) to tropical Africa and

subtropical regions. In most parts of Africa and some parts of the world, leaves are used as raw or cooked vegetable (Scher 2004). It contains various medicinally important bioactive compounds like its derivatives and saturated (aliphatic) esters, oleanolic acid, stigmasterol, triterpenes α-spinasterol, β-sitosterol and β-spinasterol (Jansen 2004). A number of registered drugs are available which contain a main ingredient taken from *A. sesselis* (Walter et al. 2014). *A. sesselis* is used for simple stomach disorders, diarrhoea and dysentery, as a plaster for diseased or wounded skin parts, against fever (Jansen 2004; Jain et al. 2007; Swapna et al. 2011), for increasing milk in mothers (Naples 2005) and as antidotes for snakebites and scorpion stings (Nadkarni 2007). Bathing oil made from sessile plant juice has a cooling effect to the body and eyes, neuritis, piles and halitosis (Sambasivam Pillai 1991). Previous literature also suggest hepatoprotective (Lin et al. 2006), antiulcer (Roy and Saraf 2008), hematinic (Arollado and Osi 2010), antipyretic (Nayak et al. 2010), antioxidant (Archana Borah and Yadav 2011), anti-inflammatory (Sahithi et al. 2011) and antidiarrhoeal (Kumar and Sanjib 2013) activities of the plant.

Bacopa monnieri *(L.) Pennell. (Water Hyssop or Brahmi; Plantaginaceae)*

Water hyssop (*Bacopa monnieri*) is an important creeping aquatic or semiaquatic herb that is native to India and Australia. However, it is widely cultivated in some Asian countries and the USA (Barrett and Strother 1978) due to its use as medicinal plant and as an important component of Ayurvedic system (Augiar and Borowski 2013). It contains essential bioactive compounds like acid A, monnierin, alkaloids (brahmin and herpestine), beta-sitosterol, betulinic acid, flavonoids (luteolin and apigenin), saponins D-mannitol and hersaponin, stigmasterol (Ali et al. 1999) and bacopasaponins (bacopasaponin F, bacopasaponin E, bacopaside III, bacopaside IV, bacopaside N1, bacopaside V; Deepak et al. 2005; Anbarsi et al. 2006; Rastogi et al. 2012). It is used as memory enhancer (Mukherjee and Dey 1996; Vijayakumar et al 2010); for treatment of anxiety or epileptic disorders, asthma, bronchitis, eczema, enlargement of the spleen, hoarseness, insanity, epilepsy, irritable bowel syndrome, gastric ulcers (Shakoor et al. 1994), leprosy, ringworms (Basu and Walia 1994), snakebites and rheumatism.

Water hyssop plant have highly important and economic biomolecules, its anti-inflammatory (Jain et al. 1994), antipyretic and diuretic (Vohora et al. 1997; Stough et al. 2001), anxiolytic (Bhattacharya and Ghosal 1998), anti-ulcerogenic (Sairam et al. 2001), anti-depressant (Sairam et al. 2002), adaptogenic (Bhatia et al. 2003), hepatoprotective (Ghosh et al. 2007), antineoplastic (Deb et al. 2008), anticonvulsant (Mathew et al. 2010), analgesic (Abbas et al. 2011), Immunostimulatory (Yamada et al. 2011) and antimicrobial (Azad et al. 2012) activities have been reported during the last two decades

Centella asiatica *(L.) Urban (Centella; Apiaceae)*

Saponin- and glycoside-rich centella (*Centella asiatica*) (Singh and Rastogi 1969; Singh and Singh 2002) is an important medicinal herb of Ayurvedic, African, Chinese and Western Orthodox medicines since centuries ago (Diwan et al. 1991; Meulenbeld and Wujastyk 2001). It has high economic importance and is cultivated on minor scales in some nontraditional parts of the world including Turkey by enthusiasts. It is a small herbaceous perennial plant of Apiaceae family that prefers to grow in swamps. It has fan-shaped leaves which are small in size, white flowers and small-sized oval fruit. It is an indigenous plant in Asia mainly found in wetlands of Southeast Asia where it is used as food (Gohil et al. 2010). Besides that, it is also present in rice fields of Madagascar and South Africa, South Pacific and Eastern Europe as weed (Chevallier 1996; Jamil et al. 2007).

Centella is one of the most widely used medicinal plants in the traditional medicinal systems against amenorrhea, diarrhoea, eczema, female genitourinary tract diseases, fever, leprosy, lupus, psoriasis, ulcers, varicose (Brinkhaus et al. 2000), chronic venous insufficiency (Suguna et al. 1996), minor wound healing (Singh and Rastogi 1969; Sunilkumar et al. 1998; Shetty et al. 2006), lactation and strangury (Tanaka and Nguyen 2007). It is also used for lepra treatments (Chaudhuri et al. 1978) and as sedative (Darnis et al. 1979; Wijeweera et al. 2006), antiviral (Yoosook et al. 2000), antipsoriatic (Sampson et al. 2001), anticonvulsant (Sudha et al. 2002), immunostimulant (Wang et al. 2003), antiulcer (Cheng et al. 2004), cytotoxic and antitumor (Bunpo et al. 1987), antibacterial (Zaidan et al. 2005), antidiabetic (Venu Gopal Rao and Mastan 2007), hepatoprotective (Pingale 2008), anti-inflammatory (George et al. 2009) and cardioprotective agent (Raghavendra et al. 2009).

Centipeda minima *A. Braun and Ascheron (Spreading Sneezeweed; Asteraceae)*

Sneezeweed is a semiaquatic prostate leafy herb with a lot of branches originating from its roots. It is found in wet places or rice fields in most of the Asian countries. It is widely used for traditional medicines because it contains compounds like alkaloid; glycoside, including sesquiterpene, lactones and triterpenes (Wu et al. 1985; Bohlmann and Chen 1984); saponin; and terpenes. It is used for nasopharyngeal carcinoma (NPC) (Cheng and Li 1998; Zhang 2000), nasal congestion, rhinitis, sinusitis, swellings and inflammation (Panda and Misra 2011). Besides that, the studies also report about anti-allergy and antibacterial (Wu et al. 1994), antiprotozoal (Yu et al. 1994), antibacterial (Taylor and Towers 1998), anti-inflammatory (Qin et al. 2005), antiproliferative (Su et al. 2009), antiasthmatic, antiulcer (Singh et al. 2012), antioxidant (Huang et al. 2013b), anticancer (Guo et al. 2015) and antiangiogenic activities (Huang et al. 2016) in the plant.

Coix lacryma-*Jobi Linn. (Job's Tears; Poaceae)*

Job's tears is a tall-growing perennial semiaquatic native plant of the Southeast Asia and is distributed throughout Asian countries. It is also a cultivated plant in most part of the world as an alternative for barley. Besides for its use as food, it has been used in Chinese medicinal system for curing illness and disorders. It is also used for treatment of chickenpox, arthritis, stomach ache menstrual disorders (Santhoshkumar and Satyanarain 2010) and as an analgesic, antispasmodic (Manosroi et al. 2016) and diuretic agent. Furthermore, anodyne, antipyretic, antiseptic, antisplasmodic, hypoglycaemic, hypotensive, sedative, vermifuge (Duke and Ayensu 1985), anti-cancer (Chiang et al. 2000), anthelmintic (Manandhar 2002), antiulcer (Chung et al. 2011), anti-mutagenic (Chen et al. 2011), anti-allergic (Chen et al. 2012) and anti-inflammatory (Seo et al. 2000) properties also increased the demand of this plant.

Enhydra fluctuans *Lour (Water cress; Asteraceae)*

Watercress is an annual herb of marshy areas of the tropical and subtropical regions of Asia and Africa (Gupta 2012). It has stalkless, serrate leaves with fleshy stem that is branched and may reach up to 30 cm in length. The plant is sensitive to low temperature and prefers to grow along water courses, ditches, rice fields and ponds (Ali et al. 2013). This plant contains high amount of protein (Krishnaswamy and Prasanna 1975) along with β-carotene (Dewanji et al. 1993), cholesterol, glycoside, kaurol, cholesterol, myricyl alcohol, a number of diterpenoid acids and their isovalerate and angelate derivatives, saponins, sesquiterpene lactone, sitosterol and stigmasterol. Generally, watercress constitute an important part of local healthcare systems to treat inflammation, skin diseases, smallpox (Kirtikar and Basu 2002), food poisoning (Jain et al. 2007), neuralgia and nervous diseases (Yusuf et al. 2009; Ali et al. 2013), ascites, dropsy and anasarca. It is also used as antibilious and cooling agent and laxative. Furthermore, the plant has compounds that have analgesic (Rahman and Gegum 2002), antimicrobial (Bhakta et al. 2009), antidiarrhoeal (Uddin et al. 2011), hepatoprotective (Sannigrahi et al. 2010), antimicrobial, cytotoxic (Amin et al. 2012) and antioxidant functions (Swain et al. 2012). The compounds are also used to treat central nervous system (CNS) depressions (Roy et al. 2011).

Hedychium coronarium *J. Koenig (White Ginger Lily; Zingiberaceae)*

White butterfly ginger lily (*Hedychium coronarium*) of the Zingiberaceae (ginger) family is an amphibian aquatic herbaceous medicinal plant of tropics and subtropics that grows in waterlogged areas very close to streams or shallow water systems

(de Souza and Correia 2007). Most of the wild gingers are enlisted as endangered (Soares and Barreto 2008) due to its extinction from its origin area. It is native to Himalayas regions and Southern China with humid submontane forest ecosystems (Van Valkenburg and Bunyapraphatsara 2001). It is an upright plant which can gain height of 1–2.5 cm with rhizomes of around 5–25 cm in diameter. Younger leaves are found in vertical position with more stomata on the abaxial side (Boeger et al. 2007). The flowers are hermaphroditic and zygomorphic with strong odour and low fruit set (de Souza and Correia 2007). Under natural conditions, the plants propagate through vegetative means by rhizome buds along with very low frequency through generative propagation with localised seed dispersion (HEAR 2004).

It has high potential as ornamental medicinal and aromatic plant that contains essential oils with strong fragrance and used in the perfume industry (Van Valkenburg and Bunyapraphatsara 2001) with a total of 75 compounds from rhizomes, of which 53 (71%) were labdane-type diterpenes and 22 (29%) were diarylheptanoids, fatty acids and steroids (Chan and Wong 2015), phenolics and sesquiterpenes. It is also used for hair and skin treatment, headache, lancinating pain and inflammatory and intense pain (Anonymous 2017a), cough and fever (Jain et al. 2007). Furthermore, a number of studies highlight that *H. coronarium* has hypertensive, antidiabetic, antisyphilitic and antifungal (Lechat-Vahirua et al. 1993; Bhandary et al. 1995; Macedo 1997), anticancer (Chinmoi et al. 2009), antioxidant, antimicrobial, larvicidal activity, analgesic (Dash et al. 2011) and anti-inflammatory (Kiem et al. 2011) characteristics.

Hydrocotyle sibthorpioides *Lam. (Lawn Marshpennywort; Araliaceae)*

Lawn marshpennywort (*Hydrocotyle sibthorpioides*) of Apiaceae family (ITIS 2017) is a small, dicotyledonous, annual herbaceous plant of Southeast Asia that shows a wide range of adaptation in different habitats like dry area to submerged under water. The leaves are simple, broad, alternate and small with average size ranging from 0.5 to 2.0 cm. The flowers are small in size with faint yellow with addition of purple colour (Anonymous 2017b). The fruit is flat and has only one seed (UCIPM 2017). The plant is an important medicinal plant that has a significant place in Chinese herbal medicinal system (Huang et al. 2008). *H. sibthorpioides* has been used against asthma, healing of bone fractures (Rahmatullah 2010), detoxification of throat pains, oedema, fever, hepatitis B (Huang et al. 2008; Huang et al. 2013a), dengue (Husin et al. 2015), dysentery (Deka and Devi 2015) and typhoid fever (Kar and Borthakur 2008). Furthermore, its antioxidant/antiproliferative (Huang et al. 2008) and antitumor/immunomodulatory (Yu et al. 2007) activities have also been reported.

Ipomea aquatica *Forssk. (Water Spinach; Convolvulaceae)*

Water spinach (*Ipomea aquatica*) is an annual or perennial aquatic or semiaquatic medicinal and food plant of the Convolvulaceae family (Pullaiah 1998). It is commonly found in moist soil, margins of stagnant water, ditches, freshwater ponds and rice fields as trailing plant. The plant has it origin in China, from where it spread to other parts of the world. It is a cultivated economic plant in Southeast Asian countries (Chen et al. 1991), where it is used as leafy vegetable or salad (Ismail et al. 2004) or as fodder (Phimmasan et al. 2004). It is an important part of Ayurvedic healthcare system as it is rich in medicinally important flavonoids (Prasad et al. 2005), alkaloids (Yajima and Yabuta 2001) and carotenes (Paul et al. (1997). Furthermore, it is rich in amino acids, lipids, reducing sugars, saponins, tannins, vitamins and minerals (Malakar and Choudhury 2015).

Water spinach is a very common and popular aquatic plant used both as food and for medicinal purposes. It is used for healing a number of diseases and disorders like liver diseases (Badruzzaman and Husain 1992), constipations, intestinal problems (Samuelsson et al. 1992), mental illness, diabetes (Malalavidhane et al. 2000), high blood pressure, nosebleeds (Duke and Ayensu 1985; Perry and Metzger 1980), otorrhoea, retinitis (Jain et al. 2007), biliousness, bronchitis (Kirtikar and Basu 1993), fever, jaundice and hypoglycaemic effects (Malalavidhane et al. 2000). It has high potential as antimicrobial (Egami et al. 1998), antidiabetic (Villasensor et al. 1998), diuretic (Mamun et al. 2003), antioxidant (Ismail et al. 2004), anticancer (Prasad et al. 2005), antiulcer (Sivaraman and Muralidaran 2008), anti-inflammatory (Dhanasekaran et al. 2010), nootropic (Sivaraman and Muralidaran 2010a), hypolipidemic (Sivaraman and Muralidaran 2010b) and anxiolytic agent (Mohd et al. 2011).

Marsilea minuta *L. (Dwarf Water Clover; Marsileaceae)*

Marsilea minuta of the family Marsileaceae is an aquatic or sub-aquatic fern (Sarker and Hossain 2009) that is used as vegetable and medicinal plant in traditional and folk medicinal systems in Southeast Asia since thousands of years ago (Okoko 2009) in India and Bangladesh (Chakraborty et al. 2013). It contains several medicinally active compounds like marceline, flavonoids and marsileagenin-A (Bhattamisra et al. 2008; Chakravarti et al. 1975). It is extensively used for healing cough and respiratory problems (Upreti et al. 2009; Sen et al. 2011), headache, diarrhoea, epilepsy, hypertension, insomnia, migraine, muscle tension, psychopathy, respiratory diseases, skin diseases, sleeping disorders, spastic condition of the legs (Sharma 2002; Sarker and Hossain 2009; Upreti et al. 2009; Sen et al. 2011; Rout et al. 2009; Rahmatullah et al. 2014), diabetes, gastrointestinal disorders (Thirumalai et al. 2009; Anbarashan et al. 2011), chronic cancer, cardiovascular diseases, diabetes, etc. (Hu 2003; Kaisoon et al. 2011). It has been reported that *M. minuta* has also hypocholesterolemic (Gupta et al. 2000), antifertility (Gupta et al. 2002), anxiolytic

(Bhattamisra et al. 2007), anti-depressant (Bhattamisra et al. 2008), anti-amnestic, antistress (Tiwari et al. 2009) and anti-aggressiveness (Tiwari et al. 2010) characteristics.

Nelumbo nucifera *Gaertn. (the Sacred Lotus; Nelumbonaceae)*

Sacred lotus also known as Indian lotus is an aquatic, perennial, rhizomatous and creeping herb (Paudel and Panth 2015) of Asia and Australia that prefers extra sunlight and space to thrive in water bodies. Its leaves extend from 20 to 90 cm in length. Both leaves and flowers are found as aerial or floating. It is also cultivated in some parts of the world due to its high commercial value as medicinal or food plant (Mehta et al. 2013; Bharadwah and Modi 2016) and as ornamental plant in China, Korea, Japan, India and Australia (Paudel and Panth 2015). Studies on phytochemical studies of *N. nucifera* revealed the presence of bioactive compounds in seeds, fruits, leaves, flowers and rhizomes (Mehta et al. 2013; Paudel and Panth 2015). The plant contains isoliensinine, liensinine, negferine, N-methylisococlaurine, N-nornuciferine, nuciferine, O-nornuciferine, pronuciferine, remerine and roemerine, different glycosidse and nelumbosides (Nagrajan et al. 1966; Nakaoki 1961). It also has different flavonoids and alkaloids like anonaine, armepavine, asimilobine, dehydroanonaine, dehydroemerine, dehydronuciferine and irinidine (Tomita et al. 1965; Shoji 1987; Kashiwada et al. 2005; Xubiao et al. 2005).

The plant is extensively used as medicinal plant due to the presence of secondary metabolites found in all parts of plants. A number of diseases and disorders like blood vomiting, burning, conception, cough, dizziness, dysentery, epistaxis, fever, hematemesis, haematuria, haemoptysis, haemorrhoids, hypertension, infections, metrorrhagia (Sridhar and Rajeev 2007; Ou 1989), healings of wounds (Punjaji 2012), sunstroke, urinary problems, diarrhoea, headache, piles (Panda and Misra 2011; Swapna et al. 2011), ringworms, skin diseases, ulcer (Gupta and Pandey 2014), diabetes, eye vision (Jain et al. 2007), etc. are treated using the extracts from the plant in parts of the world. *N. nucifera* has been reported useful for treating cardiovascular (Shoji 1987), antiarrhythmic (Wang et al. 1993), antidiarrhoeal (Mukherjee et al. 1995), psychopharmacological (Mukherjee et al. 1996b), diuretic (Mukherjee et al. 1996a), antifertility problems (Yu and Hu 1997). The plant extracts are also used as antipyretic (Sinha et al. 2000), antiviral (Kuo et al. 2005), antifibrosis (Xiao et al. 2005), anti-inflammatory (Yoshiki et al. 2005), antiproliferative (Xiao et al. 2006), lipolytic (Ohkoshi et al. 2007), antioxidant (Yang et al. 2007), antibacterial (Venkatesh and Dorai 2011), anticancer (Arjun et al.2012) and antiobesity (Vahitha 2012) agent.

Nymphaea nouchali *Burm. F. (Blue Water Lily; Nymphaeaceae)*

The Indian blue water lily (*Nymphaea nouchali*; family Nymphaeaceae) is a widely distributed aquatic plant in South Asian countries, Australia and Africa (Wiart 2006). This plant is the national flower of Sri Lanka and Bangladesh and used for different purposes (Parimala and Shoba 2013). Although it is a wild aquatic plant found in ponds and stagnant waters, it is also cultivated in Southeastern countries (Slocum 2005; Raja et al. 2010). Its leaves, roots, rhizomes, fruits, flowers and tubers are also edible (Kumar and Bohra 2005). It is an important part of Ayurvedic system for treating various diseases (Raja et al. 2010) due to the presence of alkaloids, astragalin, flavonoids (Parimala and Shoba 2013), gallic acid, sterols, kaempferol (Sachan et al. 2011), quercetin, saponins and tannins. These compounds are also used to treat heart, liver and kidney disorders (Lakshmi et al. 2014). It also used as antidiabetic (Rajagopal and Sasikala 2008), hepatotoxic (Verma and Ahmed 2009) and anti-hepatotoxic (Adewusi and Afolayan 2010), antioxidant agent (Nagavani and Rao 2010). The extracts from the plant have also effective aphrodisiac (Raja et al. 2010), anti inflammatory (Jahan et al. 2012), antimicrobial and cytotoxic charachteristics (Chowdhury et al. 2013).

Persicaria hydropiper *(L.) Delarbre (Water Pepper; Polygonaceae)*

Water pepper (*Persicaria hydropiper* (L.) Delarbre with synonym of *Polygonum hydropiper*) is an annual aquatic or semiaquatic plant with a height of 40–70 cm. It is native to temperate and tropical Asia but shows wide distribution all over the world (USDA 2014; Moyeenul Huq et al. 2014). It grows in wet areas, marshes and agricultural fields (Miyazawa and Tamura 2007). It is being used in food industry as spice, flavouring and garnishing agent (Noor Hashim et al. 2012). It contains compounds like flavonoids, phenylpropanoids, sesquiterpenes and sesquiterpenoids, which show medicinal and pharmacological properties (Moyeenul Huq et al. 2014). Its plant parts like leaves, root and plant juice is used for curing certain diseases and disorders like uterine disorders (Choudhary et al. 2011), diuretic and emmenagogue (Stuart 1979), menstrual irregularities (Blatter et al. 1998), diarrhoea, dyspepsia, excessive menstrual bleeding, haemorrhoids, itching skin (Chevallier 1996), cancer (Hartwell 1970), astringent and cicatrising gastric, pulmonary problems, uterine haemorrhages (Tita et al. 2009), headache (Bari and Rahmatulla 2009), painful carbuncles (Ghani 1998), skin diseases and wounds. It is also used to prevent ovulation and cease pregnancy (Xiao and Wang 1991). The plant extracts are anthelmintic (Duke and Ayensu 1985), carminative, diuretic and stimulant. Besides its use as food and medicinal plant, this plant has gained attention to the researchers for

anti-inflammatory (Furuta et al. 1986), anti-nociceptive (Andre et al. 2004), antioxidant and antifeedant (Das et al. 2008), antibacterial and antifungal (Duraipandiyan et al. 2010), neuroprotective (Ma et al. 2010), anti-adipogenic (Lee et al. 2011), cytotoxic (Lajter et al. 2012), anticholinesterase (Noor Hashim et al. 2012) and anthelmintic (Raihan et al. 2012) activities.

Rotula aquatica *Lour. (Aquatic Rotala; Boraginaceae)*

Rotula aquatica is an important medicinal and aromatic aquatic plant of Asia and is native to India, China and Malaysia. However, it is also available in other continents like Africa and South America (Vysakh et al. 2016). It can grow up to 2–3 m high and contains important phytochemicals like alkaloids, flavonoids, phenolic compounds and steroids (Singh et al. 2011). That is why the plant was the major component in many Ayurvedic drugs (Vysakh et al. 2016) used for curing diabetes, kidney and bladder stones (Swapna et al. 2011), piles and venereal diseases. Besides that, it is used against blood disorders, coughs, dysuria, fever, heart diseases, poisonings, ulcers and uterine diseases (Sivarajan and Balachandaran 1994; Vysakh et al. 2016). Furthermore, studies on *R. aquatica* revealed psychoactive (Nayar et al. 1999), antioxidant (Patil et al. 2003), antimitotic (Patil et al. 2004), anti-urolithiatic (Raut et al. 2008), analgesic, anti-inflammatory, antipyretic (Gupta et al. 2011), anthelminthic (Lakshmi et al. 2012), antidiarrhoeal (Singh et al. 2012) and antibacterial (Prashanthi et al. 2012) properties.

Sphaeranthus indicus *Linn. (East Indian Globe Thistle; Asteraceae)*

The Indian globeflower (*Sphaeranthus indicus* Linn.) is an important semiaquatic medicinal plant that prefers to grow in dry or wet places like rice fields and cultivated lands. It shows wide distribution in India along with Sri Lanka and other continents like Australia and Africa (Chatterjee and Pakrashi 2003; Varsha et al. 2010). It is one of the most widely used medicinal plants of Indian traditional medicinal system for curing various diseases (Kirtikar and Basu 1981). It is an annual, branched herb, with cylindrical stems with taproots (Chatterjee and Pakrashi 2003). It contains abiologically active compounds like sesquiterpene lactone (Sohoni et al. 1988), sesquiterpenoids (Rojatkar and Nagasampagi 1992), eudesmanoids (Pujar et al. 2007), glycoside (Singh et al. 1989), certain essential oils (Lodha 2003) and alkaloids (Basu and Lamsal 1946).

Seeds, leaves, flowers and roots of this important medicinal plant are in use since a long time ago for certain ailments like anaemia, asthma, bile, bowel complaints, bronchitis, chest pains, chronic skin diseases (Nadkarni 2007; Prajapati et al. 2003;

Agarwal 1997), cough (Swapna et al. 2011), dysentery, elephantiasis, epilepsy and mental disorders, epileptic convulsions, hemicrania (Kirtikar and Basu 1981), indigestion, insanity, intestinal worms, leukoderma, liver and gastric disorders, looseness of the breasts, pain in the rectum, pain in the uterus and vagina, piles, biliousness, spleen diseases, toothache, tuberculous glands, urinary discharges and vomiting. Due to its high demand as medicinal plant, researchers used the metabolites for investigating nematocidal action (Sharma and Saxena 1996); anti-inflammatory (Heinrich et al. 1998), antimicrobial (Shaikh et al. 1986), antibacterial and antifungal (Dubey et al. 2000), larvicidal (Tiwari and Saxena 2003), immunomodulatory (Bafna and Mishra 2004), anxiolytic (Ambavade et al. 2006), neuroleptic (Mhetre et al. 2006), macrofilaricidal (Nisha et al. 2007), hepatoprotective (Prabhu et al. 2008) renoprotective effect (Srinivasan et al. 2008); sedative (Galani and Patel 2009); analgesic and antipyretic effects (Nanda et al. 2009) and anti-hyperlipidemic activity (Pande and Dubey 2009). These plant extracts also act as effective bronchodilatory (Sarpate et al. 2009); antioxidant activity (Tiwari and Khosa 2009); and antiviral agents (Vimalanathan et al. 2009).

Pistia stratiotes *L. (Water Lettuce; Araceae)*

Water lettuce is a stoloniferous and floating aquatic plant commonly found in stagnant water, lakes and rivers throughout Asia. It is also available in the water environment of subtropical Asia, Africa and America (Tripathi et al. 2010). It is considered as one of the most productive water plants due to its multipurpose uses (Aasim et al. 2013) ranging from food, medicines and cleaning of water bodies (Tripathi et al. 2010). It contains medicinally important compounds like alkaloids, flavonoids, glycosides and steroids (Khare 2005). Leaves and roots of water lettuce are used for curing anaemia, bladder complaints, chronic skin diseases (Kirtikar and Basu 2001), dropsy, dysentery, eczema, haematuria, kidney afflictions, leprosy, piles, syphilis and ulcers. Water lettuce has antimicrobial (Achola and Indalo 1997), diuretic, anti-dermatophytic, antifungal (Prem Kumar and Shyamsundar 2005), antidiabetic, antiseptic, antitubercular and anti-dysenteric activities (Tripathi et al. 2010).

Polygonum glabrum *Willdenow (Dense-Flower Knotweed; Polygonaceae)*

Dense-flower knotweed is an annual semiaquatic medicinal plant of marshy areas in or near the water bodies. It is widely distributed in large areas of Africa, North and South America and islands of the Pacific region. The plant has beautiful pink-coloured flowers which attracts the insects. Besides that, it has been used for phytoremediation of heavy metals from soil and water bodies (Raja and Ramya 2016).

Several plants and their parts like leaves, rootstock, seeds, stems, or their juice are reported for colic pain (Shiddamallayya et al. 2010; Panda and Misra 2011), debility, fever, jaundice, piles, pneumonia, unlocking bone (Santhoshkumar and Satyanarain 2010), snakebite (Kadel and Jain 2008), malaria (El Tahir et al. 1999), dysentery (Soudahmini et al. 2005), rheumatism and anthelmintic activities (Shankar and Mishra 2012), cardiotonic and used as astringent (Khare 2007). Furthermore, anti-inflammatory (Singh et al. 1987), anti-depressant (Nizar et al. 2007), antipyretic (Jamal et al. 2011), anti-hepatotoxic (Babitha et al. 2012), analgesic (Kiron et al. 2012), anti-nephrotoxic (Radha et al. 2013), cytotoxic (Khan et al. 2014), antioxidant/antimicrobial (Palani et al. 2014) and anti-leishmanial (Rahman et al. 2015) activities have also been reported.

Medicinal Uses of Some Less Important Aquatic and Semiaquatic Medicinal Plants

Aquatic plants used for medicinal purposes can be divided into two groups based on their ethnobotanical uses and pharmacological properties. Minor plants are those aquatic plants that also possess medicinal properties against different diseases and disorders but still lack of availability or have very low information about pharmacological properties. This might be due to unavailability of plant material, localised uses of plants, economic importance of plants or not considered or ignored by researchers. This section provides information about these minor aquatic (Table 1) or semiaquatic plants (Table 2) used as home remedies or cure for severe diseases and disorders.

Medicinal Uses of Minor Aquatic Medicinal Plants

Results revealed that 27 aquatic plants that always prefer to live in water bodies as floater or submerged plants belongs to different families like Araceae, Asteraceae, Cyperaceae, Fabaceae, Lythraceae, Marsileaceae, Menyanthaceae, Nymphaeaceae, Onagraceae, Plantaginaceae, Polygonaceae, Pontederiaceae, Primulaceae, Scrophulariaceae, Hydrocharitaceae and Ceratophyllaceae.

Results also revealed that plant parts like the whole plant, leaves, roots, flowers, inflorescence and seeds can be used as fresh or in dry form. Similarly, these plant parts are in use as itself or used as extracts or paste made from these parts. These plant parts are successfully used for curing ailment for routine normal diseases like fever, cough, pains, headaches, wounds to serious diseases and disorders like cardiac, kidney, anti-tumour, anticancer, menstrual disorders, etc. (Table 1).

Table 1 Medicinal uses of some important aquatic medicinal plants

Common/botanical name/ family	Plant part used	Medicinal uses	References
1. *Aeschynomene aspera* Linn. (Sola pith plant; Fabaceae)	Leaf, shoots	Cold, fever, cough, increase semen consistency	Panda and Misra (2011)
2. *Ammannia auriculata* Willd. (Eared redstem; Lythraceae)	NA	Fevers, rheumatic pains	Cook (1996) efloras (2010)
3. *Caesulia axillaris* Roxb. (Pink node flower; Asteraceae)	Inflorescence, whole plant	Cold, cough, dysentery, malaria, nasal congestion, wounds	Panda and Misra (2011) Punjaji (2012)
4. *Ceratophyllum demersum* L. (Coontail; Ceratophyllaceae)	Leaves	Bile secretion, burning, dysentery, epistaxis, fever, haematemesis, haemorrhoids or piles, hyperdipsia, intrinsic haemorrhages, scorpion sting, ulcer, wounds	Taranhalli et al. (2011) Shankar and Mishra (2012)
5. *Coix aquatica* Roxb. (Southeast Asian Coix; Poaceae)	Roots	Urination, menstrual complaints	Panda and Misra (2011)
6. *Cryptocoryne retrospiralis* Kunth (Crypt Retrospiralis; Araceae)	Fresh tubers	Anti-emetic, boils, burns, vomiting during pregnancy	Kamble et al. (2010) Gupta (2011a)
7. *Eleocharis dulcis* Trinius ex Henschel (Chinese water chestnut; Cyperaceae)	Roots	Abdominal pain, amenorrhoea, cardiac risks, hair growth, hernia, liver problems, motions, nausea	Duke and Ayensu (1985) Anonymous (2017c)
8. *Hygrophila auriculata* (Long-leaved barleria; Acanthaceae)	Roots, seeds, whole plant	Anuria, blennorrhoea, catarrh, craw-craw, diuretic properties, hydropsy, menstruation, stomach ache	Ruffo et al. (2002) Burkil (2004)
9. *Hygrophila polysperma* (Roxb.) T. Anderson (Dwarf hygro; Acanthaceae)	Leaves, seeds	Facial paralysis, hemiplegia, noise in the ears with headache, stiff neck	Bowes (1982) Karataş et al. (2014)
10. *Lagenandra ovata* Thw. (Malayan sword; Araceae)	NA	Cardiac ailments, healings, kidney disorders, skin problems, swelling	Maya et al. (2003) Selvakumari and De Britto (2007)
11. *Limnophila aromatica* (Lam.) Merr (Rice paddy herb; Plantaginaceae)		Anti-mutagenic, antitumor, dysentery, elephantiasis, fever, indigestion, intestinal worms, menstrual problems, mucus removal, pain killer	Murakami et al. (1997) Nakahara et al. (2002) Bhuiyan et al. (2010)
12. *Limnophila indica* (L.) Druce (Scrophulariaceae)	Aerial parts	Anthelmintic, antiseptic, dysentery, elephantiasis	Ahmed et al. (2009) Panda and Misra (2011)

(continued)

Table 1 (continued)

Common/botanical name/ family	Plant part used	Medicinal uses	References
13. *Lindernia anagallis* (Burm. F.) Pennell (Scrophulariaceae)	Whole plant	Asthma, gonorrhoea	Panda and Misra (2011)
14. *Ludwigia adscendens* (Linn.) Hara (Water primrose; Onagraceae)	Whole plant	Antibacterial, anti-inflammatory, antimicrobial, dysentery, skin diseases, ulcers	Ghani (2003) Ahmed et al. (2005) Panda and Misra (2011)
15. *Ludwigia octovalvis* (Jacq.) Raven (Mexican primrose-willow; Onagraceae)	Whole plant	Body ache, boil, diarrhoea, fever, flatulence, heal dermatitis, toxaemia, ulcer	Chang et al. (2004) Santhoshkumar and Satyanarain (2010)
16. *Lysimachia nummularia* (L.) (Creeping jenny; Primulaceae)	NA	Anticancer, stone lin syndrome, wounds	Luczak et al. (1989) Podolak et al. (2013)
17. *Marsilea minuta* Linn. (Dwarf water clover; Marsileaceae)	Leaves, root	Biliousness, cough, head cooling, headache, hypertension, insomnia, , sleeping disorder, spastic condition of leg muscles, sperm formation	Mani (2016) Sarker and Hossain (2009) Panda and Misra (2011)
18. *Monochoria hastata* (Linn.) Solms-Laubach (Arrow leaf pondweed; Pontederiaceae)	Leaves	Boils, cooling	Gupta (2011e) Swapna et al. (2011)
19. *Monochoria vaginalis* (N. L. Burman) Kunth (Pickerelweed; Pontederiaceae)	Leaves, root	Asthma, coughs, stomach and liver disorder, toothache	Lansdown (2011) Panda and Misra (2011)
20. *Nymphaea pubescens* Willd. (Hairy water lily; Nymphaeaceae)	Rhizome, roots	Abortion, blood dysentery, dysentery, dyspepsia, jaundice, leucorrhoea, menorrhagia, piles	Gupta (2011b) Panda and Misra (2011) Rama Krishna et al. (2014)
21. *Nymphoides hydrophylla* O. Kuntze (Menyanthaceae)	Leaves, seeds, stalks	Eye diseases, fevers, insect bites, jaundice, scorpion sting, snakebite, ulcer	Cook (1996) Panda and Misra (2011)
22. *Nymphoides indica* (L.) Kuntze (White snowflake; Menyanthaceae)	Leaves, whole plant	Bile, dysentery, fever, headache, rheumatism, scabies	Panda and Misra (2011)
23. *Ottelia alismoides* Persoon (Duck lettuce; Hydrocharitaceae)	Leaves	Haemorrhoids, piles, poultices for fever	Zhuang (2011) Gritto et al. (2015) Swapna et al. (2011)

(continued)

Table 1 (continued)

Common/botanical name/ family	Plant part used	Medicinal uses	References
24. *Polygonum barbatum* Steward (Knotgrass; Polygonaceae)	Leaf, roots, seed	Bleeding from wounds, colic pain, cooling agent, ulcer	Usher (1984) Maya et al. (2003) Panda and Misra (2011) Swapna et al. (2011)
25. *Rotala indica* (Willd.) Koehne (Indian toothcup; Lythraceae)	Flower, leaves	Migraine, respiratory diseases, stomach disorder	Santhoshkumar and Satyanarain (2010) Gupta and Pandey (2014)
26. *Rotala rotundifolia* (Roxb.) Koehne (Roundleaf toothcup; Lythraceae)	Whole plant	Antipyretic, anti-swelling, cold, fever, cough, detoxication, diuresis, gonorrhoea, menstrual cramps, piles, production in HepA2 cells, suppression of HBV surface antigen (HBsAg)	Anonymous (2004) Panda and Misra (2011) Zhang et al. (2011) Karataş et al. (2014)
27. *Vallisneria spiralis* Linn. (Tape grass; Hydrocharitaceae)	Leaves	Leucorrhoea, stomach ache	Swapna et al. (2011)

Medicinal Uses of Some Important Amphibian (Semiaquatic) Medicinal Plants

Amphibian plants are mainly aquatic plants which can grow both in water bodies as submerged plant like in aquariums and survive in swamps, on land growing under open places with roots in water and near water bodies or cultivated fields. Most of these plants are considered as weeds, but in recent years, they became economically important due to their use as vegetable or medicinal plants. The results on these plants in this study comprised of 25 plants from different families (Acanthaceae, Asteraceae, Boraginaceae, Commelinaceae, Hydrophyllaceae, Lythraceae, Molluginaceae, Poaceae, Rubiaceae, Scrophulariaceae and Typhaceae). Likewise, aquatic plants are used for curing routine diseases like fever, cough, pains, headaches and wounds. They are also used to treat cardiac, kidney, anti-tumour, anticancer and menstrual disorders, etc. (Table 2).

Table 2 Medicinal uses of some important amphibian (semiaquatic) medicinal plants

Common/botanical name/ family	Plant parts used	Medicinal uses	References
1. *Ammannia baccifera* Linn. (Lythraceae)	Leaves	Antioxidant, anti-steroidogenic, fever, hepatoprotective activities, rheumatic pains, ringworm, scabies, skin diseases, skin itching, typhoid fever	Dhanapal et al. (2005) Lavanya et al. (2010) Panda and Misra (2011) Swapna et al. (2011) Rhazi et al. (2014)
2. *Coldenia procumbens* Linn. (Creeping coldenia; Boraginaceae)	Leaves, whole plant	Relief from pain and swelling, fever, piles, and scorpion sting	Senthamari et al. (2002) Panda and Misra (2011)
3. *Cyanotis axillaris* (Commelinaceae)	Leaves	Abortions, ascites, inflammation of eardrum, swellings, joint pain, rheumatism	Cook (1996) Gupta (2011c)
4. *Cyathocline purpurea* O. Kuntze (Asteraceae)	NA	Anticancer, headache, stomach ache	Cook (1996) Ma et al. (2009) Rehel (2011)
5. *Dentella repens* (Linn.) J. R. and J. G. A. Forster (Creeping dentella; Rubiaceae)	Leaves	Poulticing sores	Santhoshkumar and Satyanarain (2010)
6. *Eclipta prostrata* (Linn.) Linn. (Asteraceae)	Whole plant	Antiulcer, eczema, headache, jaundice, mental disorders, scorpion sting, skin diseases, snakebite, spleen enlargements, toothache	Panda and Misra (2011) Lansdown et al. (2014)
7. *Epaltes divaricata* Cassini (Asteraceae)	Roots	Acute dyspepsia, astringent tonic, jaundice, urethral discharges	Cook (1996) Chah et al. (2006) Glorybai et al. (2015)
8. *Glinus oppositifolius* (Linn.) A. DC. (Molluginaceae)	Leaves, whole plant	Fever, inflammations, joint pains, malaria, skin diseases, wounds	Inngjerdingen et al. (2005) Panda and Misra (2011)
9. *Grangea maderaspatana* Poiret (Asteraceae)	Leaves, whole plant	Antiseptic, antisplasmodic, deobstruent, earache, menstrual disorders, piles, stomach disorders	Panda and Misra (2011) Swapna et al. (2011)
10. *Heliotropium indicum* Linn. (Boraginaceae)	Flowers, shoots, whole plant	Asthma, boils, bronchitis, cataract, dysentery, menstrual blood loss, redness and conjunctivitis of the eyes, antiseptic, scorpion sting, ulcers	DeFilipps et al. (2004) Panda and Misra (2011)
11. *Hydrolea zeylanica* Vahl (Hydrophyllaceae)	Leaves, shoots, whole plant	Antiseptic, wounds healing	Cook (1996) Gupta (2011d) Panda and Misra (2011)

(continued)

Table 2 (continued)

Common/botanical name/ family	Plant parts used	Medicinal uses	References
12. *Hygrophila schulli* (F. Hamilton) M. R. and S. M. Almeida (Acanthaceae)	Leaves, roots, seeds, whole plant	Anaemia, jaundice, gout, hepatic obstruction, impotency, inflammation, pain, rheumatism, spermatorrhoea, urinary infections	Shanmugasundaram and Venkataraman (2005) Panda and Misra (2011)
13. *Lindernia ciliata* (Colsmann) Pennell (Hairy saltwort; Scrophulariaceae)	NA	Gonorrhoea, menorrhagia	Ipor (2001) Santhoshkumar and Satyanarain (2010)
14. *Lindernia crustacea* (Linn.) F. von Mueller (Malaysian false pimpernel; Scrophulariaceae)	Leaves, whole plant	Bile, secretion, biliousness, boils, diarrhoea, dysentery, indigestion, ringworms, snakebites and tick bites	Duke (2010) Panda and Misra (2011)
15. *Lindernia oppositifolia* (Retzius) Mukherjee (Scrophulariaceae)	Roots	Fevers	Cook (1996)
16. *Lindernia procumbens* (Krocker) Borbas (Scrophulariaceae)	Leaves	Cooling of blood, detumescence, dysentery, inflammation, liver heat, ringworm	Ahmed et al. (2009) Pan et al. (2009) Santhoshkumar and Satyanarain (2010)
17. *Murdannia nudiflora* Brenan (Doveweed; Commelinaceae)	Roots, whole plant	Asthma, astringent, giddiness, leprosy, piles, stomach	Panda and Misra (2011) Patwari et al. (2014)
18. *Polygonum plebeium* R. Brown (Small knotweed; Polygonaceae)	Roots	Bowel complaints, colic complaints, eczema, pneumonia	Katewa and Galav (2005) Swapna et al. (2011)
19. *Spilanthes calva* A. P. de Candolle (Asteraceae)	Flower head, roots, whole plant	Dysentery, psoriasis, purgative, rheumatism, scabies, stammering in children, tongue paralysis, toothache	Swapna et al. (2011)
20. *Typha domingensis* Persoon (Southern cattail; Typhaceae)	Rhizomes	Astringent, burns, diuretic, dysentery, gonorrhoea, measles, wound healing	Aliotta et al. (1990) Akkol et al. (2010) Panda and Misra (2011)
21. *Vetiveria zizanoides* (Linn.) Nash (Poaceae)	Roots, rhizome	Boil, burn, colic and obstinate vomiting, diaphoretic, epilepsy, febrifuge, fever, flatulence, headache, mouth ulcer, refrigerant, rheumatism, scorpion sting, snakebite, thirst	Santhoshkumar and Satyanarain (2010) Swapna et al. (2011) Rao and Suseela (2017)

(continued)

Table 2 (continued)

Common/botanical name/ family	Plant parts used	Medicinal uses	References
22. *Wedelia chinensis* (Osbeck) Merr. (Asteraceae)	Plant extract	Blackening of hair, gouty arthritis, hair growth, juvenile arthritis, mental tension, multiple sclerosis, osteochondritis dissecans, rheumatic fever	Suresh et al. (2010) Meena et al. (2011) Panda and Misra (2011)

Conclusion

Every terrestrial or aquatic plant growing around us is important and can be used in one way or another. This study explain role of some important aquatic or semi-aquatic plants that have potential for cultivation. Exploration of their ethnobotanical and pharmacological uses will be of great interest. Although Turkey is covered with water bodies on three sides, any type of systematic aquatic plant study is almost neglected or underutilised. This review explains parts of the plant most extensively used for medicinal or pharmacological purposes. Generally, the people are not aware of the importance of aquatic plants in the provision of sustenance, clothing and abode for human and animals. Extensive and systematic studies about these plants would change their status from weed to their actual value based on qualitative characteristics, which will help in changing the status of these plants more rationally from most exceedingly terrible weeds to plants of vital importance. These studies will help in the advancement of all the mentioned sectors described earlier in this chapter.

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Flavonoids and Their Biological Secrets

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Introduction

Flavonols

3-Hydroxy flavones or flavonols, one major subclass of flavonoids, are poly-aromatic secondary plant metabolites. Their structure consists of general three-ring backbone of flavonoids, i.e., rings A, B, and C. Flavones are classified as flavanones when there is a hydroxyl group attached to the 3-position of C ring. Structures of flavonol and flavone molecules are shown in the figure.

In higher plants, flavonols are present in glycosylated form; most abundant are the *O*-glycosides. The sugar residues commonly found in flavonols are glucose, galactose, rhamnose, and glucuronic acid. Glycosylation is reported at 3-, 7-, 3-, and 4′-positions (Table 1).

Flavonols are present in various parts of plants including leaves, fruits, and vegetables. Among different plants tested for flavonols, their highest concentration was found in strawberry (*Fragaria* spp.), peepal (*Ficus religiosa*), spinach (*Spinacia oleracea*), and cauliflower (*Brassica oleracea*) (Sultana and Anwar 2008). Like

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Table 1 The relative substitutions among the 15 different flavonoids discussed in the paper

Sr.#	Flavonols	Position								
		2′	3′	4′	5′	3	5	6	7	8
1	3-Hydroxyflavone	H	H	H	OH	H	H	H	H	H
2	Azaleatin	H	OH	OH	H	OH	OCH_3	H	OH	H
3	Fisetin	H	H	OH	OH	OH	H	H	OH	H
4	Galangin	H	H	H	H	OH	OH	H	OH	H
5	Gossypetin	H	OH	OH	H	OH	OH	H	OH	OH
6	Isorhamnetin	H	OCH_3	OH	H	OH	OH	H	OH	H
7	Kaempferide	H	H	OCH_3	H	OH	OH	H	OH	H
8	Kaempferol	H	H	OH	H	OH	OH	H	OH	H
9	Morin	OH	H	OH	H	OH	OH	H	OH	H
10	Myricetin	H	OH	OH	OH	OH	OH	H	OH	H
11	Natsudaidain	H	H	OCH_3	OCH_3	OH	OCH_3	OCH_3	OCH_3	OCH_3
12	Pachypodol	H	H	OH	OCH_3	OCH_3	OH	H	OCH_3	H
13	Quercetin	H	OH	OH	H	OH	OH	H	OH	H
14	Rhamnazin	H	OCH_3	OH	H	OH	OH	H	OCH_3	H
15	Rhamnetin	H	OH	OH	H	OH	OH	H	OCH_3	H

other flavonoids, flavonols are most apparent antioxidant in higher plants. Antioxidative activity of flavonols has been shown experimentally to prevent nuclear DNA damage by hydrogen peroxide in plants (Melidou et al. 2005). Studies on *Arabidopsis* plant have shown that flavonols are involved in providing protection to plant leaves against oxidative damage due to excessive visible radiation (Havaux and Kloppstech 2001). They are also involved in providing defense against fungal infection to plant leaves (Treutter 2006).

Despite their role in plant survival, biological activities of flavonols also contribute to human health. Antiviral activities of flavonoids were discovered in the first half of the twentieth century. Hydroxyl group at 3-position makes flavonols more effective against herpes simplex virus type 1 than flavones (Cody et al. 1986; Selway 1986). Anti-inflammatory response of flavonol has also been reported on animal models for both chronic and acute inflammation (Lee et al. 1993). Flavonols also involved in antithrombogenic effect by preventing platelet aggregation (Gryglewski et al. 1987).

Fisetin

Fisetin is a special class of flavonoid compounds defined as 3,3′,4′,7-tetrahydroxyflavone, 6-desoxyquercetin, and fisidenolon; its empirical formula is $C_{15}H_{10}O_6$. In plants it is present as glycoside fisetin-8-glucoside. Chemically it is defined as -(3,4-dihydroxyphenyl)-3,7-dihydroxy-4*H*-1-benzopyran-one and 3,3′,4,7-tetrahydroxy-2-phenylchromen-4-one. Fisetin usually is found in plants as the glycoside fisetin-8-glucoside.

It is a basic 15-carbon structure also known as diphenylpropane molecule having two aromatic rings which is linked through three carbon atoms. Flavonoids make difference because of the saturation of the heteroatomic ring C, in place of B at position C-2 or C-3 of ring C and throughout patterns of methoxylation (Nijveldt et al. 2001).

Biological Properties

Natural polyphenolic compounds including flavonoids are present in fruits, vegetables, and some beverages (Aherne and O'Brien 2002). They have role in pharmaceutics and have the potential of treating cancer and heart diseases (Havsteen 2002; Hill et al. 1989; Lopez-Lazaro 2002; Middleton et al. 2000; Monasterio et al. 2004). It has been discovered that few flavonoids have also a role in the organization of cytoskeleton especially in the assembly of tubulin.

According to the reported data, fisetin is the most active member of flavonoid compounds and shows a role in the modification of morphology of endothelial cells that is related to the stabilization of microtubule and to the α-tubulin acetylation which is known to be a distinctive marker for the stabilization of tubulin. Such type of presented data has been useful for us in the selection of food which has such type of active flavonoids acting against cancer and other diseases.

Flavonoid compounds showed responses against inflammation, allergy, and bacterial infection (Melgarejo et al. 2007; Middleton et al. 2000; Williams and Grayer 2004). Fisetin is an important class of flavonoid compounds found in a variety of fruits and vegetables which is responsible for decreasing the process of degranulation of mast cells (Arai et al. 2000).

It has been discovered that fisetin has a role in the differentiation of nerve cells and also protects them from death due to oxidative stress (Ishige et al. 2001; Sagara et al. 2004). It has been studied that fisetin possesses also the properties of antiaging. Fisetin has also a role in rising the serotonin and non-adrenaline levels in the brain which results in the production of effect against depression (Zhen et al. 2012).

Fisetin also has a role in the promotion of growth and maintenance of nerve cells without the help of neurotropic factors. These are important factors because if those factors are removed, it will result in the death of nerve cells. In the absence of those factors, fisetin is invovled promotion of growth and survival of nerve cells (Maher 2006, 2008).

NF-kappa B pathway plays an important role in inflammation which results in the progression of cancer. Fisetin and some other flavonoids perform an important role in the suppression of numerous inflammatory pathways most importantly NF-kappa B pathway which is important in many remedies against cancer (Gupta et al. 2010; Prasad et al. 2010; Sung et al. 2007).

Fisetin has also a role in the prevention of Huntington's disease which is an important neurodegenerative disorder affecting various brain functions (Maher et al. 2011a). Wnt signaling pathway has an important role in the proliferation and

progression of cancer; fisetin compound performs function in inhibition of this pathway (Teiten et al. 2012).

It has been studied that fisetin performs function against pulmonary inflammation infection, against asthma specifically doing by downregulation of NF-kappa B pathway (Wu et al. 2011). Many other flavonoids including fisetin decrease the activation of mast cell which reduces the histamine level as a result of which inhibition of many allergies occurs. Because when mast cells become active, they tend to secrete histamine and many other pro-inflammatory compounds (Park et al. 2008).

Fisetin makes the high expression of glyoxalase 1 which is an important enzyme and plays a significant role in the exclusion of substances and reducing the levels of glycated proteins responsible for diabetes (Maher et al. 2011b). In contrast if the expression of glyoxalase 1 becomes low, it leads to the increased level of glycation and complications in diabetes (Miyata et al. 2001).

Also by decreasing the secretion of glucose from the liver, fisetin hinders the hyperglycemia which is induced due to the glucose secretion from the liver (Constantin et al. 2010).

Galangin

Galangin is an important class of flavonoid compounds; they are found in honey, propolis, *Helichrysum aureonitens*, and *Alpinia officinarum* in greater amount. This compound has an important role in pharmaceutics; also it performs function against oxidation, against mutations, and against cancer (Cushnie and Lamb 2006; Gwak et al. 2011; Heo et al. 2001).

It has three hydroxyl groups on its carbon ring, and it has the capability of enzyme modulation and can decrease the chemical toxicity (Chen et al. 2008). It has been reported earlier that galangin has a role in the inhibition of aryl hydrocarbon receptor; in organisms these compounds are also involved in certain biological activities at nontoxic levels (Murray et al. 2006).

Biological Properties

By using the agar dilution assay, it was studied that galangin also showed its activity against the 17 strains of *Staphylococcus aureus* species which was resistant against quinolone. In a specific strain when there is a change in the amino acid in the GrlB subunit of topoisomerase IV, it results in its increase receptiveness toward galangin. Therefore topoisomerase IV enzyme plays an important role in the function of galangin against bacterial infection (Cushnie 2006).

The activity of galangin was also discovered against 17 strains of *Campylobacter jejuni* and several gram-positive and gram-negative strains, but the highest galangin activity was found against the 17 strains of *Campylobacter jejuni* (Campana et al. 2009).

In colorectal and liver cancer, the transcriptional process of beta-catenin is increased; galangin reduces its transcription by the elimination of beta-catenin inside the cell. This compound also decreases the levels of beta-catenin by making the mutations inactive of adenomatous polyposis coli (Gwak et al. 2011).

According to the in vivo and in vitro studies, it has been reported that galangin has the capability of performing functions in the regulation of enzyme activity and in decreasing the toxic effect of chemicals and against oxidation (Heo et al. 2001). Galangin has been found in liposomes; these liposomes have been analyzed for their activities against oxidation, and results have showed that liposomes which have greater concentration of galangin have more antioxidative activity (Landi-Librandi et al. 2011).

The effect of galangin was studied in rat liver which was fed on fructose; the high expression of plasma glucose, triglycerides, and insulin was prohibited by galangin; and furthermore it also increases the sensitivity of insulin, while galangin also plays an important role in decreasing the expression of cytokines. It also prohibited the high translocation of NF-kappa B (Sivakumar and Anuradha 2011).

HPLC and MS have been used in finding the quantification of galangin in biological samples, and results showed that these compounds are aggregated more in the nucleus than cytoplasm (Mukai et al. 2009). According to chromatographic studies, galangin is also available in propolis of *Lactobacillus fermentum* (Saavedra et al. 2011). It has been discovered after the analysis of seven different types of Slovenian honey that it contains the greater amount of galangin (Bertoncelj et al. 2011).

Extracts of different plants have galangin which is considered to be the most active compound (Yang et al. 2011b). Concentration of polyphenol in fruits and leaves of *Ficus carica* has shown the occurrence of galangin as a major constituent (El-Shobaki et al. 2010).

By using the technique of HPLC, seven different phenolic components were discovered in bee pollen sample, among which galangin was the most active and also galangin was found in different fruits which were taken from Italy (Grippi et al. 2007; Šarić et al. 2009). Galangin was also discovered from the 120 samples of Chinese propolis which were detected by using the fingerprint method (Chen et al. 2008).

Galangin was significantly found in the chemical composition of propolis which was taken from arid and semiarid areas of Sonora, Mexico, Europe, China, and Argentina (Gardana et al. 2007). Galangin was also found from the extracts of apple and parsley (Abdel-Rahim and El-Beltagi 2010).

Gossypin

Gossypin (3,3′,4′,5,7-pentahydroxy-8-*O*-glucosylflavone) is a flavonol and a derivative of gossypetin. It is a monoglucoside. On complete methylation and hydrolysis, it gives an *O*-pentamethyl gossypetin (Rao and Seshadri 1946b). The presence of

glucose moiety in the eighth position of hexahydroxyflavone makes it water soluble (Gautam and Vijayaraghavan 2007).

It was initially extracted from *Gossypium indicum* (Neelakantam and Seshadri 1936). However, the species *Gossypium indicum* did not yield sufficient amount of gossypin to carry out further experimentation. It was then found out that *Hibiscus vitifolius* was a rich source of gossypin (Rao and Seshadri 1946a). A detailed study of the structure and function of the flavonol has been carried out since.

Biological Properties

Major focus of today's research is finding a cure for cancer. To avoid the harms of chemotherapy and radiotherapy, scientists now look toward natural products with higher efficacy and fewer side effects. In this regard, the anticancer activity of gossypin has been investigated by many researchers. A study by Babu et al. (2003) demonstrated the anticarcinogenic activity of the bioflavonoid gossypin against the carcinogens such as DMBA which causes skin papillomas in mouse. Moreover it was shown to decrease the tumor burden in solid tumors and inhibition of angiogenesis. The antitumor activity of gossypin is attributed somewhat to its ability to inhibit the key enzymes in DNA replication, the topoisomerase I and II (Babu et al. 2003). In another study conducted on human glioma, U251 cells treated with gossypin showed promising results. Gossypin caused cell cycle arrest at G2/M phase involving the phosphorylation of cell division cycle 25C (Cdc25C) tyrosine phosphatase through the stimulation of checkpoint kinase 1 (Chk1) (Shi et al. 2012). Additionally gossypin has been found to block cell multiplication in L929, HT29, and K562 tumor cell lines in vitro (Babu et al. 2003). Another possible mechanism underlying anti-tumorigenic capability of gossypin was demonstrated by Kunnumakkara et al. (2007). They analyzed the effect of gossypin on NF-kappa B, a master regulator involved in inflammation, carcinogenesis, hyper-proliferation, invasion, and angiogenesis. The results supported the hypothesis of possible NF-kappa B inhibition by gossypin (Kunnumakkara et al. 2007).

Gossypin's role as a potent antioxidant was examined in a study involving lead toxicity. Lead is known to cause generation of reactive oxygen species (ROS) and destruction of antioxidant reserves in the body (Patrick 2006; Silbergeld et al. 2000). Gautam et al. demonstrated the significance of co-administrating gossypin during lead exposure. They concluded that gossypin prevents lead-induced oxidative stress by chelating lead, stimulating the enzymes involved in protecting antioxidant reserves, and by inducing delta-aminolevulinic acid dehydratase, which is primarily targeted by lead (Gautam and Flora 2010).

Not only gossypin is effective in its antitumor activities, but its potential role in alleviating many other pathologies is also under consideration. Many of the current orally administrated hypoglycemic drugs for the treatment of diabetes mellitus induce harmful side effects. A study was conducted to evaluate the antidiabetic effect of gossypin in streptozotocin (STZ)-induced experimental diabetes in rats.

Results revealed a strong antidiabetic activity of gossypin against STZ-induced experimental diabetes (Venkatesan and Sorimuthu Pillai 2012).

Epilepsy is a set of neurological disorders characterized by recurrent or single seizures accompanied by alterations in the brain (Chang and Lowenstein 2003; Fisher et al. 2005). To avoid the drug interactions caused by the many antiepileptic drugs, researchers are investigating natural alternatives with fewer side effects. In this pursuit, gossypin was used in a set of experiments to evaluate its anticonvulsant activity. The results obtained emphasized the importance of gossypin against convulsions probably by influencing the GABA aminergic and glycine inhibitory mechanism (Rasilingam et al. 2008).

Mast cell degranulation and release of histamines and other inflammatory cytokines underlie severe allergic reactions. Gossypin was shown to inhibit anaphylaxis in a rat model of allergy (Ganapaty et al. 2010). The anti-inflammatory activity of gossypin is thought to be the consequence of inhibition of arachidonic acid breakdown through blocking of the cyclooxygenase and lipoxygenase enzymes (Ferrandiz and Alcaraz 1991). Gossypin also has a potent effect against sulfur mustard (SM), a blistering agent, possibly through its anti-inflammatory action (Gautam and Vijayaraghavan 2007).

Gossypin has also been shown to relieve pain in mice, acting as an analgesic possibly through the induction of opiate receptors (Viswanathan et al. 1984).

Isorhamnetin

Isorhamnetin (3′-methoxy-3,4′,5,7-tetrahydroxyflavone) is an *O*-methylated flavonol occurring naturally in plants but is also a metabolic product of quercetin(isorhamnetin is methylated quercetin) (phytochemicals.info). It can be extracted from *Tagetes lucida* (Bohm and Stuessy 2001). It is mostly found in fruits and medicinal herbs (Kim et al. 2011). Isorhamnetin is a metabolite of quercetin, a widely distributed natural flavonol (Anderson 2004).

Biological Properties

Isorhamnetin has found its promising role in treatment of various diseases such as cardiovascular disorders, rheumatism, and hemorrhage (Gupta et al. 2010; Ma et al. 2007a; Suomela et al. 2006). Isorhamnetin is known to have cardiovascular effects. Isorhamnetin and its parent compound quercetin caused endothelium-independent vasodilatation in the aorta, mesenteric arteries, portal vein, and porcine coronary arteries of rat (Ibarra et al. 2002).

Anti-inflammatory activity of isorhamnetin was observed in murine RAW264.7. Analysis was performed based on the expression of pro-inflammatory markers in lipopolysaccharide-stimulated murine macrophages (Boesch-Saadatmandi et al.

2011). The possible way by which isorhamnetin blocks inflammation is not yet clear; however the study by Boesch-Saadatmandi et al. suggested the inhibition of NF-kappa B to have a significant role in this regard (Boesch-Saadatmandi et al. 2011).

A derivative of isorhamnetin, isorhamnetin 3-*O* neohesperidoside (I3ON), has potential antioxidant activity and protective capability against DNA damage caused by hydroxyl free radical (Bouhlel et al. 2009).

Osteoporosis is mainly attributed to estrogen deficiency in postmenopausal women (Richelson et al. 1984). Different flavonols were examined for their estrogen receptor agonist activity. Isorhamnetin along with other flavonols under study exhibited stimulatory activity for estrogen receptors, thereby producing the required osteogenic effects (Yang et al. 2011a).

Isorhamnetin has been found useful in treating obesity as it has an anti-adipogenic action. Differentiation of human adipose tissue-derived stem cells into adipocytes is controlled in different ways. Wnt signaling, being one of the chief regulatory mechanisms in the differentiation process, is targeted by isorhamnetin mainly by the stabilization of β-catenin (Lee et al. 2010).

As for the role of isorhamnetin in cancer, it is shown to exert antitumor activity. One of the possible mechanisms for this anticancerous activity was delineated by Kim et al. (2011) in a study of skin cancer. In this study isorhamnetin blocked epidermal growth factor (EGF)-induced neoplastic cell transformation by suppressing the expression of COX-2 protein. COX-2 is a major inflammatory mediator and exerts pro-tumorigenic activity (Méric et al. 2006). Furthermore it exerted a negative effect on anchorage-dependent and anchorage-independent growth of A431 human epithelial carcinoma cell line (Kim et al. 2011). Many other evidences support isorhamnetin's role in reducing cell growth and weight and size of tumors (Ma et al. 2007a; Steffen et al. 2008). Another set of experiments revealed antitumor role of isorhamnetin by inhibition of the cell cycle protein, farnesyl protein transferase (FPTase) (Oh et al. 2005). Previously, quercetin was shown to be a potent anticancer agent, but newer studies have signified the increased potential of isorhamnetin an effective anticancer entity. Such isorhamnetin showed elevated levels of cytotoxicity against cancerous cells as compared to quercetin. It induces necrosis and apoptosis in human colon cancer cell line (HCT-116) (Jaramillo et al. 2010). Moreover in another research aflatoxin B1 (AFB1)-mediated oxidative stress was lessened considerably by isorhamnetin more than quercetin in hepatocellular carcinoma cells (Choi et al. 2010).

Isorhamnetin was shown to reduce proliferation and stimulate apoptosis in gastric cancer. These functions were mediated through the activation of peroxisome proliferator-activated receptors (PPAR-γ) which is known to be involved in promoting tumorigenesis in gastric cancer. The results by Ramachandran et al. provide a strong basis for establishment of combination therapy involving the use of isorhamnetin to reduce the side effects and enhance treatment efficacy for gastric cancer (Ramachandran et al. 2012).

Kaempferol/Kaempferide

3,5,7-Trihydroxy-2-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one kaempferol is a class of flavonol compounds (Calderon-Montano et al. 2011). It is abundantly found in edible plants and one of the most important flavonoid compounds (Miean and Mohamed 2001a). These plants include tea (Park et al. 2006a), broccoli (Calderon-Montano et al. 2011), cabbage (Calderon-Montano et al. 2011), and strawberries (Calderon-Montano et al. 2011; Hakkinen et al. 1999), and other dietary plants (Calderon-Montano et al. 2011). Previously during epidemiological research, it has been observed that these dietary plants also used for human health betterment and during various preclinical and clinical trials have revealed that kaempferol-rich diet overcomes the human malignancy development (Kim et al. 2003). Many naturally occurring glycosides of kaempferol are extracted from different plants. These glycosides are kaempferitrin (kaempferol 3,7-dirhamnoside) (Vishnu Prasad et al. 2009), astragalin (Wei et al. 2011), afzelin (kaempferol 3-rhamnoside) (Markham et al. 1992), kaempferol 7-*O*-glucoside (Ibrahim et al. 2008), robinin (kaempferol-3-*O*-robinoside-7-*O*-rhamnoside) (March et al. 2004), sophoraflavonoloside (kaempferol 3-*O*-sophoroside) (Kim et al. 2012), and trifolin (kaempferol-3-*O*-galactoside) (Nowak and Wolbis 2002). Kaempferol-3-*O*-β-D-glucopyranoside-7-*O*-α-L-rhamnopyranoside is one of the most bitter-tasting glycoside compounds and has been isolated from the methanolic plants (Gohar et al. 2000; Ragasa et al. 2005). During the metabolism process by the activity of enzyme transferase, these flavonoid kaempferol compounds can transfer the product of kaempferol and *S*-adenosyl methionine to kaempferide (Calderon-Montano et al. 2011; Curir et al. 2001). Kaempferide is defined as 4′-*O*-methylkaempferol which is also included in chemical flavonoid compound (Curir et al. 2001). These isolated flavonoid and chemical compounds have an antimicrobial (Yang et al. 2010), antioxidant (Choi et al. 2013), anticancer (Calderon-Montano et al. 2011), neuroprotective (Filomeni et al. 2012), antidiabetic (Habtemariam 2011), immunomodulatory (Kim et al. 2008), anti-osteoporotic, antiestrogenic (Oh et al. 2006), anxiolytic (Vissiennon et al. 2012), analgesic (Tsiklauri et al. 2011), and anti-allergic activities (Kim et al. 2008). Therefore naturally occurring plants are usually used as medicinal plants and for pharmaceutical products. The study of immunopharmacological properties of these plants has clearly shown the result to inhibit the cell growth, oxidative low-density lipoprotein (LDLP) suppression, viral inhibition, and reduction of apoptosis and strengthen the immune system (Kim et al. 2008). Kaempferol and kaempferide are developed by the metabolic activities of bioactive plants and are agents to treat many disorders (Kim et al. 2008). *Ginkgo biloba*, *Moringa oleifera*, *Equisetum* spp., *Tilia* spp., propolis, and *Sophora japonica* are the species of medicinal productivity (Calderon-Montano et al. 2011). For the cure of free radical damages and different infectious diseases, these pharmacokinetics species have often been utilized (Calderon-Montano et al. 2011). It was indicated that kaempferol compound in plants can be used as an agent of chemo-protection (Chen and Chen 2013). Recently it was observed that hypertension stress that associated with cardiac risks

has been suppressed by the consumption of this anticancer compound in tea and broccoli(Calderon-Montano et al. 2011). It has been concluded that kaempferol also plays a vital role to overcome the inflammatory response (Choi et al. 2013). Kaempferol also suppresses the translational activity of particular protein that may help to inhibit the growth of inflammatory lesions (Choi et al. 2013). Kaempferol has been isolated from *B. pinnatum* which is a medicinal herb used as drug for the antimicrobial activity (Tatsimo et al. 2012). It also inhibits the aggregation of the foam-producing cells, and these foam-producing cells increase the low-density lipoprotein oxidation (Li et al. 2013). These naturally occurring compounds are also used to eliminate cholesterol and lipids from macrophages (Li et al. 2013). Therefore it can reduce the effect of atherosclerotic disorder (Li et al. 2013) and toxicity of neurodegenerative Parkinson's disease (Filomeni et al. 2012). Secondary glycosidic metabolites of kaempferoid have the ability to develop products like kaempferol as anticancer, antioxidant, and anti-glycine (Al-Musayeib et al. 2011). While these metabolites not only target the tumorous cells but are also capable of minimizing the side effects of the combination of both radio- and chemotherapies (Al-Musayeib et al. 2011). Productivity of kaempferol and phytochemically active compound kaempferide has a key importance to act as antiestrogenic property (Hung 2004). Photochemicals inhibit the estrogen and progesterone receptors to control the proliferation of inflammatory cancerous cells (Frigo et al. 2002). Kaempferide has structural capability to suppress the attack of fungal infections (Curir et al. 2001). It was hypothesized that kaempferol reduces the effect of vascular endothelial growth factor receptors too. VEGF receptor increases the risk of ovarian cancer; it can be controlled by taking dietary fruits and vegetables having flavonoid products (Luo et al. 2010). In vivo investigations induced the role of flavonoid kaempferide acting as an antioxidant to treat the liver patients by the use of isoforms such as P450 (Otake and Walle 2002). Another side in vitro studies show that kaempferide has anti-plasmodium and antimalarial activity against the strains of *Plasmodium falciparum* (De Monbrison et al. 2006). Therefore in this study it was analyzed that naturally occurring flavonol and chemically active compounds can be used as therapeutic agents. These therapeutic products play an important role to save human life and suppress the activity of various infectious diseases with the help of development of these compounds.

These plants include tea (Park et al. 2006b), broccoli, cabbage (Calderon-Montano et al. 2011), and strawberries (Häkkinen et al. 1999; Calderon-Montano et al. 2011). These isolated flavonoid and chemical compounds have an antimicrobial (Yang et al. 2010), antioxidant (Choi et al. 2013), anticancer (Calderon-Montano et al. 2011), neuroprotective (Filomeni et al. 2012), antidiabetic (Habtemariam 2011), immunomodulatory (Kim et al. 2008), anti-osteoporotic, antiestrogenic (Oh et al. 2006), anxiolytic (Vissiennon et al. 2012), analgesic (Tsiklauri et al. 2011), and anti-allergic activities (Kim et al. 2008). Therefore in this study it was analyzed that naturally occurring flavonoid and chemically active compounds can be used as therapeutic agents. These therapeutic products play an important role to save human life and suppress the activity of various infectious diseases with the help of development of these compounds.

Rhamnetin and Rhamnazin

Rhamnetin is also one of the chemical *O*-methylated flavonoid compounds (Ozipek et al. 1994; Yun et al. 2000). It was recognized as rhamnetin 3-*O*-[3‴″-*O*-(*p*-coumaroyl)-alpha-L-rhamnopyranosyl(1→3)-alpha-L-rhamnopyranosyl(1→6)]-beta-D-galactopyranoside (Ozipek et al. 1994). It can be isolated from various plant sources such as cloves, green vegetables, and fruits (Yun et al. 2000). Chemical structure of this natural compound has been discovered by Austrian chemist Josef Herzig. Basically this molecule having flavonol nuclei consisted of two benzene rings (Zhen et al. 2012). And these rings have been combined by O_2-containing pyran rings as shown in Fig. 1 (Zhen et al. 2012).

Biological Properties

Rhamnazin was synthesized through the activity of enzyme 3-methylquercetin 7-*O*-methyltransferase (Khouri et al. 1988; Ozipek et al. 1994). This transferase enzyme uses *S*-adenosyl methionine and isorhamnetin to produce *S*-adenosylhomocysteine and rhamnazin (Khouri et al. 1988). Basically rhamnazin is known as 3,5-dihydroxy-2-(4-hydroxy-3-methoxyphenyl)-7-methoxychromen-4-one (Khouri et al. 1988). It is also the naturally occurring 3′,7-dimethylquercetin flavonoid compound (Joe et al. 2010). Like other naturally occurring chemical compounds, rhamnetin and rhamnazin also have anticancerous (Lee et al. 2011; Ma et al. 2012), antioxidant (Pande 2001), and anti-infectious activities (Ahmed et al. 2001), etc. The *O*-methylated flavonoid compounds are chemically methylated on hydroxyl groups. Chemical formation of this methoxy bond is difficult because methoxylation is possible in any position of molecule (Lee et al. 2011). So the usage of specific enzyme *O*-methyltransferase plays an important role which interacts substrate on specific position of molecule (Mattarei et al. 2010). That enzyme implies the *O*-methylation on a specific hydroxyl (3-OH) position. Multile hydroxal functional groups greatly contribute towards the therapeutic potential of polyphenols (Mattarei et al. 2010). In the metabolism these substrate molecules are rapidly being converted into sulfates, glucuronides, and methyl ethers (Biasutto et al. 2007;

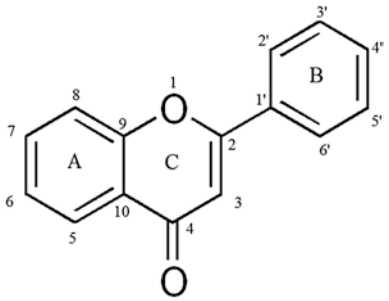

Fig. 1 Core structure of flavonols

Mattarei et al. 2010). The effect of *O*-methylation depends on the solubility of flavonoids (Mattarei et al. 2010). In vivo experiments induced that chemical modification of these molecules were to enhance the solubility effect while reduces the metabolic effects to provide the low bioavailability of polyphenols (Biasutto et al. 2007; Manach et al. 2005; Silberberg et al. 2006; Williamson and Manach 2005). These studies observed the overall survival of polyphenol OHs (Mattarei et al. 2010). As shown in Fig. 1, structurally rhamnetin is a monoethyl ether of quercetin while rhamnazin is identified as quercetin dimethyl ether (Martini et al. 2004). Therapeutic potential could be attributed to functional groups i.e. C-ring of 3′-hydroxyl and 4′-hydroxyl groups contribute towards redox activity (Metodiewa et al. 1999), while 3-OH act as inhibitor (Sarno et al. 2002). On the other hand, 7-OH and 5-OH are having weak and less acidic activity because of intramolecular hydrogen bonding to the 4-carbon of carbonyl compound (Van Dijk et al. 2000). This study reports that acetylated 3-OH bond has enzymatic activity and is used as protective chemical reaction of catecholic OHs (Mattarei et al. 2010). They also suggest that this mitochondrial-targeted compound has a free OH at the specific 3-position (Mattarei et al. 2010).

Quercetin

Quercetin is one of the major dietary flavonoids. It is present in various plant parts including fruits, vegetables, and beans. Although exact concentration of quercetin in food stuff is not known, it is estimated that it makes 50% of the total dietary flavonoids. Depending on various factors including plant varieties, growth conditions, processing, etc., quercetin content may vary, but onions are experimentally shown to have highest concentrations of quercetin, i.e., about 200–600 mg/kg.

Quercetin contains five hydroxyl residues which are responsible for its activity and possible derivatives. Quercetin has two main groups of derivatives, i.e., glycosides and ethers. Some quercetin derivatives also contain sulfate and prenyl substituents but they are less frequent (Williams and Grayer 2004).

Quercetin *O*-glycosides are widely distributed in plants. They may either contain one or two *O*-glycoside residues. The most common derivative is quercetin 3-*O*-glycosides which contain OH-group at C-3 carbon. The commonly found sugar residues in quercetin 3-*O*-glycoside derivatives include glucose, galactose, rhamnose, and xylose. Another derivative is quercetin 7-*O*-glucoside which contains glucose residue at the hydroxyl group of C-7 carbon (Chang and Wong 2004).

Quercetin ethers make the second major group of quercetin derivatives. They may contain up to five ether bonds along with other substituents such as sugar residues and alkyl groups. Quercetin molecules are lipophilic but become hydrophilic by the glycosidation of at least one OH-group (Materska 2008).

Biological Properties

Quercetin is an ubiquitous antioxidant and is shown beneficial in maintaining good health. Quercetin acts as anticancer agent by regulating cell cycle human breast cancer MCF-7 cells (Chou et al. 2010). Antiviral activity of quercetin has been reported against various viral trains along with other flavonoids (Cody et al. 1986). Pharmacologic effects of quercetin in various diseases including neurodegenerative disorders, cardiovascular diseases, inflammation, bacterial and fungal infections, and liver disorders have also been reported (Tanwar and Modgil 2012).

Morin

Morin (2′,3,4′,5,7-pentahydroxyflavone) is a yellow-colored naturally occurring substance in *Maclura tinctoria* (old fustic) and *Maclura pomifera* (Osage orange) wood and from *Psidium guajava* (common guava) leaves (Rattanachaikunsopon and Phumkhachorn 2007). By the circular dichroism spectrum, the change in both confirmations after the binding of morin with high affinity to site II (subdomain IIIA) of bovine serum albumin (BSA) has been observed (Hu et al. 2012).

Morin having comparatively high bimolecular rate constant (k_2) value for its interaction with the 1,4-dinitrobenzene (1,4-DNB) electrochemical system presents to its less intermolecular hydrogen bonding and more acidic nature (Arshad et al. 2012). Circular dichroism (CD) and UV-vis spectroscopy results showed that the binding of bovine serum albumin (BSA) to morin and other flavonol compounds induces some conformational changes in BSA (Shahabadi and Mohammadpour 2012).

Biological Properties

Morin is known to have the antihypertensive and antioxidant effects in deoxycorticosterone acetate (DOCA)-salt-induced hypertension in rats (Prahalathan et al. 2012a, b). Morin has found its role in the treatment of many diseases. A significant interaction of flavonoid drug or flavonoid xenobiotic has been observed during test regarding to b5 reductase inhibition that shows a promising role in therapeutic and toxicological outcomes for certain drugs and xenobiotic (Çelik and Koşar 2012; Çelik et al. 2013). In cases of colon cancer and hepatocellular carcinoma, 3,5,7,2′,4′-pentahydroxyflavone has been observed to possess chemopreventive potential in animal models. Antiproliferative and anticarcinogenic effects also have been determined against 7,12-dimethylbenz(*a*)-anthracene (DMBA)-induced experimental mammary carcinogenesis (Nandhakumar et al., 2012). In tumor cells, downregulation of STAT3-dependent hemosensitization and gene expression was led by the suppression of the signal transducer and activator of transcription 3 (STAT3) pathway after the application of morin (Gupta et al. 2012).

Morin inhibited the expression of matrix metalloproteinase-3 (MMP-3) and matrix metalloproteinase-13 (MMP-13), and it also has increased the expression of tissue inhibitors of metalloproteinase-1 (TIMP-1) in interleukin-1β (IL-1β) which induced rat chondrocytes (Chen et al. 2012a). Morin has decreased in asymmetric dimethylarginine (ADMA) level, while dimethylarginine dimethylaminohydrolase (DDAH) activity in the liver was significantly higher in rats (Merwid-Ląd et al. 2013). Morin is also been reported to be indirectly involved in insuline signalling and functionality (Paoli et al. 2013).

In vivo study in murine model, for osteoarthritis (OA) induced by anterior cruciate ligament transection (ACLT), the results clearly indicated suppression of cartilage degradation by orally administered morin. So morin has been observed to be used for the treatment of osteoarthritis (OA) as therapeutic agent (Chen et al. 2012a). Human inhibits the formation of amyloid by hydrate of morin (2′,3,4′,5,7-pentahyd roxyflavone). The polypeptide hormone islet amyloid polypeptide (IAPP, amylin) and disaggregates preformed IAPP amyloid fibers observed under right-angle light scattering and transmission electron microscopy (TEM) (Noor et al. 2012). Nitric oxide (NO) and prostaglandin E2 (PGE-2) production was inhibited by morin as well as the expression of inducible NO synthase (iNOS) and cyclooxygenase (COX-2) in interleukin-1-beta (IL-1β)-induced chondrocytes. Morin also suppressed the degradation of inhibitor of nuclear factor-κB (IκB-α) as well as the translocation of nuclear factor kappa B (NF-kappa B).

In rats, an IL-1β-induced osteoarthritis (OA) model, morin also exerted anti-inflammatory properties during in vivo study (Chen et al. 2012b). Morin exhibits antioxidant potential and offers enhancement in antioxidant levels simultaneously showing protection that clearly reduce in urea, ammonia, lipid peroxidation (Subash and Subramanian 2009). It has also been clarified that a lower concentration of morin in carcinomas than normal oral mucosa inhibited the activation of activated protein kinase AKT, whereas Jun N-terminal kinase (JNK), p38 kinase, and polyclonal antibodies (GADD45) all induced the same dose-response parallel curves in normal oral mucosa and carcinomas (Brown et al. 2003).

Myricetin

Myricetin (3,5,7-trihydroxy-2-(3,4,5-trihydroxyphenyl)chromen-4-one) (figure) is a major plant secondary metabolite; these are commonly found particularly in the whole plant kingdom and in majority of human foods, i.e., different fruits, berries, grapes, herbs, vegetables, and many other plants. A rich source of myricetin is walnuts; traces can be found as glycosides (Miean and Mohamed 2001b).

Myricetin is one of the phenolic compounds which are found in red wine (Maggiolini et al. 2005). It is found on the leaf surface of wild tomato (*Solanum habrochaites*) plants that contain 3,7,3′,5′-tetramethyl myricetin, 3,7,3′-trimethyl myricetin, and 3,7,3′,4′,5′-pentamethyl myricetin, with secreting glandular trichomes (gland types 1 and 4) containing abundantly than storage glandular tri-

chomes (type 6) and with the tetramethylated compound predominating in all types 1, 4, and 6 (Schmidt et al. 2011). Myricetin contains a three-ring structure with a central oxygenated heterocyclic and two aromatic centers (Gee and Johnson 2001) that serve as multiple functions like antioxidant activities and pigmentation (Hertog et al. 1994).

Biological Properties

Myricetin and other polyphenolic compounds are absorbed in human gut, remaining larger fraction in the lumen, thus the major proportion of mucosa from gastrointestinal. These compounds also show considerable biological effects at cellular level. These myricetin with some other phenolic compounds control the cellular cycles, apoptosis (programmed cell death), and differentiation after interaction with cellular signal pathways (Gee and Johnson 2001). A wide range of bioactivities have been reported for this molecule i.e. Allelochemic; Antioxidant; Antibacterial; Anti-feedant; Anti-HIV; Antihistaminic; Anti-gingivitic; Antiallergenic; Anti-gastric; Anti-gonadotrophic; COMP-Inhibitor; Antihistaminic; Antiseptic; Anti-inflammatory; Anti-mutagenic; Anti-periodontic; Antiplaque; Antiviral; Diuretic;Topoisomerase-I-Inhibitor; Hypoglycemic; Vasodilator; Cancer-Preventive; Mutagenic; Candidicide; Larvistat; Lipoxygenase-Inhibitor; Oxidase-Inhibitor; Quinone-Reductase-Inducer; Pesticide; Tyrosine-Kinase-Inhibitor; Topoisomerase-II-Inhibitor (http://www.ars-grin.gov/duke/, accessed).

Myricetin can cause muscle paralysis by inhibiting acetylcholine release at the neuromuscular junction. When compared to *Clostridium botulinum* neurotoxin (BoNT/A) the myricetin effect on muscle paralysis was unpretentious (Yang et al. 2011c). It is also claimed myricetin has antibiotic effects on *R. leguminosarum* bv *trifolii* (Fottrell et al. 1964). Myricetin is known to have DNA damage (strand breakdown and oxidized pyrimidines/purines) effect in human hepatocellular carcinoma (HepG2) cells which is induced by taking it as a diet to have a significant protective effect against *N*-nitrosopyrrolidine (NPYR)-, *N*-nitrosodimethylamine (NDMA)-, and benzo(*a*)pyrene (BaP)-induced DNA damage (Delgado et al. 2008). Myricetin and rosmarinic acid are inhibited by amyloid-β (Aβ) protein, and by site-specific binding, there were also observed aggregation and synaptic dysfunction (Ono et al. 2012).

As for the role of myricetin in cancer, myricetin and scutellarin are potently shown to inhibit the severe acute respiratory syndrome coronavirus (SARS-CoV) helicase protein (Yu et al. 2012). Myricetin has been found to protect neurons' discrete and multiple pathways and inhibited glutamate-induced excitotoxicity (Shimmyo et al. 2008). Myricetin was exerted as potent chemopreventive activity mainly by targeting activity of Fyn kinase straightly and afterward attenuated UVB-induced cyclooxygenase-2 (COX-2) expression (skin carcinogenesis) (Jung et al. 2008). Myricetin (20 μM) when treated with macrophages derived from U937 has been significantly observed to inhibit the expression of mRNA and surface protein CD36 cells, which means myricetin might play an important role in ameliorating

atherosclerosis (Lian et al. 2008). During the experimental result of Perls' iron staining, it has made an evidence in the substantial nigra by the enhancement of iron-staining cells; myricetin prevented the 6-hydroxydopamine (6-OHDA) (Ma et al. 2007b). It has also been observed that antiproliferative potential of flavonoids decreased in the order isorhamnetin > kaempferol > myricetin > rutin, while their antioxidant properties decreased in the order rutin > myricetin > kaempferol > isorhamnetin. When combined the treatment of isorhamnetin, kaempferol, and myricetin with AraC has led to synergism in their antiproliferative activities (Nadova et al. 2007).

However, the result of myricetin on pharmacokinetics of carvedilol has not been reported in vivo. The enhanced oral bioavailability of carvedilol may result from both inhibition of CYP2C9 or CYP2D6-mediated metabolism and P-gp-mediated efflux of carvedilol in the small intestine and/or in liver by myricetin rather than reducing renal elimination (Lee et al. 2012).

Natsudaidain

Natsudaidain (2-(3,4-dimethoxyphenyl)-3-hydroxy-5,6,7,8-tetramethoxychromen-4-one) was isolated from *Citrus reticulata* for the first time (Qian and Chen 1998). The name of the molecule comes from *Citrus natsudaidai* (Natsumikan, lit. "summer tangerine") (Matsui et al. 2009).

Biological Properties

Natsudaidain exhibited less inhibitory effect on the pro-matrix metalloproteinase-9 (proMMP-9)/in HT-1080 cells and progelatinase B production (Miyata et al. 2008). Natsudaidain has been shown to inhibit cyclooxygenase-2 and tumor necrosis factor-alpha production by p38 MAPK phosphorylation suppression, while there was no p65 NF-kappa B phosphorylation suppression observed, and that inflammatory diseases were also mitigated by natsudaidain (Matsui et al. 2009).

Two flavonoids, natsudaidain isolated and 3,5,6,7,8,3′,4′ heptamethoxyflavone (HEPTA) that are extracted from *Citrus* plants, in guinea pig papillary muscle produced a positive inotropic effect (PIE). It has also been observed (pD2 4.98 ± 0.07) that natsudaidain was more intense than (pD2 4.33 ± 0.08) HEPTA (Itoigawa et al. 1994). Hydroxyl C-3 and C-8 methoxyl groups were necessary for efficient activity of natsudaidain and other flavonols; on the other hand in B-ring ortho-catechol moiety and C-2, C-3 bonds were essential for the antiproliferative activity (Kawaii et al. 1999a). Natsudaidain when treated with HL 60 cells in dose-dependent manner has exerted its activity as to differentiate into repertoire of macrophage and monocytes (Kawaii et al. 1999b).

Drug Leads and Pharmacophores from Flavonols

Association of different bioactivities to flavonols has triggered computational studies to study and understand the mechanisms and interactions involved for obtaining new drug leads. Such studies involved molecular docking, three-dimensional structure-activity relationship, and pharmacophore modeling. Investigations have reported the interactions and pharmacophoric models for flavonols based on their interactions with cellular proteins. Using theoretical and computational approaches and the antioxidant activity of structure-activity relationship of flavonoids has been calculated (Butkovic et al. 2004; Ghiotto et al. 2004; Lee et al. 2009; Om and Kim 2008; Teixeira et al. 2005). A pharmacophore map based on flavonols suggested anti-angiogenic and thus antitumor drug leads as human vascular endothelial growth factor receptor 2 (hVEGFR2) antagonists (Yang et al. 2008). Therapeutic potential of flavonoids has been explained for genetic/metabolic disorders (i.e. xanthinuria, gout, and diabetes mellitus by inhibition of respective enzymes xanthine oxidase, aldose reductase, and liposygenase), but also for viral infections as well using computational approaches (Alves et al. 2001; Liu et al. 2012).

A study focused on inhibition of hVEGFR2 signaling for antitumor effects developed receptor-based pharmacophore model using crystal structures of inhibitor-hVEGFR2 complex and cyclin-dependent kinase 6 (CDK6) and flavonoid fisetin complex. Superimposition of these complexes helped in the identification of interactions between fisetin and hVEGFR2 and resulted in pharmacophore map. Hydrogen bond acceptors (HBAs), hydrogen bond donors (HBDs), and lipophilicity (Lipo) features were used to conclude the map. Resultant map had four features, two HBD, one Lipo, and one HBA. Virtual screening was performed, and the model yielded five out of nine hits with each hit flavonol having hydrogen bonding 3- and 4′-OH interacting with ATP binding site of hVEGFR2 (Yang et al. 2008).

A NS5B inhibitor pharmacophore model (Hypo 1) was developed using common feature-based pharmacophore and structure-based docking approaches for identification of novel antivirals for HCV. Discovery Studio's Common Feature pharmacophore generation protocol was used to develop the model with best conformational generation choice. The model was evaluated using decoy set of 1040 molecules of which 40, active against NS5B, were selected from the literature, while 1000 were randomly selected may bridge database. The model Hypo 1 was used to screen in-house database commercially available natural products with 3D structures and yielded 246 hits. These hits were investigated by docking studies and the list was reduced to 31. These results were validated in wet lab and showed inhibition of NS5B HCV enzyme (Liu et al. 2012).

Conclusions

Attaining the spotlight since the 2000s, flavonols have been rigorously investigated for exploring their roles in metabolism, as antioxidant and also as potential drug leads. Being natural products, these are considered as much safer than the other pharmaceutical products. Current studies are focusing on evaluating the effects of flavonols on human/mammalian cells for developing more effective therapeutic agents. Sophisticated and sensitive techniques have enabled us to mine out and exploit more and more information shedding lights on curing of diseases. Multiple studies conducted have revealed the possibilities of flavonoids as leading to improved drugs for most of the clinically difficult to treat diseases in future.

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Impact of Electron Beam Irradiation on the Nutritional Attributes of Seeds of Coastal Sand Dune Wild Legume *Canavalia cathartica*

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Introduction

According to the World Bank estimates, about 967 million of the global population are malnourished, and about 149.6 million children show retardation of growth due to inadequate supply of protein and nutrients (http://www.reliefweb.int/rw/rwb.nsf/db900SID/MCOI-7KGM87?OpenDocument). The undernourished populations mainly rely on monocarbohydrate diet like maize or rice without adequate supply of protein, fat, vitamin A, iodine, zinc, and iron (Boye et al. 2010). There is an immediate need to address the human malnutrition by adequate supply of inexpensive protein-energy alternative to expensive meat and animal products through edible legumes (e.g., peas, lentils, and beans) along with indigenous wild legumes (e.g., bay bean, cluster bean, velvet bean, and winged bean) (Singh et al. 2007; Boye et al. 2010). The edible legumes are of immense value and potential for blending with monocarbohydrate diets to meet the protein-energy requirement. Legume seeds are 2–3-fold rich in proteins than the cereals (NAS 1979), encompass considerable quantity of carbohydrates as energy reservoir, and play a vital role in the food formulations.

Intense search for novel underutilized wild legumes continued to exploit them as nutraceutical source in India (Vadivel and Janardhanan 2000; Siddhuraju and Becker 2001; Thangadurai et al. 2001, 2006; Siddhuraju et al. 2002a, b; Murthy et al. 2003; Seena and Sridhar 2006; Sridhar and Seena 2006; Vijayakumari et al. 2007; Bhat et al. 2008a, b). Among the wild legumes of the coastal habitats of southwest India, *Canavalia cathartica* Thouars is widely distributed especially in the coastal sand dunes and mangroves. This legume has valuable qualities like fast growth, production of large quantity of seeds, tolerance to adverse conditions, and resistance to pests

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(Seena and Sridhar 2006). Seeds of *C. cathartica* possess low fat, high protein, high colorific value, essential minerals, and essential fatty acids. These seeds are also endowed with some antinutritional principles (e.g., phenolics, canavanine, and concanavalin), which limit their use in human diets. Various methods of seed processing (e.g., cooking, roasting, and sprouting) have been tried to eliminate or reduce the antinutritional principles to desired levels (Bhat et al. 2008b; D'Cunha et al. 2009a, b; D'Cunha and Sridhar 2010). Gamma and electron irradiations have improved the nutritional quality of the seeds of *Mucuna*, *Sesbania*, and *Vigna* up to some extent (Siddhuraju et al. 2002b; Bhat et al. 2008b, 2009); hence, the present study explores the possibilities of improvement of nutritional qualities of seeds of *C. cathartica* of the coastal sand dunes of the southwest coast of India through electron beam irradiation.

Seeds and Processing

Canavalia cathartica in coastal sand dunes of southwest coast of India as a dominant legume spreads horizontally and serves as a good sand binder (Fig. 1). During summer, leaves become senescent (yellow) and consist of ripened (light-yellow) and dry (dark-brown) pods. The color of seeds includes light brown to dark brown, and some light-brown seeds possess longitudinal striations. Dry seeds are large and subcylindric and possess long hilum with 71% cotyledon and 29% seed coat (Table 1).

The dry pods of *C. cathartica* were harvested during summer months (February–April) from the coastal sand dunes of Someshwara, southwest coast of India (12°47′ N, 74°52′ E) (Fig. 1). Healthy seeds were separated in the laboratory and sun-dried for 2–3 days, and dimensions (calipers) and weight (gravimetric) of seeds were determined. Seeds (about 15–20 g) were packed in Ziploc polyethylene bags (6 × 6 cm) and were evenly exposed to EB irradiation (2.5, 5.0, 10, and 15 kGy) at room temperature (25 ± 2 °C) from the Microtron source (Microtron Centre, Mangalore University). The conditions of the Microtron accelerator were dose rate, 2 kGy/min; beam energy, 8 MeV (MeV is the unit of energy, 10^6 or 1000 keV; 1 eV is the energy received by an electron when it crosses a potential difference of 1 V); beam current,

Table 1 Seed characteristic features of *Canavalia cathartica* ($n = 25$; mean ± SD)

	Mean	Range
Length (cm)	1.71 ± 0.08	1.56–1.87
Width (cm)	1.09 ± 0.06	0.95–1.18
Thickness (cm)	0.88 ± 0.06	0.78–0.97
L/*B* ratio	1.58 ± 0.08	1.45–1.77
Hilum length (cm)	0.98 ± 0.05	0.90–1.07
Dry weight/seed (g)	0.73 ± 0.09	0.55–0.92
Dry weight of cotyledons (g)	0.52 ± 0.07	0.36–0.67
Dry weight of seed coat (g)	0.21 ± 0.02	0.17–0.24

Fig. 1 Horizontal spread of *Canavalia cathartica* on the coastal sand dune of Someshwara (Mangalore, Southwest coast of India) showing inflorescence and tender pods (**a**), dry pods (**b**), light-brown seeds (**c**), dark-brown seeds (**d**), and light-brown seeds with striations (**e**)

30 mA; and distance between the sample and the beam port, 30 cm (Table 2) (Siddappa et al. 1998). The absorbed dose was measured employing a current integrator calibrated with chemical dosimeters (Gupta et al. 1999). Seed samples packed in polythene bags without irradiation served as control (0 kGy). Cotyledons of control and irradiated seeds were separated from seed coat, powdered (Wiley Mill, mesh # 30), and preserved in airtight glass containers.

Table 2 Salient features of Microtron accelerator used to expose *Canavalia cathartica* seeds

	Details
Beam energy	8 MeV
Beam current	30 mA (max)
Dose rate	2 kGy/min
Distance between sample and the beam port	30 cm
Number of electron orbits	14
Beam size	3 mm × 5 mm
Pulse duration	2.5 μs
Pulse repetition rate	250 Hz (max)
Average beam power	375 W (max)
Magnetic field strength	1927.5 G
Magnetron power	2 MW
Operation frequency	2998 MHz

Nutritional Assessment

Proximal Analysis

The moisture content of the seed flour was estimated gravimetrically by drying at 100 °C in an oven (Scientronic SBIM-25; New Delhi, India) until a constant weight is attained. It was expressed in percentage based on the difference between initial and final weights of the flours. The total nitrogen and crude protein (N × 6.25) of the flours were evaluated by micro-Kjeldahl method (Humphries 1956). Total lipid extraction of the seed flours (1 g; moisture, <10%: 5.5–7%) was carried out in thimbles covered with glass wool in a Soxhlet extractor, and the lipid was extracted using 200 mL of petroleum ether at 60–80 °C (AOAC 1995). The condensation rate was fixed (150 drops/min for 7 h); the samples were allowed to cool, transferred to pre-weighed beaker, and evaporated to dryness at room temperature (28 ± 2 °C); and the lipid content was gravimetrically estimated. The crude fiber and ash contents were also determined gravimetrically following the AOAC (1995) methods.

The carbohydrates were calculated based on Müller and Tobin (1980):

$$\text{Carbohydrates } (\%) = \left[100 - \left(\text{Crude protein } (\%) + \text{Crude lipid } (\%) + \text{Crude fibre } (\%) + \text{Ash } (\%)\right)\right]$$

The gross energy was calculated using the formula by Ekanayake et al. (1999):

$$\text{Gross energy } \left(\text{kJ}/100\ \text{g}\right) = \left[\left(\text{Protein} \times 16.7\right) + \left(\text{Lipid} \times 37.7\right) + \left(\text{Carbohydrates} \times 16.7\right)\right]$$

Mineral Analysis

The mineral contents (sodium, potassium, calcium, magnesium, iron, copper, zinc, manganese, and selenium) of the seed flours were determined by atomic absorption spectrophotometry (GBC 932AA; Australia) by digesting in a mixture of concentrated nitric acid, sulfuric acid, and perchloric acid (10:0.5:2 v/v) (AOAC 1995). The vanadomolybdophosphoric acid method was employed to determine the total phosphorus by measuring the absorbance at 420 nm using KH_2PO_4 as standard (AOAC 1995). The ratios of Na/K and Ca/P were calculated.

Protein Fractions

Protein fractions were extracted as described by Gheyasuddin et al. (1970). One gram of the seed flour in 10 mL of chilled acetone (–20 °C) was ground in pre-chilled pestle and mortar. The seed protein was extracted at room temperature for 1 h using the acetone, flour to the solvent ratio of 1:10 (w/v) in a magnetic stirrer with the solvents: distilled water to extract albumins, 4% (w/v) NaCl solution for globulins, 60% isopropanol for prolamines, and 0.4% (w/v) NaOH solution for glutelins. After each extraction the slurry was centrifuged (4000 rpm, 10 min), and the residue obtained was further subjected to extraction. The soluble nitrogen content of each extract was estimated by micro-Kjeldahl method (Humphries 1956). The ratio of albumin-globulin was estimated.

To estimate nonprotein nitrogen, the sample (100 mg) was extracted using 10 mL of 10% ice-cold trichloroacetic acid (10%, 10 mL) (Merck) to precipitate protein (Sadasivam and Manickam 1992). The supernatant was collected, the precipitate centrifuged, and the process repeated. The volume of the pooled supernatant was made up to 25 mL with TCA (10%). Micro-Kjeldahl method (Humphries 1956) was employed to estimate the nitrogen in the supernatant.

Amino Acid Analysis

The amino acids of seed flours were assessed by the method outlined by Hofmann et al. (1997, 2003). A known quantity of seed flours was hydrolyzed with HCl (6 N, 15 mL) for 4 h at 145 °C. On cooling, HCl was eliminated using a rotoevaporator (Büchi Laboratoriumstechnik AG RE121; Switzerland) combined with a diaphragm vacuum pump (MC2C; Vacuubrand GmbH, Germany). The internal standard, trans-4-(Aminomethyl)-cyclohexanecarboxylic acid (Aldrich, purity, 97%) was added to each sample. The derivatization consisted of esterification with trifluoroacetylation (Brand et al. 1994).

The samples of standard amino acids were weighed in reaction vials and dried using CH_2Cl_2 under a gentle stream of helium with slow heating in an oil bath (40–60 °C) to remove any water traces. A 12 mL of fresh acidified isopropanol (acetyl chloride, 3 mL + 2-propanol, 12 mL) was added, and the mixture was heated at 100 °C for 1 h. After cooling, the reagent was removed by a gentle stream of helium at 60 °C. To remove propanol and water, evaporation with three successive aliquots of CH_2Cl_2 was followed. The dry residue was trifluoroacetylated with 200 mL trifluoroacetic anhydride overnight at room temperature. An aliquot of this solution was used without treatment for gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS).

The measurements of GC-C-IRMS were carried out using a Hewlett-Packard 58590 II gas chromatograph, connected via a split with a combustion interface to the IRMS system (GC-C-II to MAT 252, Finnigan MAT; Germany) for the isotopic determination of nitrogen and via a transfer line with a mass spectrometer (GCQ, Finnigan MAT; Germany) for qualitative and quantitative analysis of the amino acids. The capillary column of GC was a 50 m × 0.32 mm i.d. × 0.5 μm BPX5 (SGE), operating with the carrier gas flow of 1.5 mL/min with following temperature and pressure: initial 50 °C (1 min), increased to 100 °C at 10 °C/min (10 min), increased to 175 °C at 3 °C/min (10 min), and increased to 250 °C/min (10 min); head pressure, 13 psi (90 kpa).

The ratio of essential amino acids (EAA)-total amino acids (TAA) was calculated:

$$\text{EAA / TAA ratio } (\%) = \left[\left(\text{Total EAA / TAA}\right) \times 100\right]$$

Protein Digestibility, EAA Score, PDCAAS, and PER

The in vitro protein digestibility (IVPD) was estimated according to Akeson and Stahmann (1964). Subsamples of defatted flours (100 mg each) were incubated (37 °C, 3 h) with pepsin (Sigma, 3165 units/mg protein) (1.5 mg/2.5 mL 0.1 N HCl) followed by inactivation (0.25 mL 1 N NaOH). Further incubation was continued (24 h, 37 °C) with trypsin (Sigma, 16,100 units/mg protein) and α-chymotrypsin (Sigma, 76 units/mg protein) (2 mg each/2.5 mL potassium phosphate buffer, pH 8.0, 0.1 M) followed by inactivation (0.7 mL 100% TCA). The zero-time control was maintained by inactivating the enzyme before the addition of substrate. After centrifuging the inactivated mixture, the supernatant was collected, and the residue was washed (2 mL 10% TCA) and centrifuged. The combined supernatant was extracted with 10 mL diethyl ether twice, and ether layer was removed by aspiration. The aqueous layer was kept in boiling water bath (15 min) to remove traces of ether. After attaining room temperature, the solution was made up to 25 mL with distilled water. Nitrogen (in 5 mL aliquots) content was determined by micro-Kjeldahl method (Humphries 1956) to estimate protein in the digest. The in vitro protein digestibility was expressed in percentage:

$$\text{IVPD } (\%) = \left(\text{Protein in digest} \div \text{Protein in defatted flour}\right) \times 100$$

The essential amino acid (EAA) score was calculated:

$$\text{EAA score} = \left[\left(\text{mg of EAA in 100 mg test protein} / \text{mg of EAA in 100 mg reference FAO WHO pattern}\right) \times 100\right]$$

The protein digestibility-corrected amino acid score (PDCAAS) of EAA requirement for adults (FAO-WHO 1991) was estimated:

$$\text{PDCAAS} = \left[\left(\text{EAA in food protein} / \text{EAA reference pattern of FAO WHO}\right) \times \text{IVPD } (\%)\right]$$

The protein efficiency ratio (PER) was calculated from the amino acid composition of seed flours based on Alsmeyer et al. (1974):

$$\text{PER}_1 = \left[-0.684 + 0.456 \times \text{Leu} - 0.047 \times \text{Pro}\right]$$
$$\text{PER}_2 = \left[-0.468 + 0.454 \times \text{Leu} - 0.105 \times \text{Tyr}\right]$$
$$\text{PER}_3 = \left[-1.816 + 0.435 \times \text{Met} + 0.78 \times \text{Leu} + 0.211 \times \text{His} - 0.944 \times \text{Tyr}\right]$$

Data Analysis

One-way ANOVA (ORIGIN Pro 8.1) was employed to determine the variation between the control (0 kGy) vs. irradiated (2.5, 5, 10, 15 kGy) seed flours of each parameter assessed.

Nutritional Qualities

Proximal Features

Moisture content and crude lipid of unirradiated seeds showed significant dose-dependent decrease in irradiated seeds (Table 3). The crude protein of irradiated seeds although slightly decreased was not significant, while crude fiber significantly decreased at 2 kGy ($p < 0.05$), 10 kGy ($p < 0.01$), and 15 kGy ($p < 0.05$). The crude lipid decreased significantly on irradiation. The ash content of irradiated seeds slightly increased without significant difference. The carbohydrate content of unirradiated seeds showed dose-dependent significant increase on irradiation, while the calorific value significantly decreased at 5 and 15 kGy ($p < 0.05$).

Table 3 Proximate composition of unirradiated and irradiated seeds of *Canavalia cathartica* on dry weight basis ($n = 5$; mean ± SD)

		Irradiated (kGy)			
	Unirradiated	2.5	5.0	10.0	15.0
Moisture (%)	7.69 ± 0.03	6.16 ± 0.26**	5.96 ± 0.26**	5.86 ± 0.31**	5.86 ± 0.72**
Crude protein (g/100 g)	29.48 ± 0.51	28.9 ± 0.88	28.02 ± 1.75	28.31 ± 0.51	25.1 ± 0.51
Crude lipid (g/100 g)	5.9 ± 0.82	3.88 ± 0.71*	3.09 ± 0.29*	3.52 ± 0.23*	3.01 ± 0.45*
Crude fiber (g/100 g)	2.12 ± 0.23	1.22 ± 0.13*	1.35 ± 0.34	0.99 ± 0.29**	1.26 ± 0.30*
Ash (g/100 g)	3.30 ± 0.20	3.35 ± 0.05	3.35 ± 0.05	3.40 ± 0.21	3.23 ± 0.06
Carbohydrates (g/100 g)	60.54 ± 2.71	64.34 ± 1.2*	63.31 ± 1.94**	65.11 ± 0.55**	65.44 ± 0.67***
Calorific value (kJ/100 g)	1715 ± 22	1675 ± 16	1657 ± 11*	1671 ± 12	1658 ± 13*

Asterisks across the columns between unirradiated and irradiated seeds are significantly different (one-way ANOVA: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$)

Minerals

Sodium, potassium, calcium, zinc, and manganese did not show significant difference between unirradiated and irradiated seeds (Table 4). Phosphorus showed dose-dependent significant decrease up to 10 kGy ($p < 0.001$) but significantly increased at 15 kGy ($p < 0.001$). Magnesium showed significant increase only at 5 kGy ($p < 0.05$), while iron in 2.5 kGy ($p < 0.05$). Copper significantly increased at 5 and 10 kGy ($p < 0.001$), while selenium decreased at 2.5, 5, and 15 kGy ($p < 0.001$). Potassium (1039–1171 vs. 500–700 mg/100 g), magnesium (94–131.5 vs. 60 mg/100 g), iron (11.4–16.6 vs. 10 mg/100 g), copper (0.7–0.89 vs. 0.6–0.7 mg/100 g), and manganese (0.9–1.4 vs. 0.3–1 mg/100 g) were higher than NRC-NAS standard for infants. Selenium was higher than the required limits proposed by Pennington and Young (1990) (0.48–0.89 vs. 0.05–0.2 mg/100 g). The Na/K ratio was decreased in all irradiated doses except for 15 kGy, while Ca/P ratio was elevated in all doses.

Protein Fractions

The true protein of unirradiated seeds was significantly increased only at 5 kGy (16.8 vs. 23.6 g/100 g; $p < 0.001$) (Table 5). Albumin in unirradiated seeds showed dose-dependent significant decrease (7.3 vs. 1.04–3.6 g/100 g), while globulin significantly decreased at 5 kGy (4.8 vs. 1.2 g/100 g, $p < 0.001$) and elevated at 15 kGy (4.8 vs. 9.2 g/100 g, $p < 0.001$). Prolamin and glutelin were significantly raised in

Table 4 Mineral composition of unirradiated and irradiated seeds of *Canavalia cathartica* (mg/100 g dry mass) ($n = 5$; mean ± SD)

Mineral	Unirradiated	Irradiated (kGy)				NRC-NAS pattern[a]; Pennington and Young[b]
		2.5	5.0	10.0	15.0	
Sodium	34.00 ± 0.25	26.00 ± 0.27	25.60 ± 0.62	25.23 ± 0.88	26.22 ± 0.25	120–200[a]
Potassium	1124 ± 0.92	1125 ± 0.14	1087 ± 2.5	1171 ± 0.39	1039 ± 1.02	500–700[a]
Calcium	76.39 ± 0.94	84.10 ± 1.05	70.76 ± 0.42	87.09 ± 0.08	95.65 ± 0.37	600[a]
Phosphorus	206.15 ± 0.49	183.91 ± 0.27**	179.66 ± 0.07**	178.97 ± 0.26**	198.15 ± 0.07**	500[a]
Magnesium	93.83 ± 0.07	105.32 ± 0.03	131.52 ± 0.02*	127.25 ± 0.06	116.08 ± 0.03	60[a]
Iron	16.64 ± 0.02	11.36 ± 0.03*	13.23 ± 0.02	15.39 ± 0.04	12.37 ± 0.03	10[a]
Copper	0.74 ± 0.01	0.70 ± 0.01	0.89 ± 0.02**	0.86 ± 0.01**	0.77 ± 0.03	0.6–0.7[a]
Zinc	2.50 ± 0.01	2.28 ± 0.04	2.92 ± 0.07	2.98 ± 0.02	2.74 ± 0.05	5.0[a]
Manganese	0.95 ± 0.04	0.90 ± 0.05	1.13 ± 0.01	1.38 ± 0.04	1.22 ± 0.04	0.3–1.0[a]
Selenium	0.86 ± 0.01	0.48 ± 0.01**	0.59 ± 0.01**	0.89 ± 0.01	0.59 ± 0.01**	0.05–0.2[b]
Na/K ratio	0.03	0.02	0.02	0.02	0.03	–
Ca/P ratio	0.37	0.46	0.39	0.49	0.48	–

Asterisks across the columns between unirradiated and irradiated seeds are significantly different (one-way ANOVA: $*p < 0.05$, $**p < 0.001$)

[a]NRC-NAS (1989) recommended pattern for infants

[b]Pennington and Young (1990)

all doses of irradiation except for glutelin at 15 kGy. The nonprotein nitrogen was significantly increased at 2.5 kGy ($p < 0.001$), while at 5, 10, and 15 kGy, it was significantly decreased ($p < 0.001$). The albumin/globulin ratio decreased in all doses (1.53 vs. 0.11–0.7) except for 5 kGy (2.26).

Amino Acids

Glutamic acid was highest followed by aspartic acid in irradiated seeds, while tryptophan was absent (Table 6). Irradiation did not significantly change the concentration of amino acids with a few exceptions. Aspartic acid and proline were significantly decreased at 2.5 and 5 kGy; methionine and lysine were significantly decreased at 2.5 kGy. The EAA/TAA ratio was increased in irradiated seeds (0.41 vs. 0.44–0.45).

Table 5 True protein, protein fractions, and nonprotein nitrogen of unirradiated and irradiated seeds of *Canavalia cathartica* (g/100 g) ($n = 5$; mean ± SD)

		Irradiated (kGy)			
	Unirradiated	2.5	5.0	10.0	15.0
True protein	16.82 ± 1.82	17.50 ± 0.75	23.61 ± 0.19***	18.24 ± 1.02	17.25 ± 0.14
Albumin	7.30 ± 1.53	3.60 ± 0.70**	2.64 ± 0.24***	2.24 ± 0.01***	1.04 ± 0.43***
Globulin	4.76 ± 0.64	5.15 ± 0.72	1.17 ± 0.24***	3.35 ± 0.01*	9.15 ± 0.29***
Prolamin	1.30 ± 0.01	3.10 ± 0.01*	7.48 ± 0.32**	4.09 ± 0.56*	2.35 ± 0.01*
Glutelin	3.46 ± 0.67	5.66 ± 0.73*	12.32 ± 0.25**	8.56 ± 0.46*	4.71 ± 0.01
Nonprotein nitrogen	12.56 ± 0.44	14.95 ± 0.44***	4.40 ± 0.01***	10.04 ± 0.72***	7.84 ± 0.01***
Albumin/globulin ratio	1.53	0.70	2.26	0.67	0.11

Asterisks across the columns between unirradiated and irradiated seeds are significantly different (one-way ANOVA: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$)

Among EAA, threonine was comparable or higher than soybean, wheat, and FAO-WHO pattern, while valine was higher or comparable to wheat and FAO-WHO pattern. Cystine and methionine of unirradiated seeds were comparable to soybean, wheat, and FAO-WHO pattern. Isoleucine in unirradiated and irradiated seeds was higher than FAO-WHO pattern, while tyrosine was higher than soybean and comparable to wheat. The lysine in unirradiated and irradiated seeds was higher than soybean, wheat, and FAO-WHO pattern, while histidine was comparable or higher than wheat and FAO-WHO pattern.

IVPD, EAA, PDCAAS, and PER

The IVPD was substantially high in unirradiated seeds, which did not change significantly on irradiation at 2.5 and 5 kGy (Table 7). The EAA score of unirradiated and irradiated seeds (2.5 and 5 kGy) was high in threonine, valine, isoleucine, lysine, and histidine. The EAA of threonine showed dose-dependent increase. The PDCAAS of threonine, valine, isoleucine, leucine, and histidine were higher in 5 kGy irradiated than unirradiated and 2.5 kGy irradiated seeds. The PER_1 and PER_2 were elevated in seeds irradiated with 5 kGy.

Table 6 Amino acid comparison of unirradiated and irradiated seeds of *Canavalia cathartica* in comparison with other food sources (mg/100 mg protein; $n = 5$, mean ± SD)

Amino acid	Unirradiated	Irradiated (kGy)		Soybean[a]	Wheat[b]	FAO-WHO[c]
		2.5	5.0			
Glutamic acid	13.06 ± 0.27	10.23 ± 0.3	10.95 ± 0.69	16.90	35.5–36.9	
Aspartic acid	14.28 ± 1.15	8.81 ± 0.27**	10.02 ± 0.63**	11.30	3.7–4.2	
Serine	3.89 ± 0.45	4.28 ± 0.13	4.49 ± 0.28	5.67	3.7–4.8	
Threonine	3.71 ± 0.13	3.83 ± 0.12	3.94 ± 0.25	3.76	2.2–3	3.4
Proline	3.10 ± 0.14	2.28 ± 0.07**	2.74 ± 0.17*	4.86	11.4–11.7	
Alanine	4.09 ± 0.09	4.11 ± 0.13	4.21 ± 0.26	4.23	2.8–3	
Glycine	3.38 ± 0.20	3.35 ± 0.10	3.45 ± 0.22	4.01	3.2–3.5	
Valine	3.96 ± 0.19	3.8 ± 0.12	4.00 ± 0.25	4.59	3.7–4.5	3.5
Cystine	1.83 ± 0.35	0.63 ± 0.02	0.62 ± 0.04	1.70	1.6–2.6	2.5[d]
Methionine	1.07 ± 0.15	0.70 ± 0.02*	0.84 ± 0.05	1.22	0.9–1.5	
Isoleucine	3.29 ± 0.17	3.05 ± 0.09	3.22 ± 0.20	4.62	3.4–4.1	2.8
Leucine	6.00 ± 0.14	5.68 ± 0.17	6.25 ± 0.39	7.72	6.5–7.2	6.6
Tyrosine	2.32 ± 0.29	2.50 ± 0.08	2.5 ± 0.16	1.24	1.8–3.2	6.3[e]
Phenylalanine	3.52 ± 0.15	3.44 ± 0.10	3.62 ± 0.23	4.84	4.5–4.9	
Tryptophan	ND	ND	ND	3.39	0.7–1	1.1
Lysine	6.43 ± 0.17	4.97 ± 0.15*	5.13 ± 0.32	6.08	1.8–2.4	5.8
Histidine	2.33 ± 0.06	2.39 ± 0.07	2.39 ± 0.15	2.50	1.9–2.6	1.9
Arginine	4.10 ± 0.20	3.86 ± 0.12	4.34 ± 0.27	7.13	3.1–3.8	
EAA/TAA ratio[f]	0.406	0.447	0.439			

Asterisks across the columns between unirradiated and irradiated seeds are significantly different (one-way ANOVA: *$p < 0.05$, **$p < 0.001$)
ND not detectable
[a]Bau et al. (1994)
[b]USDA (1999)
[c]FAO-WHO (1991)
[d]Cystine + methionine
[e]Tyrosine + phenylalanine
[f]Essential amino acid/total amino acid

Discussion

Seeds and Proximal Features

Seeds of *C. cathartica* are sufficiently large, heavy, and dormant and develop seed bank as they withstand extreme salinity, pH, and sand burial on the coastal sand dunes of southwest coast of India (Arun et al. 2001). The dose-dependent significant loss of moisture in *C. cathartica* seeds on irradiation supported the observation by Warchalewski et al. (1998). The crude protein did not show any significant variation

Table 7 In vitro protein digestibility (IVPD) (n = 5; mean ± SD), essential amino acid (EAA) score, protein digestibility corrected to amino acid score (PDCAAS) protein efficiency ratio (PER) of unirradiated and irradiated seeds of *Canavalia cathartica*

		Irradiated (kGy)	
	Unirradiated	2.5	5.0
IVPD (%)	67.41 ± 9.74	57.01 ± 8.40	73.46 ± 5.79
EAA Score			
Threonine	109.18	112.66	115.89
Valine	113.14	109.36	114.26
Cystine + methionine	116.00	53.25	58.28
Isoleucine	117.36	109.08	114.89
Leucine	90.97	86.03	94.62
Tyrosine + phenylalanine	92.70	39.62	40.95
Tryptophan	0	0	0
Lysine	110.86	84.47	88.45
Histidine	122.42	126.0	125.97
PDCAAS			
Threonine	73.60	64.23	85.13
Valine	76.27	62.35	83.94
Cystine + methionine	78.20	30.36	42.81
Isoleucine	79.11	62.18	84.40
Leucine	61.32	49.05	69.51
Tyrosine + phenylalanine	62.49	22.59	30.08
Tryptophan	0	0	0
Lysine	74.73	48.16	64.97
Histidine	82.52	71.84	92.54
PER			
PER_1	1.91	1.80	2.05
PER_2	2.01	1.85	2.10
PER_3	1.64	1.07	1.49

on irradiation of seeds of *C. cathartica*; however, linear dose-dependent increase in crude protein was seen on EB-irradiated *Mucuna pruriens* seeds (Bhat and Sridhar 2007; Bhat et al. 2008a) and in gamma-irradiated cowpea seeds (Dario and Salgado 1994; Bhat and Sridhar 2007). According to Taghinejad et al. (2010), EB irradiation had no effect on the protein composition of the canola meal even at higher doses (15, 30, and 45 kGy) of irradiation. The dose-dependent significant decrease in crude lipid in *C. cathartica* seeds corroborates the earlier study of *Mucuna* seeds (Bhat et al. 2008a; Bhat and Sridhar 2007). The quantity of crude lipid extracted at 2.5 kGy was higher than many wild legumes (3.9 vs. 1.2–1.9 g/100 g) (Viano et al. 1995). The crude fiber significantly decreased especially at 2.5, 10, and 15 kGy, and such decrease was also evident in *Mucuna* seeds (Bhat et al. 2008b; Bhat and Sridhar 2007). The decrease in fiber was predicted due to the depolymerization and delignification (Sandev and Karaivanov 1977; Campbell et al. 1987). Reduction of fiber

by EB irradiation is nutritionally advantageous as they trap less protein and carbohydrates (Balogun and Fetuga 1986). The ash content of *C. cathartica* seeds did not alter significantly, and it reflects in minimum loss of minerals on EB irradiation. However, EB irradiation of *Mucuna* seeds resulted in significant loss of ash content (Bhat and Sridhar 2007). The carbohydrates showed almost significant dose-dependent elevation between irradiated seeds of *C. cathartica* as seen in *Mucuna* seeds (Bhat et al. 2007, 2008b). Usually carbohydrates exist in a combined state with other molecules, and the EB irradiation facilitated the breakdown of such compounds into simple free sugars. The carbohydrate content of *C. cathartica* is higher than *Mucuna* seeds (Siddhuraju et al. 2000; Adebowale et al. 2005; Bhat et al. 2008b). The calorific value of *C. cathartica* seeds is higher than cultivated pulses (Kuzayali et al. 1966), which decreased on EB irradiation as seen in *Mucuna* seeds (Bhat and Sridhar 2007; Bhat et al. 2008b).

Mineral Profile

Legumes are generally rich in potassium, magnesium, iron, zinc, and calcium (Salunkhe et al. 1985). However, seeds of *C. cathartica* showed higher potassium, magnesium, and iron content than NRC-NAS (NRC-NAS 1989) recommended pattern for infants. Potassium, magnesium, iron, zinc, and calcium were not significantly differed on irradiation of *C. cathartica* seeds (except for iron at 2.5 kGy, 16.6 vs. 11.4 mg/100 g). Selenium showed significant decrease in all doses except for 10 kGy and is higher than the recommended dose by Pennington and Young (1990). Iron, selenium, zinc, and manganese are well-known antioxidants (Talwar et al. 1989), and they also are involved in strengthening the immune system. Selenium serves as an antioxidant in protecting cells against free radicals (Combs and Gray 1998). Magnesium, zinc, and selenium are also known to prevent cardiomyopathy, muscle degeneration, immunological dysfunction, and congenital malformations (Chaturvedi et al. 2004). By and large, the EB irradiation of *C. cathartica* did not significantly influence the overall composition of minerals. Interestingly minerals were not drastically altered by EB irradiation of *Mucuna* seeds (Bhat et al. 2008b). At the recommended dose of radiation (10 kGy), only phosphorus significantly elevated and copper significantly decreased without significant effects on other minerals of *C. cathartica* seeds. This result corroborates with studies on EB irradiation of *Mucuna* seeds (Bhat et al. 2008b). The ratio of Na/K lies in favorable range (0.02–0.03) in unirradiated as well as irradiated seeds of *C. cathartica* as the low ratio (<1) is known to control high blood pressure (Yusuf et al. 2007). The Ca/P ratio was lower than desired (0.37–0.49 vs. >1), and low Ca/P ratio (<1) will not prevent the loss of calcium in urine and restore calcium in bones (Shills and Young 1988). However, in irradiated seeds there is an increasing trend of Ca/P ratio (0.37 vs. 0.39–0.49) as phosphorus significantly decreased on irradiation.

Protein Fractions

Although crude protein did not elevate significantly at 5 kGy, the true protein significantly elevated (16.8% vs. 23.6%). Albumin showed dose-dependent decrease and resulted in the loss of sulfur amino acids. The globulin stooped to the lowest level at 5 kGy and elevated to the highest at 15 kGy but did not result in decrease of hemagglutinin activity. The albumin/globulin ratio was elevated at 5 kGy (1.53 vs. 2.26) and seems to be a desirable dose for improvement of seed nutritional quality. The nonprotein nitrogen decreased significantly in all doses of irradiation with elevation of prolamin and glutelin.

Amino Acid Profile

Except for a few amino acids, almost all amino acids were not altered by EB irradiation. Aspartic acid, proline, methionine, and lysine were significantly decreased on irradiation. The major amino acid, aspartic acid, showed a dose-dependent decrease as seen in *Mucuna* seeds (Bhat et al. 2008b). Quantities of many EAA are comparable to soybean (Bau et al. 1994), wheat (USDA 1999), and FAO-WHO (1991) standard. Legumes are known to possess high lysine and are limiting in sulfur amino acids (Norton et al. 1985; Jansman 1996). In the seeds of *C. cathartica*, lysine in unirradiated seeds was higher than FAO-WHO (1991) pattern as well as wheat (USDA 1999) but showed significant loss at 2.5 kGy. Sulfur amino acids of legumes are susceptible for irradiation (Khattak and Kloppenstein 1989) due to radiation-induced damage of sulfhydryl and disulfide groups in proteins (Lee 1962). Although cystine + methionine of unirradiated seeds are comparable to soybean, wheat, and FAO-WHO pattern, EB irradiation decreased methionine significantly (2.5 kGy, 1.1 vs. 0.7).

IVPD, EAA Score, PDCAAS, and PER

According to Saunders et al. (1973), IVPD is comparable to in vivo protein digestibility in the rat model. The IVPD of unirradiated seeds of *C. cathartica* (67.41 ± 9.74) was not significantly altered on irradiation at 2.5 kGy (57.01 ± 8.40), 5 kGy (73.46 ± 5.79), and 10 kGy (56 ± 5.52), but it was significantly decreased at 15 kGy (46.67 ± 5.77; $p = 0.041$). Decrease in the IVPD may be possible due to the formation of cross-links and aggregates by the unfolded proteins which are less susceptible to enzyme hydrolysis (Cho et al. 1999). This clearly shows that the recommended dose 10 kGy will not change the protein digestibility drastically. Interestingly, the IVPD of unirradiated *Mucuna* seeds showed significant EB irradiation

dose-dependent increase up to 15 kGy; however it reduced significantly at 30 kGy as seen in *C. cathartica* seeds at 15 kGy. Such increase in IVPD has also been reported in some cereals and legumes (Nene et al. 1975; Reddy et al. 1979). According to Abu-Tarboush (1998), increase in IVPD by irradiation is due to the degradation of proteins into small peptides and their susceptibility to enzyme attack. Decrease in protein digestibility at high doses of irradiation was attributed to increased total phenolics in *Mucuna* seeds as phenolics inactivate by binding to digestive enzymes (Bhat et al. 2008b).

The EAA score was adequate in six and four EAA of unirradiated and irradiated seeds, respectively. It was deficient in leucine and tyrosine + phenylalanine and tryptophan in unirradiated seeds. The EAA score of threonine and histidine increased on irradiation, while it decreased in cystine + methionine, isoleucine, leucine, tyrosine + phenylalanine, and lysine. The EAA score of valine, although decreased at 2.5 kGy, was elevated higher than unirradiated seeds at 5 kGy (113.1 vs. 114.3). Drastic reduction in EAA score was seen in cystine + methionine, tyrosine + phenylalanine, and lysine. The EAA score of cystine + methionine in *Mucuna* seeds was also decreased significantly by EB irradiation. The PDCAAS of nine EAA of *C. cathartica* showed drastic decrease at 2.5 kGy, but at 5 kGy, threonine, valine, isoleucine, leucine, and histidine were elevated. Interestingly, the PDCAAS did not show significant change up to 15 kGy of EB irradiation in *Mucuna* seeds, while the gamma irradiation resulted in significant elevation in all irradiated doses (5, 10, 15, and 30 kGy) (Bhat and Sridhar 2007; Bhat et al. 2008b). The PER_1, PER_2, and PER_3 of unirradiated seeds decreased at 2.5 kGy, while PER_1 and PER_2 were elevated at 5 kGy. Increase in PER_1 and PER_2 of seeds irradiated at 5 kGy shows its superiority. According to Friedman (1996), PER below 1.5 is of poor-quality proteins, between 1.5 and 2 as moderate quality, and PER over 2 of high quality. In the present study with one exception (PER_3 at 2.5 kGy, 1.1), the rest were moderate ($\geq$1.5) to high ($\geq$2) indicating the desired PER value of *C. cathartica* seeds.

Fatty Acid Profile

Supriya et al. (2012) demonstrated selective changes in fatty acid methyl esters (FAMEs) of *C. cathartica* seeds, and the low doses of EB irradiation (e.g., 2.5 and 5 kGy) serve as hormetic doses in selective enhancement of fatty acids. Considerable variation in FAMEs profile on subjecting to EB irradiation was seen by the Soxhlet (hot) and Bligh and Dyer (cold) methods of extraction of total lipids. Changes in medium-chain fatty acids, long-chain saturated fatty acids, and mono- and polyunsaturated fatty acids and ratios of fatty acids were dependent on the seed material, the dose EB irradiation, and the method of lipid extraction.

Conclusions

This study revealed selective changes of proximal features, minerals, protein fractions, and amino acids of seeds of wild legume *Canavalia cathartica* on EB irradiation. The promising features of EB irradiation include decrease in quantities of crude fiber, globulin, and Na/K ratio while increase in carbohydrates, Ca/P ratio, albumin/globulin ratio, EAA/TAA ratio, EAA score (threonine, valine, leucine, and histidine), PDCAAS (threonine, valine, isoleucine, leucine, and histidine), and PER. The EB irradiation did not significantly change contents of crude protein, minerals (e.g., sodium, potassium, calcium, magnesium, zinc, and manganese), and EAA (threonine, cystine, isoleucine, leucine, tyrosine, phenylalanine, and histidine). Such selective impact of EB irradiation on the constituents of *C. cathartica* seeds provides a wide scope to utilize for value-added food stuffs with nutritional and functional significance in future. To follow the more specific impact and application of EB irradiation on *C. cathartica* seeds, further studies need to focus on changes in carbohydrates, albumin, globulin, EAA, IVPD, and hemagglutinin activity.

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Phytochemical Profile and Therapeutic Properties of Leafy Vegetables

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Introduction

Consumption of fruits and vegetables are predicted to prevent many deadly diseases and disorders; though no direct evidence is available (UNESCO 2008), these can be due to the presence of various antioxidants, phenolic compounds, minerals, vitamins, and other pigments in it. Still consumption of these fruits and vegetables are found to be very less. Among these green leafy vegetables (GLV) are considered best instant sources of fibers, essential amino acids, vitamins, and minerals (Sharma and Kumar 2013; Adenipekun and Oyetunji 2010) producing various biological effects likes antimicrobial effect (Kubo et al. 2004; Hedges and Lister 2009; Dhiman et al. 2012), antihistaminic (Kesari et al. 2005), hypolipidemic (Khanna et al. 2002), anticarcinogenic (Rajeshkumar et al. 2002), and antidiabetic properties (Yamamura et al. 1998) to cure and prevent many lifestyle and harmful diseases such as insomnia, hypertension, diabetes, age-related ailments, oxidative strains, hypertension, and cardiovascular diseases (Iyer Shanti et al. 2012; Vishwakarma and Dubey 2011; Patro et al. 2011). However, these bioactive compounds are present in low amount; higher consumption of these GLVs along with physical exercise can prevent age-related disorders.

GLVs are also used as medicine to produce physiological actions on the human health because of the presence of their bioactive compounds, which produce immune system repair, antibacterial, antioxidant, antiviral, detoxification, and anti-inflammatory activities (Lampe 2003; Raju et al. 2007). Vitamins and secondary metabolites may be commonly termed as "phytochemicals," considering certain vitamins as primary metabolites whereas provitamins such as β-cryptoxanthin or β-carotene, etc. as a part of secondary metabolites (Poiroux-Gonord et al. 2010).

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These provitamins are converted to vitamins in the animal body producing the essential affect. GLVs are also rich source of antinutrient, reducing many diseases like CVD, high blood pressure, stroke, etc. (Aletor and Adeogun 1995).

Plant Metabolites

The level of plant metabolites are greatly influenced by genetic and environmental factors as well as storage and transportation conditions. Growth factors including temperature, humidity, light, type of soil, damage by microorganisms and insects, stress induced by UV radiation, application of fertilizers, pesticides, and heavy metals alter the metabolite composition of plants (Orcutt and Nilsen 2000).

Primary Metabolites

The primary metabolites such as amino acids, carbohydrates, fatty acids, and organic acids are commonly found in all species across broad range of phylogenetic groups. These compounds are directly related to the growth and development, hormone and protein synthesis, respiration, and photosynthesis. The biochemical pathways used for modifying and synthesizing these primary metabolites, which are found essentially same in all organisms, apart from minor variations (Hounsome et al. 2008). Classification of primary metabolites is given in Fig. 1.

Protein

Proteins are complex molecules having various compositions of amino acids. They play a vital role in regulating body metabolism, cellular function, and structure. Hence, they add value to daily diet of consumers. Green leafy vegetables are rich and inexpensive sources of proteins because of their synthesizing ability of amino acids (Aletor et al. 2002). Ribulose-1, 5-bisphosphate carboxylase/oxygenase (RUBISCO) is a major leaf cell protein (accounts to about 50%) which plays a critical role in carbon fixation during photosynthesis (Kawashima Nobumaro and Wildman 1970). This is a similar protein found in leaf chloroplasts of all green leafy vegetables with minor changes in amino acid base for different species. Recent research reported that the green leafy vegetables such as broccoli (*Brassica oleracea* var. italica), duckweed (*Lemna perpusilla*), and spinach (*Spinacia oleracea*) render all the essential amino acids that meet the FAO nutrition standards (Edelman and Colt 2016). Studies have also proven that cassava (*Manihot esculenta*) leaves have amino acid profile balanced with pulse and dairy products (Fasuyi 2005). The protein content in African leafy vegetables such as green leaves of septic weed (*Senna occidentalis*) and cassava (7 g/100 g of fresh weight) are greater than exotic

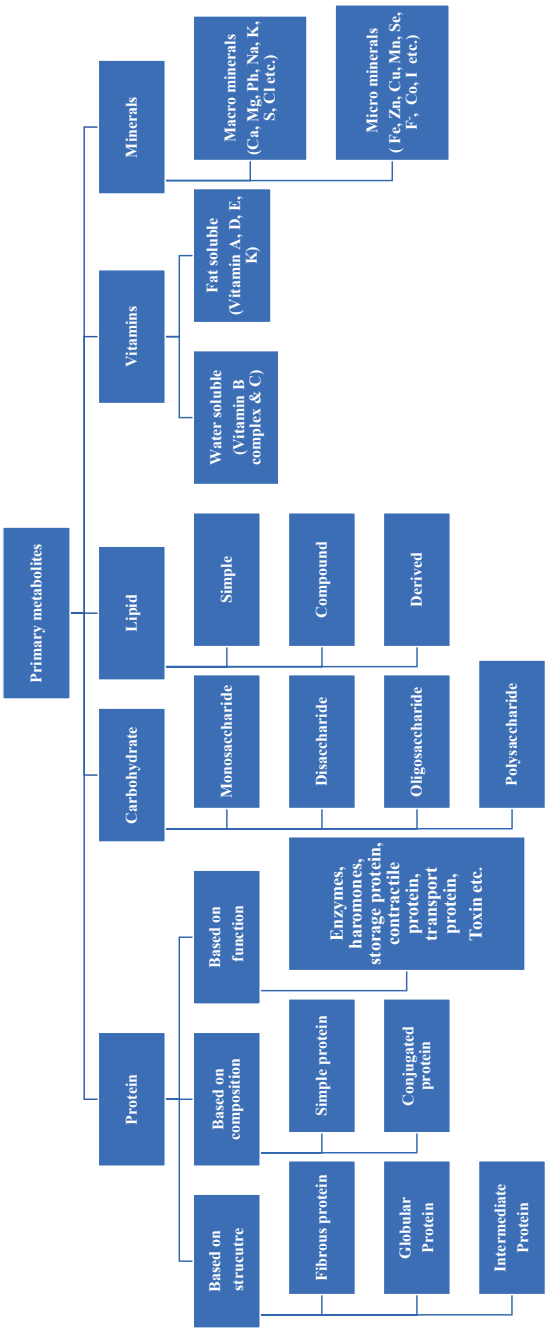

Fig. 1 Classification of primary metabolites, Boyle 2005; Belitz et al. 2009

leafy vegetables such as *Brassica oleracea* subsp. capitata (1 g/100 g of fresh weight) (Uusiku et al. 2010). However, African leafy vegetables have relatively less protein content than legume proteins (white lupine (*Lupinus albus*) with 11.5 g protein/100 g of fresh weight) (Kalogeropoulos et al. 2010).

According to prevailing environmental conditions and farming practices, the amount of protein in leafy vegetables may vary (Odhav et al. 2007). Thermal processing inactivates heat-labile anti-nutritional factors such as lectins, goitrogens, thiaminases, and protease inhibitors.

It improves digestibility of proteins and starch but leads to protein denaturation and hence effects the bioavailability of proteins in leafy vegetables (Gibson et al. 2006).

Dietary Fiber

Dietary fiber is considered a class of compound, which comprises a mixture of plant carbohydrate polymer, noncarbohydrate component, polysaccharides, non-starch polysaccharides, and oligosaccharides (Elluech et al. 2010). In these mixtures, non-starch polysaccharides and oligosaccharides are considered a major component of dietary fiber. Based on their solubility, the dietary fiber can be classified into insoluble fibers (cellulose, lignin, hemicellulose) and soluble fibers (β-glucan, pectin, gums) (Natesh et al. 2017). Not all these components can be digested by human enzyme, and it is passed through the gastrointestinal tract as bulk fiber. Later this bulk fiber undergoes digestion and modification by the enzymes of colon microbes (Blaut 2002). The consumption of dietary fiber may reduce the cardiovascular diseases, colon cancer, diabetes, diverticulosis, obesity, and constipation (Jenkins et al. 2001; Spiller 2001). According to the Food and Nutritional Board, Institute of Medicine (2001), the daily requirement of dietary fiber is 30 g/day for men older than age 50 and 38 g/day for men younger than age 50. The daily requirement for women is 25 g/day (age below 50) and 21 g/day (age above 50).

The green leafy vegetables are considered as good source of dietary fiber. For example, the soluble fiber content of curry leaf is 4.4% and insoluble fiber is 55.6% (Bako et al. 2002). But the amount of dietary fiber may vary based on species, climate condition, geographical condition, maturity stages, and fertilizers used (Natesh et al. 2017). According to the literature, those Indian green leafy vegetables such as hibiscus (*Hibiscus cannabinus*), cabbage (*Brassica oleracea*), spinach (*Spinacia oleracea*), coriander (*Coriandrum sativum*), fenugreek (*Trigonella foenum-graecum*), and basella (*Basella rubra*) are rich sources of soluble dietary fiber (Jenkins et al. 2001). African green leafy vegetables such as *Portulaca oleracea*, *Galinsoga parviflora, Justicia flava*, *Adansonia digitata*, *Amaranthus* sp., *Arachis hypogaea*, *Bidens pilosa*, *Brassica* sp., *Ceratotheca triloba*, *Vigna unguiculata*, *Chenopodium album*, *Emex australis*, *Cleome* sp., *Cucurbita pepo*, *Senna occidentalis*, *Bidens pilosa*, *Manihot esculenta*, *Solanum* sp., and *Chenopodium album* are good sources of dietary fiber. The fiber content of African leafy vegetables may range from 1 g/100 g to 8 g/100 g (Uusiku et al. 2010). Leafy vegetables such as

water leaf (*Talinum triangulare*), *Telfairia occidentalis*, and *Amaranthus hybridus* are found in different parts of Nigeria. Literature reveals that the African green leafy vegetables lose their dietary fiber content when cooking at high temperature, because it breaks the weak bonds between polysaccharides and cleavage of glycosidic linkages which leads to solubilization of the dietary fiber (Svanberg et al. 1997). The cooking effect is also examined in Indian leafy vegetables, in that there is a minute increase in total dietary content which may be due to the polymerization or hydration of total dietary fiber fractions (Kala and Prakash 2004). Puupponen Pimiä et al. (2003) reported that there is no consequential change in insoluble, soluble, and total dietary fiber of freezer and blanched stored spinach. Determination of dietary fiber in green leafy vegetables can be estimated by enzymatic or gravimetric methods given by AOAC.

Vitamins

Vitamins are available in the form of precursors in vegetables and are essential for proper functioning of bones, vision, hairs, teeth, skin, and the mucous membranes. Calcium and phosphorous help in the growth and maintenance of bones. Vitamins play a key role for the normal functioning of nervous system, the endocrine glands, and clotting of blood. They are also essential for macromolecule metabolism (Randhawa et al. 2015). The WHO (2009) reported 15 million pregnant women and 190 million young children from developing countries are suffering from vitamin A deficiency. This deficiency can be reduced by consumption of vegetables and green leafy vegetables instead of antioxidant supplements.

Raw green leafy vegetables have considerable amount of various vitamins such as fat-soluble vitamins (vitamin E and vitamin A) and water-soluble vitamins (vitamins C, B1, B2, B3, B5, B7, B9). Green leafy vegetables have high vitamin K contents when compared to fruits and vegetables because of their direct involvement in the photosynthetic process. Leafy vegetables like spinach have sufficient vitamin C content to prevent and cure scurvy (Randhawa et al. 2015). Green leafy vegetables such as spinach, asparagus, Brussels sprouts, turnip, lettuce, and cauliflower are reasonably good sources of B vitamins (USDA 2005).

Vitamin A

In leaves, vitamin A is in the form of provitamin A carotenoid such as α-carotene, β-carotene (abundant source), γ-carotene, β-cryptoxanthin, and non-provitamin A carotenoids such as neoxanthin, lutein, and violaxanthin. Beta-carotene is one of the most important *provitamin* carotenoids with respect to its quantitative contribution to the diet and relative provitamin A activity (SACN 2005). The level of vitamin A in the diet relies on the amount of beta-carotene of the African green leafy vegetables (ALVs), amount consumed, bioavailability, and bioefficacy (West et al. 2002). Vitamin A content is expressed in terms of retinol equivalents (RE), where 1 RE is

equivalent to 6 μg of β-carotene and 12 μg of the other provitamin carotenoids such as α-carotene, β-cryptoxanthin, and γ-carotene. The US Institute of Medicine has recently replaced retinol equivalents (RE) with "retinol activity equivalent" (IOM 2000). According to the Institute of Medicine dietary reference, intake of vitamin A content is recommended to be 700 and 900 μg RAE for an adult female and male, respectively.

The β-carotene content of African leafy vegetables varies according to the species from 99 mg RE in *Vigna unguiculata* to 1970 mg RE (per 100 g edible portion) for *M. esculenta*. Considering the most recent edition of Recommended Nutrient Intakes (RNI), 1 RE is equivalent to 6 mg β-carotene (FAO/WHO 2001). According to the RNI, 300 g fresh African leafy vegetables intake would satisfy the dietary requirements of vitamin A for children. But for adults, 300 g of fresh *Cucurbita pepo* would provide 97% of male RNI and 116% of female RNI, while 300 g of fresh *V. unguiculata* would provide only 50 and 59% of male and female daily requirements, respectively. The stability and retention of carotenoids in various foods are affected by their food particle size, chemical nature, storage time and storage conditions, and the method and severity of processing (Cheynier 2005). Cooking of leafy vegetables enhances the bioavailability of α-carotene and β-carotene (Khachik et al. 1992). Heat treatment such as steaming and boiling enables to release carotenoids which are bound by protein and helps them to be extracted readily (Howard et al. 1999). Higher retention of β-carotene in the vegetables was achieved by microwave steaming and stir-frying with oil compared to stir-frying with water or boiled (Masrizal et al. 1997). Addition of oil to *Ceiba* sp. and *Manihot* sp. has shown to improve serum retinol in Ghanaian preschool children (Takyi 1999). When compared to the raw vegetables, cooked and pureed spinach provides higher plasma total β-carotene concentrations (Rock et al. 1998). While cooking, extractability increases because of enzyme destruction which could otherwise lead to carotene degradation (Kala and Prakash 2004).

Riboflavin

Riboflavin characterized as vitamin B2 which may have neuroprotective effects in neurological disorders like migraine, multiple sclerosis, and Parkinson disease. It is also having an antioxidant property which can prevent lipid peroxidation and reperfusion oxidative injury. Riboflavin is found in various plants and animal foods such as milk, eggs, and green leafy vegetables (Saedisomeolia and Ashoori 2018). African green leafy vegetables (ALVs) contain reasonably good concentrations of riboflavin. The riboflavin content in ALVs ranges from 0.04 mg/100 g (*Brassica* species) (Kruger et al. 1998) to 0.6 mg/100 g (*M. esculenta*) (FAO 1990). Intake of 300 g of fresh *Cleome* sp. would give 33–60% RNI of riboflavin for children, 30% for adult women, and 23% for adult men. Riboflavin deficiency commonly arises with lactose intolerance (FAO/WHO 2001), and this condition is majorly found in African populations. The deficiency of this vitamin is related to the increased risk of cardiovascular diseases (Powers 2003).

Folic Acid

Folic acid characterized as vitamin B9 also known as pteroylmonoglutamic acid or simply as "folates." It is also an essential part of B complex (Cossins 2000). GLV contains abundant amount of folic acid and related compounds. Folic acid contains two to eight glutamic acids (essential amino acids) in the primary structure of pteroylmonoglutamic acids (Herbert et al. 1999). It is an important part of transport of amino acids to specific location in the protein synthesis pathway (Kelly 1998) and for the methylation of RNA, DNA, and amino acids (Lucock et al. 1996; Ma et al. 1997). Methionine is regenerated from homocysteine by folic acid to maintain the cardiovascular working (Leclerc et al. 1998). Being an essential part of cell proliferation and DNA synthesis, it is also essential for the cell division to regulate sleep, mood, appetite, and working of central nervous system (Bottiglieri et al. 2000). For the same reason, this is very important for the growth of fetus and during gestation (FDA 1997; Antony 2007). RDA suggests 400 μg/day of folate for the nonpregnant women, whereas 4 mg/day of folate is suggested for women having history of delivering babies with neural tube defects. RDA also suggests 600 μg/day for the development of maternal tissues, the placenta, and the fetus. Deficiency of folates also leads to many serious issues like neural tube defects in babies such as spina anencephaly and bifida and neurocristopathies. Studies have shown that daily intake of folic acid in women belonging to low-economic status of rural and urban areas of India is just 75–165 μg (Misra et al. 2002), which is very much less than that of FDA recommendations. Reports are not available on toxicity of folic acids with no to consumption of folic acid-supplemented food or folic acid-fortified foods. This might be due to the fact that folic acids are solubilized in water and are excreted from the body in the form of urine (Hathcock 1997). Scientists are continuously working for increasing such health-promoting compounds in the fruits and GLV by new methods of genetics and plant breeding. They proved that use of good quality soil for growing fruits and GLVs can abundantly improve the concentration of health-promoting compounds (Lester and Eischen 1996; Lester and Crosby 2002).

Vitamin C

The ascorbic acid content in some of the unprocessed ALVs varies from 2 to 311 mg (per 100 g edible portion) in *Solanum nigrum* and *M. esculenta*, respectively. It is evident from the literature that this vitamin is immensely affected by processing. Ascorbic acid reduced by 19, 61, and almost 100% in cooked amaranth, dried *Vernonia amygdalina*, and dried *Adansonia digitata* respectively (FAO 1990). In order to minimize the degradation of ascorbic acid and browning, it is advisable to store the leafy vegetables at low temperature (Negi and Roy 2001).

The ascorbic acid intake from green leafy vegetables in sub-Saharan Africa is often determined by various seasonal factors, time and temperature of storage, as well as postharvest storage, chlorination of water, and cooking practices (FAO 2001). When vegetables are blanched, the loss of ascorbic acid is relatively higher

than those under frozen storage. These losses are primarily due to the leaching effect occurring during thermal processing rather than the chemical degradation (Sreeramulu et al. 1983; Wallace et al. 1998; Howard et al. 1999; Mepba et al. 2007; Rawson et al. 2012; Rawson et al. 2013) and reported a significant reduction in ascorbic acid content of several African green leafy vegetables after thermal treatment. The most effective preservation methods reported in retaining the ascorbic acid content is steam blanching followed by dehydration (Schippers 2000). In comparison with sun drying, shade drying, and vacuum drying, freeze-drying is found to be the most suitable method for retaining ascorbic acid content of African leafy vegetables (Shitanda and Wanjala 2006).

Minerals

Green leafy vegetables are good sources of calcium, magnesium, iron, zinc, sodium, and potassium. The absorption of these minerals is influenced by the presence of various inhibitors such as phytate and oxalates (Kumari et al. 2004). During processing, minerals have higher stability as compared to vitamins and proteins (Kala and Prakash 2004). But mineral contents in green leafy vegetables are affected by several factors including climatic conditions, water availability, soil type and pH, plant variety and age, and especially the application of fertilizers.

For instance, mineral profile of spinach has higher calcium content (1036 mg/100 g), followed by magnesium (827 mg/100 g), sodium (827 mg/100 g), and iron (28.4 mg/100 g), while duck weed is rich in zinc (15 mg/100 g). However, soy seed also has notable calcium content (195 mg/100 g), magnesium (407 mg/100 g), iron (6 mg/100 g), phosphorus (469 mg/100 g), sodium (12.3 mg/100 g), potassium (2387 mg/100 g), and zinc (3.7 mg/100 g) when compared to all the seeds but relatively higher in the green leafy vegetables (Natesh et al. 2017).

Iron

Iron deficiency in women and children results in the development of anemia (Galloway 2003). The iron content of African leafy vegetables ranges between 0.2 and 12.8 mg/100 g edible portion for *Solanum nigrum* and *Lesianthera africana*, respectively. Intake of 300 g of fresh *L. africana* and *S. nigrum* would give 7 and 431% of RNI for children and 2 and 131% of RNI for adult women, respectively (Uusiku et al. 2010).

Zinc

Zinc deficiency leads to impaired gastrointestinal and immune functions. It varies within the same species and ranges from 0.03 to 3.1 mg/100 g edible portion for *Ipomoea batatas*, 1.4 to 18.5 mg/100 g for *C. album*, and 0.02 to 8.4 mg/100 g for

Amaranthus species. Zinc bioavailability is adversely influenced by phytate concentration (Turnlund et al. 1984).

Calcium and Magnesium

The calcium content in African leafy vegetables is 668 mg/100 g in *U. urens* and 15 mg/100 g edible portion in some *Chenopodium* species, while the magnesium content is 225 mg/100 g in *J. flava* and 13 mg/100 g in *Brassica* species. The bioavailability of these minerals is affected by the presence of anti-nutritional factors, age and sex of an individual, and also the fat content in the diet (Uusiku et al. 2010) (Table 1).

Secondary Metabolites

Biologically active substances like secondary metabolites are majorly found in fruits and vegetables (Tables 2 and 3). These metabolites do not directly participate in the growth and development of the human body but are important for all the biochemical processes and producing positive effects in human life. British Nutrition Foundation divided these secondary metabolites into four categories – alkaloids (12,000 compounds), terpenoids (25,000 compounds), phenolic compounds (8000 compounds), and sulfur-containing compounds (Goldberg 2008). Plant samples tested for secondary metabolites were also found to be used for body building, cell growth, and repair (Kubmarawa et al. 2008). Classification of secondary metabolites is shown in Fig. 2.

Phenolic Compounds

Polyphenols are considered as main antioxidant having in vitro effect on the human health including vitamins and carotenoids (Gardner et al. 2000). Correlation between the polyphenol content and antioxidant activity was found for the African GLVs (Mai et al. 2007), whereas no such correlation was found in Indian (Dasgupta and De 2007) and Malasian GLVs (Ismail et al. 2004). This correlation depends on the methodology and type of the vegetable, which is due to contribution of antioxidant component with different antioxidant activity (Ismail et al. 2004). Similar work done by (Modi 2007) on boiled *Amaranthus* proves the correlation between the growth temperature and plant age with respect to antioxidant activity. He found that *Amaranthus* harvested 60 days after sowing had higher antioxidant capacity than that harvested after 20 or 40 days of sowing. Correlation was also found between the food processing or cooking method on the phenolic content and thus affecting the antioxidant activity. Increase and decrease in flavonoids were observed with the cooking method. While increase in flavonoids may be due to inhibition of oxidative enzymes and increased release of flavonoids from the breakdown of cell wall, which

Table 1 Primary metabolite composition of selected green leafy vegetables (Fresh weight)

Botanical name	Common name	Protein	Fiber	Vitamins				Minerals			
				A (µg RE)	B2 (mg)	B9 (µg)	C (mg)	Iron (mg)	Zinc (mg)	Calcium (mg)	Magnesium (mg)
Adansonia digitata	Baobab	4[a]	3 [a]	–	–	–	52[a]	–	–	410[a]	–
Amaranthus hybridus	Smooth pigweed	4–6[b]	3[b]	327[c]	0.1–0.4[a,c]	64[c]	46–126[c,d]	0.3–3.8[b,e]	0.02–8.4[b,e]	253–425[a,b,c]	105–224[b,c]
Arachis hypogaea	Peanut	4[f]	8[f]	–	–	–	87[f]	1.0[f]	2.9[f]	–	–
Bidens pilosa	Black-jack	3–5[a,b,c]	3–6[a,b,c]	301–985[a,c]	0.2[c]	351[c]	23[c,d]	2.0–6.0[b,c]	0.9–2.6[b,c]	162–340[a,b,c]	79–135[b,c]
Brassica sp.	–	1–2[f]	2–4[f]	–	0.0–0.2[c,f]	16[c]	30–113[c,f]	0.5–3.5[f]	0.9–1.3[f]	27–31[c,e]	13[c]
Ceratotheca triloba	Wild foxglove	2[b]	2[b]	–	–	–	–	2.85[b]	0.45[b]	105.75[b]	64.2[b]
Chenopodium album	Fat hen	4–5[b,c]	2[b,c]	917[c]	0.3[c]	30[c]	31[c]	2.2–6.1[b,c]	1.4–18.5[b,c]	15–226[c,e]	155–211[b,c]
Cleome sp.		5[a,b,c]	1–[5a,b,c]	1200[c]	0.1[c]	346[c]	13–[50a,c]	2.6–2.9[b,c]	0.6–0.8 [b,c]	31–288 [b,c]	44–76[a,b,c]
Cucurbita pepo	Sweet dumpling squash	3[c]	2[c]	194[c]	0.1[c]	36[c]	11[c]	1.5 [c]	0.06–0.2 [c]	39 [c]	38 [c]
Emex australis	Doublegee	5[b]	2 [b]	–	–	–	–	1.7 [b]	2.2 [b]	17.6 [b]	111.98 [b]
Galinsoga parviflora	Mielcilla	4 [b]	1 [b]	–	–	–	–	3.0 [b]	1.5 [b]	17.82 [b]	74.91 [b]
Justicia flava	Impela	3 [b]	1 [b]	–	–	–	–	2.6 [b]	1.8 [b]	331.68 [b]	225 [b]
Lesianthera Africana		3[g]	4 [g]	–	–	–	–	0.2 [g]	0.1 [g]	–	–
Manihot esculenta	Cassava	7[b]	4 [b]	1970 [b]	0.6 [b]	–	311 [b]	–	–	30–303 [b]	–
Momordica sp.	–	5[a]	3[a]	–	–	–	4[d]	3.5[b]	1.8 [b]	403.2 [b]	91.95 [b]
Portulaca oleracea	Parsley	3 [b]	1 [b]	–	–	–	–	2.9 [b]	2.4 [b]	95.27 [b]	72.59 [b]

Senna occidentalis	Septicweed	7 [b]	3 [b]	–	–	–	–	2.5 [b]	2.1 [b]	513 [b]	196.42 [b]
Solanum sp.	–	3–5 [b]	1 [b]	1070[c]	0.3 [c]	404 [c]	2 [c]	8.5–12.8[b,c]	0.8–3.5[b,c,h]	278–310[b,c]	84[c]
Spinacia oleracea	Spinach	3 [c]	3 [c]	669 [c]	0.2 [c]	194 [c]	28 [c]	2.7 [c]	0.4 [c]	99 [c]	79 [c]
Vernonia sp.	–	3–5[a,h]	2–5 [a,h]	–	0.3 [a]	457 [a]	51–198 [a,h]	0.8–3.2 [h]	0.08[e]	145 [a]	–
Vigna unguiculata	Cowpea	5[c]	4 [c]	99 [c]	0.2 [c]	141 [c]	50 [c]	0.3–3[c,d]	0.23[d]	188 [c]	60 [c]

[a]FAO (1990)
[b]Odhav et al. (2007)
[c]Kruger et al. (1998)
[d]Steyn et al. (2001)
[e]Orech et al. (2007)
[f]Mosha and Gaga (1999)
[g]Isong and Idiong (1997)
[h]Ejoh et al. (2007)

Table 2 Secondary metabolite composition of selected green leafy vegetables (fresh weight)

Botanical name	Common name	Total phenolics (mg/g)	Total carotenoids (mg/g)	Total flavonoids (mg/g)
Amaranthus hybridus	Smooth pigweed	3.7 ± 0.23[h]	–	1.2 ± 0.35[h]
B. napus cv.	Summer rape	3.58 ± 0.08 [g]	–	0.78 ± 1.52 [g]
Brassica oleracea	Cauliflower	0.62[d]	0.32 [d]	2.99 [d]
C. gynandra	Stinkweed	3.94 ± 0.09 [g]	–	2.19 ± 0.11 [g]
Coriandrum sativum	Coriander	0.49[d]	0.42 [d]	2.26 [d]
Cucurbita maxima	Pumpkin	2.68 ± 0.03 [g]	2.05 ± 0.01 [g]	1.55 ± 0.44 [g]
Lactuca sativa	Lettuce	0.11[d]	0.43 [d]	0.22 [d]
Manihot esculenta	Cassava	2.94 ± 0.11 [g]	2.19 ± 0.01 [g]	–
Murraya koenigii	Curry leaves	18.40 ± 1.23[c]	0.214[f]	–
Petroselinum crispum	Parsley	0.30[d]	0.33 [d]	1.63 [d]
Spinacia oleracea L.	Spinach	2.09 ± 0.03[b]	0.12 ± 0.01[b]	2.92[d]
Telfairia occidentalis	Fluted gourd	1.27 [g]	–	7.68 [g]
Trigonella corniculata L.	Kasuri methi	17.30[a]	–	7.40 [a]
Trigonella foenum-graecum L.	Fenugreek	8.75[a]	0.24 [a]	6.42 [a]

[a]Pasricha and Gupta (2014)
[b]Bunea et al. (2008)
[c]Kamath et al. (2015)
[d]Shehata et al. (2014)
[e]Okonwu et al. (2017)
[f]Jain et al. (2012)
[g]Moyo et al. (2013)
[h]Jimenez-Aguilar and Grusak (2017)

is binding site flavonoids and decrease was observed due to heat lability or leaching of specific flavonoids (Yamaguchi et al. 2001). Neugart et al. (2015) in his study found indigenous African leafy vegetables as high source of flavonoid glycosides and hydroxycinnamic acid derivatives, which is evident from the fragmentation patterns, retention time, maximum absorption wavelength, and molecular masses. He also observed high concentration of phenolic compounds in Ethiopian kale (17,206 and12,228 μg/g DW), African nightshade (16,677 and 16,387 μg/g DW), and cowpea (16,185 and 17,408 μg/g DW), whereas medium concentration was observed in common kale (6690 and 12,143 μg/g DW) and amaranth (14,221 and 9229 μg/g DW) and low concentrations in spider plant (5297 and 6295 μg/g DW). Vietnamese plants used for making drinks showed highest antioxidant activity and amount of polyphenol compared to that of edible wild vegetables, herbs, and dark green vegetables (Mai et al. 2007).

Leaves of sub-Saharan Africa plants of *P. oleracea*, *Momordica balsamina*, and *J. flava* had high antioxidant activity and 96, 94, and 96% scavenging capacity, respectively (Odhav et al. 2007). Amaranth, a famous and highly nutritious leaf, also showed good content of polyphenolics including anthocyanin in amaranth

Table 3 Anti-nutritional content of selected green leafy vegetables (fresh weight)

Botanical name	Common name	Oxalic acid (mg/100 g)	Phytate (mg/100 g)	Tannins (mg/100 g)	Saponins (mg/100 g)	Trypsin inhibitors (mg/100 g)
Amaranthus sp.	–	40–50[a]	140 [a]	–	–	4–14 [f]
Boscia senegalensis	Bokkhelli	–	–	–	–	17 [f]
Celosia argentea	Plumed cockscomb	20 [a]	–	–	–	7 [f]
Crassocephalum sp.	–	10–20 [a]	–	–	–	–
Euphorbia hirta	Asthma plant	1115[b]	655 [b]	1222 [b]	6.7 [b]	–
Hibiscus esculentus	Okra	10 [a]	–	–	–	–
Ipomoea involucrata	Moon flower	913 [b]	176 [b]	869 [b]	386 [b]	–
Launaea sp.	–	108 [b]	9 [b]	168 [b]	424 [b]	–
Leptadenia hastate	–	–	–	–	–	1 [f]
Lesianthera Africana	–	2[c]	–	–	–	–
Maerua crassifolia sp.	–	–	–	–	–	82 [f]
Manihot esculenta	Cassava	20 [a]	100 [a]	–	–	–
Solanum sp.	–	–	40[e]	–	–	–
T. occidentalis	Fluted pumpkin	0.3814 [g]	0.7131 [g]	0.1436 [g]	0.4175 [g]	0.959 [g]
Talinum triangulare	Philippine spinach	20 [a]	190 [a]	–	–	–
Telfairia occidentalis	Fluted gourd	40 [a]	–	–	–	–
Trigonella corniculata L.	Kasuri methi	–	–	–	2.79 [f]	–
Trigonella foenum-graecum L.	Fenugreek	–	–	–	2.37 [f]	–
Vernonia sp.	–	1–2[d]	120 [a]	0.1–0.3 [d]	–	–
Xanthosoma sp.	–	654 [b]	131 [b]	481 [b]	655 [b]	–

[a]Aletor and Adeogun (1995)
[b]Wallace et al. (1998)
[c]Isong and Idiong (1997)
[d]Ejoh et al. (2007)
[e]Oboh et al. (2005)
[f]Vanderjagt et al. (2000)
[g]Okonwu et al. (2017)

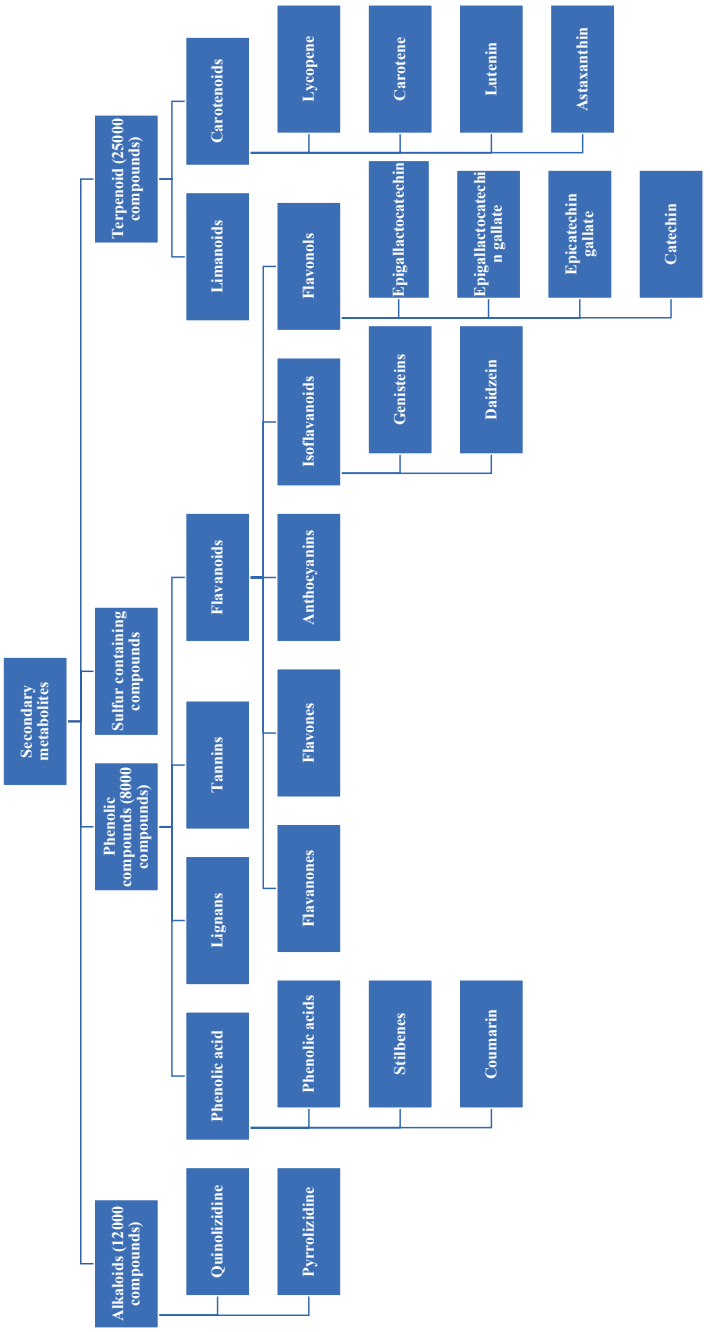

Fig. 2 Classification of secondary metabolites, Randhawa et al. (2015); Goldberg (2008); Croteau et al. (2000); Naczk and Shahidi (2004)

grains also called as Rama's grain (Rajgira) (Paśko et al. 2009). These high contents of bioactive compounds are responsible for the antioxidant potential of flowers and leaves of *Amaranthus* spp. and their extracts. This might be due to the presence of free radical scavenger rutin (Kraujalis et al. 2013). A comparison study proved that *A. tricolor* (red stem) had higher phenolic content and antioxidant capacity than *A. viridis* (green stem) due to the presence of anthocyanin, a red pigment (Routrey et al. 2013). Higher and lower scavenging activities were also found in *Xanthosoma mafafa*, *Celosia argentea*, *Manihot utilissima*, *Ocimum gratissimum* sp., and *Stractium* sp. as 99, 90, 90, 11, and 22%, respectively (Akindahunsi and Salawu 2005).

Tannins, a group of phenolic compounds, are anti-nutritional elements which react with digestive enzymes, proteins, and starches, reducing their nutritional value (Chung et al. 1998; Serrano et al. 2009). Tannins are also observed to hinder the absorption of protein and availability of iron (Bravo 1998). These were found to be high in African leafy vegetables, and their concentration was found to range between 655 mg/100 g in *Xanthosoma* sp. and 1222 mg/100 g in *Euphorbia hirta* (Wallace et al. 1998).

Alkaloids

Lower acceptability of leafy vegetables is due to the bitterness which is due to the presence of alkaloids (Wallace et al. 1998). Effect on transit time in the small intestine of human proves its microbial property, being used for medicinal purpose (Cowan 1999). Two major groups of alkaloids often studied are quinolizidine and pyrrolizidine, where the former is mostly found in genus *Lupinus* and the latter is mostly found in the family of Asteraceae and in the Boraginaceae (Croteau et al. 2000). Positive test for the presence of alkaloids was found in *Sida acuta*, *Asystasia mysorensis*, *Amaranthus* sp., *Portulaca quadrifida*, and *Crotalaria ochroleuca* spp. (Orech et al. 2005).

Carotenoids

Plants impart their yellow, red, or orange color from the carotenoids a subclass of terpenoids. Absorption of this compound requires little of fat in their cooking method after being pureed and chopped. This class of pigment is widely accepted to prevent heart disease, cancer, and eye disease. Vitamin A is mainly produced by these carotenoids. Some other examples of these pigments are zeaxanthin, lycopene, lutein, and β-carotene, where lutein and zeaxanthin are obtained from dark green and leafy vegetables that are important for preventing CVD, oxidative damage to eyes, and age-related macular degeneration. Lycopene is obtained from the processed and cooked tomato for producing red color and preventing cancer and heart diseases. Spider plants contained high amount of β-carotene a provitamin of vitamin A (up to 64.7 μg/g DW). High concentration of carotenoids was also

observed in amaranth (up to101.7 μg/g DW), whereas *A. cruentus* was found to be potentially a good source of carotenoid, a provitamin of vitamin A. It was found that the highest amount of β-carotene is present in the leaves of this species followed by seeds, stem, and roots. Highest content of antitumor agent, canthaxanthin, was found followed by a retardant for age-related eye problems – b-carotene and lutein. This species has 28.5 mg/100 g of β-carotene which is sevenfold higher than tomato and thus can be used to treat anemia reported in the African countries (Dlamini et al. 2010).

Flavonoids

Flavonoids are produced by plants and belong to the group of phenolic compounds or phytochemical compounds. This compound is proved to reduce the risk of CVD (Ali et al. 2000). There is inverse relation between the incident of CVD and consumption of fruits and vegetables (Ness and Powles 1997). Consuming F&V also reduces the blood pressure in the human body (Alonso et al. 2004). He et al. (2006) observed that consumption of 600 g/day of fruits and vegetables can decrease the threat of CVD by 31% and the risk of stroke by 19%.

Anti-nutritional Components

Oxalic Acid

The presence of oxalic acid in various GLVs like *Vernonia* sp., *E. hirta*, *Xanthosoma* sp., *Celosia argentea*, *Ipomoea involucrata*, *M. esculenta*, *Amaranthus* sp., and *Telfairia occidentalis* was reported (Aletor and Adeogun 1995; Isong and Idiong 1997; Wallace et al. 1998; Ejoh et al. 2007). This is present in various species in the soluble or insoluble form, while the former is present as potassium or sodium salt, being excreted from the body, and the latter is present as salts of magnesium, calcium, or iron or combination of any two (Noonan and Savage 1999). Soluble oxalate forms a strong chelating bond with calcium, making it unavailable for absorption and assimilation (Gupta et al. 2005; Radek and Savage 2008), but its high intake can lead to kidney stone (Radek and Savage 2008). Dietary supplement of divalent minerals or addition of source of calcium can lead to unavailability of intestinal oxalate from such foods (Ponka et al. 2006; Radek and Savage 2008).

Phytic Acid

Phytic acid also known as myoinositol 1,2,3,4,5,6 hexakis-dihydrogen phosphate is an antioxidant and source of phosphorous in GLVs. It inhibits the absorption of multivalent metal ions such as calcium, zinc, and iron, inhibiting its free radicals (Schlemmer et al. 2009) and producing adverse effect in digestion of starch and

protein (Reddy and Pierson 1994). Phytic acid also has beneficial effect by enhancing immunity by increasing natural killer cell's function and activity, inhibiting iron-mediated oxidative reactions, serving as antioxidant, and stimulating bacterial killing by neutrophils (Bohn et al. 2008). This also lowers the phosphate donor/acceptor capabilities and reducing inositol's involvement in the signaling mechanism (Bohn et al. 2008; Schlemmer et al. 2009). Phytic acid ranges between 9 mg/100 g and 655 mg/100 g in *Launaea* sp. and *E. hirta*, respectively (Wallace et al. 1998).

Glucosinolates

Glucosinolate precursors of isothiocyanate are used for its therapeutic and prophylactic properties (Fahey et al. 2001).Majorly glucosinolate are found in Capparaceae, Brassicaceae, and Caricaceae family and in *L. Africana*, which belong to Icacineae family (Isong and Idiong 1997). Since it is thermal and stable, they can only be enzymatically hydrolyzed or more specifically driven by myrosinase (Verkerk et al. 2009). After cell rapture, contact of myrosinase and glucosinolates lead to hydrolysis of the thioglucosidic bond and formation of various ranges of bioactive compounds (Baghurst et al. 1999; Verkerk et al. 2009) having chemopreventive and carcinogenic properties, depending on dosage (Fahey et al. 2001; Verkerk et al. 2009).

Saponin

Saponins are poorly absorbed, but it has cholesterol-lowering (Van Duyn and Pivonka 2000), antiparasitic, antifungal/antiyeast, antiviral, antibacterial/antimicrobial, hemolytic, anti-inflammatory, molluscicidal, antitumor, cytotoxicity, and other biological activities (Sparg et al. 2004). The effect of saponin mainly depends on the hydrophobic/hydrophilic asymmetry which in turn affects their capacity to reduce interfacial tension (Champ 2002). Higher saponin concentration of 481 mg/100 g, 424 mg/100 g, and 384 mg/100 g was found in *Xanthosoma* sp., *Launaea* sp., and *Ipomoea involucrata*, respectively, while low concentration of 6.7 mg/100 g and 0.1–0.3 mg/100 g (Ejoh et al. 2007) was found in *Euphorbia hirta* and *Vernonia* sp., respectively (Wallace et al. 1998).

Protease Inhibitor

Trypsin and chymotrypsin inhibitors bind the proteolytic enzymes and inhibit the activity of these enzymes, thus reducing the availability and absorption of proteins and amino acids present (Mosha and Gaga 1999; Glew et al. 2005). Temperature, amount of water, and rate of heating period influence the activity of these inhibitors (Mosha and Gaga 1999). Various works were done to reduce the activity of these

inhibitors. Mosha and Gaga (1999) in his study proved conventional blanching to be better than microwave blanching to inactivate these inhibitors. Heat stability after 5 min boiling was found for these trypsin and chymotrypsin inhibitors, which result in poor protein utilization in human (Vanderjagt et al. 2000) (Tables 2 and 3).

Therapeutic Values and Health Benefits of GLVs

Antidiabetic Properties

Diabetes mellitus (DM) is most prevalent and noncommunicable disease found in every country in the world. The number of affected person is expected to reach 300 million people by 2025 (King et al. 1998). The two types of DM are type I and type II. Type I DM is also known as insulin-dependent diabetes or juvenile-onset disease, and type II is known as non-insulin-dependent diabetes or adult-onset diabetes. The former category is identified as autoimmune-mediated slow or faster destruction of beta-cells produced by the pancreas (Zimmet et al. 1994), while the latter category is due to deficiency of insulin in individual, and they are also known to be resistant to actions of insulin (Defronzo et al. 2015; Lillioja et al. 1993). Type II DM is most familiar, and it is expected to increase the effect at rate of 7.7% by 2030 (Shaw et al. 2010).

This disease has two conditions like hyperglycemia and hypoglycemia. Many oral medicines like alpha-amylase and beta-amylase are given to patients to prevent digestion of complex carbohydrate (Nishikawa et al. 2000). The body frequently produces free oxygen radicals, and reactive oxygen species leads to oxidative damage (Giugliano et al. 1996) in all biochemical process, which in turn leads to increase in lipid peroxidation and adverse effects, specifically in type II DM patients. Prevention of such complications and their effects is the most important target for type II DM patient (Stanely Mainzen Prince and Menon 2001). Almost all GLVs have minerals, vitamins, antioxidants, phytochemicals, and carbohydrates which prevent CVDs and diabetes. GLVs also have low energy intake, low glycemic loads, and high fiber content along with high concentration of proteins, phytochemicals, antioxidant vitamins, magnesium, and potassium, which play vital role in reducing the risk of type II DM. Also, dark yellow and green leafy vegetables prevent this risk in the overweight women (Liu et al. 2004). Polyphenolic antioxidants including ascorbic acids, b-carotene, phenolics, and alpha-tocopherol of GLVs (Kwon et al. 2006) have high therapeutic properties and good amount of antioxidant, antidiabetic, and antihypertensive activity (Chu et al. 2002; Oboh and Rocha 2007; Oboh et al. 2008). Wild basil and jute contains higher quantity of phenolic content than chaya; fluted pumpkin, bitter leaf, and waterleaf contain the lowest. Ferric-reducing property of GLV leads to accumulation of ferric in acinar cells and islets of Langerhans of the pancreas; this in turn destructs the beta cells and prevents type II DM (Pulido et al. 2000). Reddish leaves, lettuce leaves, and omum leaves are the

cheaper and richer source of many micronutrients and phytochemicals having good antioxidant capacity (Tarwadi and Agte 2003). This property makes GLV a cheaper alternative for diabetes mellitus disease and hypertension than synthetic drugs (Saliu and Oboh 2013).

In vivo clinical studies were carried out to prove their antidiabetic effect. Streptozotocin (STZ)-induced diabetic rats showed a significant reduction in the blood glucose level and cholesterol level with methanolic extracts of *A. viridis*, *A. spinosus*, and *A. caudatus* leaves, proving it to be good antidiabetic and anti-cholesterolemic agent (Girija et al. 2011). Similarly, 50% ethanolic extract of *A. spinosus* showed similar reduction in blood glucose level on the STZ-induced albino mice, while, in STZ-induced diabetic rats, significant reduction in degeneration of pancreatic cells was observed. This extract also increased the activity of nonenzymatic and enzymatic oxidant (Mishra et al. 2012).

Antimicrobial and Anti-inflammatory Activity

Human consumption of fresh and processed GLVs are generally recognized as safe (GRAS). The bioactive components of GLVs possess many biological activities such as antimicrobial and anti-inflammatory activity (Faller and Fialho 2009). These are famous among the population due to their esthetic features like flavor, color, and therapeutic values (Gutierrez et al. 2008). Due to their GRAS status, food industry makes use of these GLVs as antimicrobial especially antibacterial agent. These vegetables also exhibit many pharmacological effects like enhancing fertility in females, reducing blood pressure, and using antibiotics in the human body (Mensah et al. 2008). Important GLVs such as *Lactuca sativa*, *Coriandrum sativum*, *Mentha piperita*, *Raphanus sativus*, and *Portulaca oleracea* in methanolic extract have high antibacterial activity (Bhat and Al-Daihan 2014). Freeze-dried cilantro (*C. sativum*) and parsley (*Petroselinum crispum*) were used on *Escherichia Coli* and *Bacillus subtilis* with effect cell damaged and growth inhibition. These GLVs having high antibacterial activity and being the future of food system need to be studied for their potential role against specific microbes (García-Lafuente et al. 2009).

Phenolics and flavonoids of GLVs are studied from many decades since they function like plant hormone regulators, which is natural protectant against many microbes in the plant cell. Phenolic compounds of GLVs like spinach and the mustard plant are also studied for their anti-inflammatory potential (García-Lafuente et al. 2009). Activation of NF-6B is very important for many inflammatory processes which can be inhibited by the use of extract of *Urtica dioica* (Guil-Guerrero et al. 2003). Saponins present in *Euphorbia hirta* and *Ipomoea involucrata* have antifungal/antiyeast, anti-inflammatory, antibacterial, cytotoxicity, antiparasitic, antiviral, antitumor, and many other biological functions (Sparg et al. 2004). Hydroalcoholic leaf extract of *A. tricolor* showed an antinociceptive and anti-inflammatory activity in the clinical studies on the induced rats (Bihani et al. 2013). Seed and leaf extract was obtained from *A. viridis*, and it showed significant inhibition of targeted bacte-

rial and fungal strains, thus proving to be an effective antimicrobial agent (Ahmed et al. 2013).

Antioxidant Property

Cells pretreated with *A. lividus* and *A. tricolor* extracts showed decrease in toxicity and suppression in the production of oxidative genes like RAGE, HMOX-1, and RelA/NF-jB in SH-SY5Y cells. This property is helpful in preventing neurodegenerative disorders and age-related diseases (Amornrit and Santiyanont 2016). Partially purified alkaloids (PPA) from *A. viridis* were used on human erythrocytes and were observed to reduce the lipid peroxidase activity while maintaining the antioxidant concentration from decline. This proves that alkaloid can be effectively used to prevent age-related problems and other free radical-mediated oxidative damages in the body (Sasikumar et al. 2015).

Cardiovascular Disease and Leafy Vegetables

In various countries of America and South Africa as well as non-African countries, the extent of obesity among children and adults are increasing in the tremendous rate. This might be due to less consumption of fruits and vegetables, implementing western lifestyle and lack of physical exercise (Mauriello et al. 2006). Thus, addition of GLVs in their diet along with physical activity and change of lifestyle has proved to reduce the risk of CVD. Higher intake of fiber from GLVs and weight of human have shown an inverse relation (Tohill et al. 2004). Similar relation is found between the evidence of coronary heart disease (CHD) as well as stroke and intake of GLVs (He et al. 2006). It was proved by meta-analysis that incidents of CVD can be reduced by 4% with the addition of GLV (Dauchet et al. 2006). Soares et al. (2015) in his study used three peptides from *A. cruentus* showing a significant reduction of cholesterol enzyme like HMG-CoA reductase, proving to be an effective anti-cholesterolemic agent.

Hypertension and Leafy Vegetables

Israili et al. (2007) reported that the major reasons for visit of patients in hospital are hypertension and their rate of increase in many countries are exponential. Direct relationship between the conditions such as stroke, CHD, cerebrovascular accident (CVA), end-stage renal disease, myocardial infarction, and congestive heart failure and the rate of morbidity and mortality is observed. Patients with diabetes and kidney disease have added problems with low blood pressure; thus its control is more

harmful. The main reason for this disorder is improper diet, and thus it can be treated with diet high in magnesium and potassium, while the intake of sodium should be reduced. Direct relation is observed between the occurrence of CVD, stroke, and BP with sodium, whereas inverse relation is observed when diet with potassium is consumed.

Risk of hypertension is one in every three persons in the highly industrialized countries where people consume diet high in refined carbohydrates, trans fats, saturated fats, processed foods, low fiber, reduced dietary magnesium, potassium, and large amounts of dietary sodium (Adrogué and Madias 2007). Epidemiological studies prove that people consuming vegetarian diet/primeval diet have low risk of CVD and blood pressure in the industrial area (Fujiwara et al. 2000).

Homeostatic balance maintained by low level of sodium and high level of potassium leads to vasodilatation of the blood vessels for sufficient flow rate of blood to heart. When such conditions are not maintained, it leads to vasoconstriction and low supply of blood to heart. Calcium also plays an important role in maintaining such balance for the healthy life. Increasing vasodilation by altering potassium is possible with many mechanisms such as reduced vasoconstrictive sensitivity to norepinephrine, intracellular sodium and tonicity, and natriuresis, angiotensin II, increased sodium/potassium ATPase activity, and urinary kallikrein, increased serum, proliferation in vascular smooth muscle, improved insulin sensitivity, alteration in DNA synthesis, sympathetic nervous system cells, reduction in cardiac diastolic dysfunction, decrease in vascular intracellular sodium and tonicity, reduction in transforming growth factor (TGF)-beta, decrease in NADPH oxidase, decrease in neointimal formation, oxidative stress and inflammation (Randhawa et al. 2015).

Fertility and LV

Dark colored GLVs are rich in chlorophyll, antioxidants, vitamins, fibers, phytochemicals, and minerals. These vegetables can be eaten raw, semi-cooked, or cooked. But it is recommended to eat raw because it prevents the loss of vitamins and certain important minerals. Minerals which are important for fertility in women are folate, vitamin C, and zinc, which depletes on cooking. The presence of diindolylmethane in certain GLV like Brussels sprouts, broccoli, and cabbage not only retains nutritional factors in the body but also helps the body to get rid of "bad" estrogen, which is very important for fertility. Thus, it is recommended to consume these vegetables at least twice in a week. Folic acid is very important for the development of fetus even before the women gets the positive pregnancy test. Spinach and asparagus are rich source of iron and are proved to reduce the condition of anovulation and possibly poor egg health during pregnancy. Inhibition of pregnancy-related issues is 60% reduced with consumption of iron-rich food than those with insufficient iron in their blood. Vitamin C is very important for absorption of iron, and being an antioxidant, it is also important to protect cells from the damage caused by the free radicals. GLVs also provide humans with sufficient amount of vitamins,

minerals, and other nutrients while maintaining acidic/alkaline balance. Spinach being rich in vitamins, nutrients, iron, and folic acid is a very good source for good health of fetus, fertility, and reproduction. Where iron promotes oxygen levels in fetus, organs, and cells, folic acid is important for preventing neural tube defects in the growing fetus. Healthy alkaline environment is important for the survival of sperm and its journey to egg. Minerals serve as a medium for activating whipping of sperm tail in the vaginal fluid, which is important for mating and creating healthy new born.

Anticancerous Properties

Antiproliferative and chemopreventive effects of polyphenols are available with in vitro studies. *A. cruentus* is suggested to be cheap, commercial, and biocompatible alternative on peripheral lymphocytes than already available antiproliferative therapeutics (Gandhi and Niraj 2011). Ethyl acetate, hexane, and methanolic extracts of *A. tristis Roxb.* were also found to be effective against colon adenocarcinoma cell line with minimum side effects (Baskar et al. 2012). *A. hybridus* and *A. caudatus* reduce micronuclei formation and safeguard the detoxifying enzymes like alkaline phosphatase (ALP) and GGT in sodium arsenite-treated albino Wistar rats, thus proving it to be an affective carcinogen (Adewale and Olorunju 2013). Stem extract of *A. lividus* and seed extract of *A. hybridus* inhibited the growth of EAC cells by 43 and 45%, respectively. This also showed downregulation of Bcl-2 mRNA in treated mice and upregulation of Bax, p53, and caspase-3, which clearly proves the mitochondrial apoptosis of EAC cells when compared with control sample (Al-Mamun et al. 2016). *A. viridis Linn*. leaves extract was prepared by 50% ethanolic solution which had better antiproliferative effect against CEM, Jurkat, and HL-603 (human leukemic cell lines) when compared with the stem. It was observed in the study that standard curcumin showed antiproliferative effect on both normal cells and leukemic cells, while both stem and leaves extracts had positive effect by enhancing the growth of normal cells (Larbie et al. 2015).

Hepatoprotective

Hepatotoxicity can be caused due to excess of alcohol consumption, autoimmune response, toxic effect of curative drugs, or any other infections. This can be prevented by giving a hepatoprotective medicine, allergy medicine, or natural extracts. The hepatoprotective effect was shown by the extract of *A. tricolor* roots against the biochemical, physical, functional, and histological changes produced by paracetamol in Wistar albino rats. This effect is evident by reduced serum enzyme activities such

as SGOT, SGPT, TB, and ALP, and this was compared with standard drug silymarin which is hepatotoxic drug against paracetamol. This was also supported by histopathological studies of the liver (Aneja et al. 2013)

Gastroprotective

Gastroprotectors of plant origin have positive effect on the mucosa of gastrointestinal tract and are used clinically. Gastric ulcers were healed by using poly-herbal formulation having *A. tricolor* as one of the ingredient (Devaraj and Krishna 2013). Similar work with hydroalcoholic extract of piperine and *A. roxburghianus* roots showed hemorrhage, minimal ulceration, necrosis, and decreased levels of myeloperoxidase, leucocyte infiltration in histopathological observation, and MDA and increased glutathione levels in colon and blood tissue in rats having ulcerative colitis (Nirmal et al. 2013). Cysteamine-induced duodenal ulcers and ethanol-induced gastric ulcers can be significantly treated by *A. spinosus* leaves powder (Mitra et al. 2013).

Antimalarial

The development of resistance by the exposed species, nontarget specificity of chemical insecticides, and eco-hazardous nature of chemical pesticides provoke us to find an eco-friendly alternative in terms of usage of herbs, their extract, or powders. Utilizing ethanolic extract of *A. spinosus* leads to increased hemoglobin content, gain of BW, and blood schizonticidal activity in *Plasmodium berghei*-infected mice. This antimalarial effect was significantly comparable to synthetic drug, chloroquine (Susantiningsih et al. 2012). Another study was carried out on *A. hybridus L.* for treating malaria. This species is used from ancient times by Msambweni community of Kenyan South Coast (Nguta et al. 2010).

Other Effects

Extracts from various species of Amaranthus like *A. spinosus* were found to have spasmolytic, laxative, and bronchodilator properties (Koffuor et al. 2017), where ethanolic extract of up to 240 mg/kg had no adverse effect proving to be a better alternative against ischuria. While the higher dosage was found to show certain physiological changes when compared with that of escitalopram and imipramine effects (Kumar et al. 2014).

Different Methods of Processing Green Leafy Vegetables

Green leafy vegetables generally grow amply during the monsoon season; however, they are greatly perishable which affects its shelf life, and sometimes raw leafy vegetables are unpalatable (Chinyere and Obasi 2011).Various processing treatments such as sun drying, shade drying, freeze-drying, blanching, and vacuum cooling are employed to improve the palatability and bioavailability and extend the shelf life of the green leaves. The quality of green leafy vegetables can be improved by suitable processing treatment which prevents the loss of nutrient content and processing should be done after immediate harvest. The safety of green leafy vegetables can be achieved by proper handling during harvesting and appropriate refrigeration condition. For example, cooling the crops at 4 °C prevents the growth of microbes such as *E. coli* O157:H7, *L. monocytogenes*, and *Salmonella* (Solomon et al. 2009). In freeze-drying, dehydration takes place by the sublimation of water from frozen product. It improves the quality of vegetables and increases the shelf life of the product. Because at low temperature and in the absence of liquid water, microbial reaction and deterioration are arrested this improves the final quality of the product. It also prevents the loss of flavor and bioactive compound and protects the primary structure and shape of the product (Sadikoglu and Liapis 1997; Tambunan et al. 2001; Rawson et al. 2011).

Oboh and Akindahunsi (2004) examined the effect of sun drying on ascorbic acid, total phenol, and antioxidant activity of common green leaves in Nigeria such as *Telfairia occidentalis* (Ugu), *Amaranthus cruentus* (Atetedaye), *Corchorus olitorius* (Ewedu), *Solanum macrocarpon* (gbagba), *Structium sparejanophora* (Ewuro-odo), *Ocimum gratissimum* (Efinrin), *Baselia allia* (Amunu tutu), and *Vernonia amygdalina* (Ewuro). The results revealed that sun drying of green leafy vegetables reduces the ascorbic acid content (16.67–64.68% loss), but contrarily it increases the total phenol content (6.45–223.08% gain). It also increases the antioxidant activity of green leafy vegetables (126.00–5757.00% gain). Even though the ascorbic acid content decreases, the antioxidant activity of the green leafy vegetables won't be reduced by the sun drying, because the phenol content of leaves contributes more to the antioxidant properties than ascorbic acid of green leafy vegetables. Suffo et al. 2016 reported that the shade drying and sun drying improve the palatability of green leafy vegetables by reducing their anti-nutritional content. And it also extends their shelf life. They also examined the impact of processing method on chemical composition and antioxidant activity of two *Amaranthus* sp. (*A. hybridus and A. cruentus*). The results of shade drying showed significant increase in phenol content and antioxidant activity and also caused significant decrease in ascorbic acid of green leafy vegetables.

Blanching of vegetables at high temperature may inactivate some microorganism and destroy the enzymes in the tissue. It also inhibits the enzyme action, changes the color, eliminates the anti-nutritional factor and acid component, and reduces the dehydration and drying time (Akindahunsi and Oboh 1999). Green leafy vegetables are good source of minerals (iron, magnesium, zinc, and calcium) and vitamins (A,

Table 4 Effect of processing on phytochemicals (Adopted from Kuriakose and Rawson 2015)

Sl no	Type of leafy vegetable	Type of processing	Treatment conditions	Effect on phytochemicals	References
1.	*Structium sparejanophora*	Blanching	100 °C 5 min	TP↑, vit C↓	Oboh and Akindahunsi 2004
2.	*Amaranthus cruentus*	Blanching	100 °C 5 min	TP↔, vit C↓	Oboh and Akindahunsi 2004
3.	*Telfairia occidentalis*	Blanching	100 °C 5 min	TP↑, vit C↓	Oboh and Akindahunsi 2004
4.	*Basella alba*	Blanching	100 °C 5 min	TP↑, vit C↓	Oboh and Akindahunsi 2004
5.	*Solanum macrocarpon*	Blanching	100 °C 5 min	TP↑, vit C↓	Oboh and Akindahunsi 2004
6.	*Corchorus olitorius*	Blanching	100 °C 5 min	TP↑, vit C↓	Oboh and Akindahunsi 2004
7.	*Vernonia amygdalina*	Blanching	100 °C 5 min	TP↔, vit C↓	Oboh and Akindahunsi 2004
8.	*Ocimum gratissimum*	Blanching	100 °C 5 min	TP↑, vit C↓	Oboh and Akindahunsi 2004
9.	*Lactuca sativa L.*				
	Green variety – iceberg, romaine and continental	Fresh sample (analysis of vitamin C)	–	Caffeic acid and vitamin C present in larger amounts in green variety	Llorach et al. 2008
	Red variety –red oak leaf and lollo rosso	Freeze-dried sample (analysis of antioxidant)	–	Flavonols present largely in red variety,anthocyanins present only in red variety	
10.	*Amaranthus* leaves	Finely chopped leaves		Moisture (90.35) and ash content (1.36), vitamin C (1.57 mg), and iron (535.84 ppm)	Funke 2011
		Steamed and then chopped	2 min	Crude fat per gram of sample (2.31), protein (4.35), and fiber (1.09)	
		Blanched and then chopped	2 min	Carbohydrate (4.89)	

B complex, and C). The processing treatment like blanching has an adverse effect on minerals and vitamin availability. The iron availability of green leafy vegetables is reduced by processing due to the loss of ascorbic acid content (iron enhancer)

because vitamin C is light and temperature sensitive. Latunde-Dada 1990 also reported that blanching and squeeze washing of vegetables result in reduction of dialyzable iron. Effects of processing on phytochemicals of green leafy vegetables are mentioned in Table 4 (Kuriakose and Rawson 2015).

Conclusions

Green leafy vegetables are significant source of primary and secondary metabolites. And their consumption in human diet could increase the chances of various health benefits and may play a role in preventing human disease in which free radicals are involved, such as cancer, cardiovascular diseases, and aging. Processing also affects the composition of primary and secondary metabolites in leafy vegetables, and a low-temperature treatment may be able to retain the metabolites in leafy vegetables.

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Phenolic Acids and Their Health-Promoting Activity

Sana Khatri, Additiya Paramanya, and Ahmad Ali

Abbreviations

AGE	Advanced glycation end product
PRP	Proline-rich proteins
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SOM	Soil organic matter

Introduction

Phenolics are a diverse group of compounds which are identified by the presence of a hydroxyl group (–OH) linked to an aromatic hydrocarbon group. The structural diversity of these compounds ranges from simple structure (phenolic acids) polyphenols (flavonoids) to polymeric compounds. The simplest member of this group is carbolic acid or phenol (C_6H_5OH).

Phenolic compounds are polyhydroxylated phytochemicals, which have common structures. These can be further divided into three main subclasses – the flavonoids, the phenolic acids, and the tannins (Fig. 1). Flavonoids contains two or more aromatic rings, each bearing one or more phenolic hydroxyl groups which are connected by a carbon bridge, consisting of three carbon atoms. Most flavonoids bear this type of structure which have been further divided into subclasses, based on the position of the second ring relative to the C ring, as well as the functional groups (ketones, hydroxyls) and presence or absence of a double bond in the C ring (Ho 1993). These subclasses are flavones, isoflavones and isoflavonoids, flavanones, flavanols, anthocyanidins, chalcones, and dihydrochalcones.

Phenolic acids are further subdivided in two main groups – benzoic acid derivatives and cinnamic acid derivatives containing seven and nine carbon atoms,

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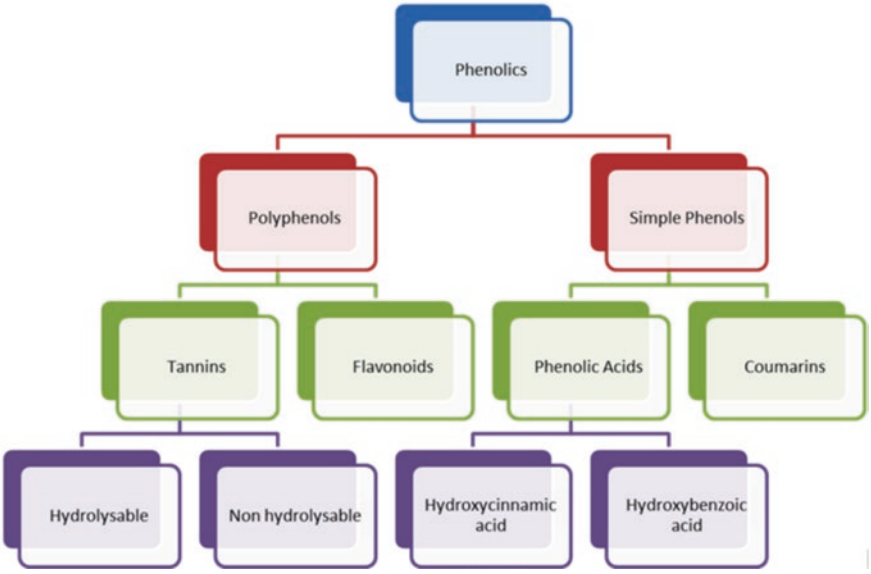

Fig. 1 Classification of phenolic compounds

respectively. All these compounds are hydroxylated. The main representative isolated compounds are caffeic acid, chlorogenic acid, ferulic acid, gallic acid, gentisic acid, and *p*-coumaric acid. Stilbenoids – the smaller subclass – include the polyhydroxylated stilbenes, resveratrol being the main representative (Castelluccio et al. 1995).

Polyphenols are considered as potent antioxidants since they are able to scavenge free radicals. It was first thought that the health benefits associated with the consumption of dietary polyphenols were due to antioxidant mechanisms but sometimes may not act as antioxidants, as they may exert modulatory actions through protein and lipid kinase signaling pathways in cells (Decker 1995). In addition to these antioxidant activities, phenolics have also been shown to possess antimicrobial, anticancer, and anti-inflammatory properties. Recent reports suggest their potential role as antiglycating agent in the prevention of glycation, a process which has been implicated in most of the secondary complications of diabetes. This chapter deals with the phenolic acids in plant, food, and soil and their health-promoting activities.

Plant Phenolics

Introduction

Plants have a diverse composition of organic compounds which can be classified as primary or secondary metabolite. Primary metabolites include nucleic acids, proteins, and other such components which are omnipresent in all plants and are

required for their growth and development, while the secondary metabolites have not directly been linked to the basic photosynthesis and respiration system but play an important role in plant's survival in the environment.

The diversity in the nature of the secondary metabolites possessed by plants is known to the world since long, and human being have used plants for all purposes including medicine. Along with food, plants produce certain nonessential and non-nutritive chemicals known as phytochemicals. These compounds have been proven to perform protective functions in plants as well as humans. Some of the important classes of compounds which have been characterized and explored for the benefit of human and other animals include alkaloids, phenolics, tannins, and terpenes.

All types of polyphenols are thought to be found in plants, soils, and food. In plants, the phenols are esterified with glucose and carbohydrates as free aglycones. Dietary polyphenols isolated from fruits (berries, apples, citrus, cherries); vegetables (onion, celery, beer hops, soybeans); herbs, roots, and spices (gingko, turmeric); green and black tea; and red wine when consumed result in benefits on human health and lower incidence of heart disease, cancer, gastrointestinal and neurological diseases, liver diseases, atherosclerosis, obesity, and allergies (Buendia et al. 2010).

Phenolic acids that are most common in plant tissues are hydroxycinnamic acids (Fig. 2). Caffeic acid; chlorogenic acid; *o*-, *m*-, and *p*-coumaric acids; ferulic acids; and sinapic acids are included in this broad class (Castelluccio et al. 1995). All these acids in plants occur mostly as esters or glycosides, in the associated form, for example, as the lignin component. Particular hydroxycinnamic acids occur in the ester form associated with carboxylic acids (Fernández de et al. 1992). Caffeic acid is one of the most widespread hydroxycinnamic acids, which is found mainly in coffee, apples, potatoes, spinach lettuce, cabbage, olive oil, wine, and tobacco leaves (Breinholt 1999). Hydroxybenzoic acid (Fig. 2; such as gallic acid, *p*-hydroxybenzoic acid, protocatechuic acid, vanillic and syringic acids) is another important group of phenolic acids (Bravo 1998). The hydroxybenzoic acids occur mostly in the glycoside form. In plant tissues, phenolic acids can be bound to various compounds, e.g., flavonoids, fatty acids, sterols, and cell wall polymers (Heleno et al. 2015).

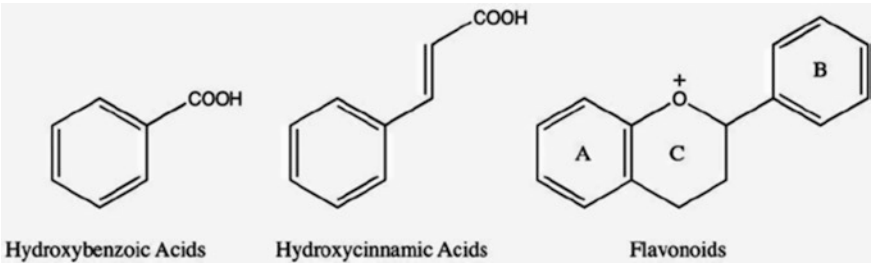

Fig. 2 Representative structures of phenolic acids

Flavonoids

Flavonoids are ubiquitous in plants; almost all plant tissues are able to synthesize flavonoids. There are also a wide variety of types – at least 2000 naturally occurring flavonoids. They are considered as the largest and most diverse group of phenolic compounds in plants. These compounds usually occur as glycosides which can be dissolved in the vacuolar juice (mainly in the *O*-glycoside form, rather than *C*-glycosides). They are crystallized around the epidermis. Anthocyanins in many species of plant are accumulated in the vesicles which get developed in the vacuole. Flavonoids are very often, the only metabolites in dicotyledonous plants, showing the presence of pharmacological activity (Arct and Pytkowska 2008). Fruits and vegetables are the main sources of flavonoids in the diet. They also occur in certain grains, seeds, and spices, as well as in wine, tea, coffee, cocoa, and herbal essences (Aherne and O'Brien 2002). They can be classified into seven groups: flavones, flavanones, flavonols, flavanonols, isoflavones, flavanols (catechins), and anthocyanidins.

In general, the leaves, flowers, and fruits of the plant contain flavonoid glycosides, woody tissues contain aglycones, and seeds may contain both. As a result of their ubiquity in plants, flavonoids are an integral part of the human diet. It is estimated that the average American's daily intake of flavonoids is close to 1 g per person. Hertog et al. (1993) also found that the major sources of flavonoid intake were tea (61%), onion (13%), and apple (10%). Certain later studies provided more precise individual data concerning the intake of various classes of flavonoids. For example, the consumption of flavonols for American has been estimated at 20–25 mg/d.

Stilbenes

Stilbenes are phenolic compounds which contain two benzene rings separated by an ethane bridge. They are widely distributed in higher plants, and their main physiological roles relate to their action as phytoalexins and growth regulators (Gotham 1989). Stilbenes had not caught the attention of food and nutritionists until one of its family members, resveratrol (3,5,4′-trihydroxystilbene), was reported to demonstrate a preventing effect on cancer (Jang et al. 1997). Resveratrol is present in many plant species, including those that are popular components of the human diet such as grapes, peanuts, and berries. Plants synthesize it which can be used as a part of defense mechanisms against mechanical injury, pathogen infection, and UV radiation (Ososki and Kennelly 2009).

Lignans

Lignans are dimers of phenylpropanoid units linked by the central carbons of their side chains. These lignans and their higher oligomers in plants act as defensive substances. Lignan-rich plant products were found to be active ingredients in the

treatment of disease in Chinese folk medicine. Unfortunately, many of the active ingredients of these plant products have not been scientifically tested as therapeutic agents (Ayres and Loike 1990). However, flax and sesame lignans have been considered as important components with health benefits (Kang et al. 1998).The main lignan compounds include lariciresinol, pinoresinol, and matairesinol – all of which are phenylpropanoid dimmers and mostly occur in the seed of flax and sunflower (Kurzer and Xu 1997); but also small amounts of them can be found in grains, vegetables, fruits, nuts, tea, and coffee (Milder et al. 2005).

Tannins

Depending on their structures, tannins are widely distributed in plants and may occur as hydrolysable tannins (formed in the pathway of the phenolic acids with sugar polymerization) and condensed tannins (combination of flavonoids) (Ashok and Upadhyaya 2012). Hydrolysable tannins are glycosylated gallic acids (Ho 1993). Condensed tannins also known as proanthocyanidins are linear polymers of flavan-3-ol (catechin and gallocatechin) and flavan-3,4-diol units. In the condensed tannins, the units are linked through the interflavonoid bonds between C-4 and C-8 or C-6. Tannins occur widely in different foods and are often concentrated in the skin of fruits and seed coats, among others. The plant phenolic compounds present a variety of colors from colorless to intense vibrant dyes such as red or violet. In the plant cells, phenolics are stored near the chloroplast, or they are accumulated in vacuoles, where they may polymerize and strengthen the cell wall.

Variability Related to Plant Samples

Botanical and geographical origin, cultivation conditions, maturity, and post-harvest storage conditions are considered as additional variation sources. Varietal differences in phenolic composition are well documented in numerous fruits, including apple (Wojdylo et al. 2008), strawberry (Buendia et al. 2010), grapes (Mattivi et al. 2006), or banana (Ucle's et al. 2010). Varietal differences are also observed in the phenolic distribution between the various parts of fruits, as illustrated for grape. Biosynthesis of proanthocyanidins and phenolic acids takes place at early stages of fruit development, while accumulation of anthocyanins occurs during ripening. Formation of flavanols in fruits takes place mostly at two stages, i.e., at flowering and in the ripe fruit. Increase in resistance to thiolytic degradation of grape seed procyanidins occurs during ripening attributes to oxidation reactions (Kennedy et al. 2001). Arabidopsis seeds lacking brown coloration contained much higher levels of procyanidins (especially extractable ones) than the wild type seeds, confirming that seed coat browning is caused by the oxidation of flavan-3-ols (Pourcel et al. 2007). In the formation of flavan-3-ol-flavonol adducts observed during aging of pinto beans, although the ethyl bonds resulting from the latter reaction are cleaved

by thiolysis, additional derivatives are yielded that are usually not considered when analyzing proanthocyanidins (Beninger et al. 2007).

Phenolic Compounds in Cultivated Plants

Heldt et al. (1997) have mentioned that plants synthesize primary carbohydrates, lipids, and proteins. Lipid precursors and aromatic amino acids synthesize secondary plant compounds. Phenolic compounds are responsible to build an important portion of the secondary plant compounds. The most important phenolic compound in plants is lignin – a complex polymer of phenylpropane units. Another abundant compound class includes the flavonoids, stilbenes, coumarins, and polyflavonoids (condensed tannins). Phenolic acids in plants have diverse functions – stabilization of the structure, protection from herbivory, protection from ultraviolet (UV) light, coloration of blossoms, exchange of information with symbionts, and biocidal effects against bacteria and fungi. After the death of plants, phenolics may persist for weeks or months affecting the decomposer organisms and decomposition processes in soils (Horner et al. 1988). Therefore, their effects are not restricted to single plants but may extend to the functioning of whole ecosystems.

Phenolic compounds may have both beneficial (Bitsch 1996) and toxic (Schroder 2007) effects on human health. Toxicologic effects of most phenolic compounds in plants are limited as compared to pesticides and other anthropogenic chemicals. External stimuli are responsible modulate the synthesis and therefore change the chemical composition or quantities of phenolic compounds in the plants. External stimuli include microbial infections, UV light, and mechanical wounding of the plant as well as chemical stressors such as heavy metals and pesticides (Gotham 1989).

Composition of Phenolics in Common Foods

Food phenolic composition is highly diverse which reflects the diversity of plant composition and additional complexity brought by reactions of plant phenolics in its processing and storage. Some families are widespread in the diet, while others are restricted to or particularly abundant in specific foods (e.g., isoflavones in soybeans, dihydrochalcones in apple). Proanthocyanidins are present in most fruits, as well as in fruits juices, wines, and beers. They are the most abundant phenolics in commonly consumed fruits such as grape (Mane et al. 2007), apple (Wojdylo et al. 2008), or strawberry (Buendia et al. 2010) and also found in large amounts in transformed food products such as red wine or cider. Dietary exposures to hydrolysable tannins are rather limited (Clifford and Scalbert 2000). Their major dietary sources are berries such as raspberry, blackberry, and strawberry (Torronen 2009), but the levels reported are rather low, ranging from 10 to 22 mg/100 g fresh weight in

strawberry (Buendia et al. 2010) and from 68 to 331 mg/100 g fresh weight in berries consumed in Finland, namely, cloudberry, rose hip, raspberry, and strawberry (Koponen et al. 2007).

Ellagitannins are abundant in pomegranate but found mostly in the nonedible fruit rind. However, punicalagin isomers and other ellagic acid derivatives were detected (1500–1900 mg/L) in industrial juices as a result of extraction from the rind (Gil et al. 2000). They can also be present in low amounts in beverages such as wine or whisky, due to their release from wood barrels. As most flavonoids are present as a number of glycosides, they vary in their chemical and biological properties. For instance, the anthocyanin profiles of foods are based on 6 common anthocyanidins but nonetheless highly diverse, the number of anthocyanins varying from 2 (in peach or pistachio) to 34 in radishes (Wu and Prior 2005a, b).

Effect of Phenolic Compounds on Food Quality

Phenolic compounds share some common properties inherent to the phenol ring (Quideau et al. 2011). They also exhibit a variety of properties linked to their particular structural features, which affect food quality and can be modified during processing.

Phenolic compounds are closely associated with the sensory and nutritional quality of fresh and processed plant foods (Macheix et al. 1990). The enzymatic browning reaction of phenolic compounds, catalyzed by polyphenol oxidase, is of vital importance to fruit and vegetable processing due to the formation of undesirable color and flavor and the loss of nutrients. For examples, polyphenol oxidase was found to be responsible for the browning of grapes (Sapis et al. 1983), and catechin, polyphenol oxidase, and oxygen were reported to be required for the browning of yams (Ozo and Caygill 1986).

A common approach for the prevention of the browning of food and beverages has been the use of anti-browning agents. The most widespread anti-browning agents used in the food and beverage industries are sulfites. Due to the health risks of sulfating agents (Taylor et al. 1986), the FDA has banned or limited the use of sulfites in certain foods. Oxidative changes of polyphenols during processing are important for the development of color and flavor in certain foods. Browning of polyphenols is a natural process of cocoa fermentation.

Changes Induced by Food Processing

Food phenolic composition is usually different from that of the raw material, due to selective extraction, especially in the manufacture of beverages but also in peeling or deseeding operations and conversion of plant phenolics to new and mostly unknown derivatives. For example, the phenolic content of strawberry juices

increased from clear juices to cloudy juices and purees, only by about 20% for anthocyanins but for up to threefold to fourfold for proanthocyanidins (Oszmianski and Wojdylo 2009). aDP values of proanthocyanidin were also higher in puree (4.1–7.1) than in clear juice (2.6–3.2), as expected from proanthocyanidins associated with plant cell walls and other solid plant material. Large losses of anthocyanins and proanthocyanidins occurred during storage, especially at higher temperature, but some protective effect was observed in the puree and turbid juices, presumably due adsorption of phenolics on the solid material and association with pectins or formation of anthocyanin-proanthocyanidin complexes.

Changes taking place during processing have been particularly investigated in black tea and in red wine (Harbowy and Balentine 1997). Other examples include reactions of ellagitannins in food processing (Bakkalbasi et al. 2009) and in whisky aged in oak barrels (Tanaka et al. 2010). Conversion of flavanol monomers, abundant in green tea, to the black tea pigments is driven by enzymatic oxidation, catalyzed by polyphenol oxidase (Roberts et al. 1959), and yields two major groups of pigments, namely, theaflavins and thearubigins. Theaflavins, based on a benzotropolone structure (Ollis et al. 1966), and numerous oxidation products formed from them have been identified (Drynan et al. 2010), while little is known about thearubigin structures (Haslam 2003). As mentioned above, recent studies suggest that they are rather low molecular weight compounds (in the range 1000–2100 Da) formed by successive steps involving oxidation of *o*-diphenol groups to *o*-quinones followed by nucleophilic addition of either another phenolic ring or water (Kuhnert et al. 2010).

Phenolic Acids in Soils

Phenolics consist of more than one aromatic ring, bearing one or more hydroxyl functional groups. They are originated from plant materials and industrial products/wastes, thereafter entering the soil either as leachates or as particulate matter (Hättenschwiler and Vitousek 2000). Once integrated into the soil, phenolics can control belowground processes, including SOM decomposition (Freeman et al. 2004) and nutrient cycling (Kraus et al. 2004). Decomposition of phenolics in soils, modification of the rate of SOM decomposition, and current environmental changes influence the fate of phenolics in soils (Appel 1993). Phenolics affect the cycling key nutrients to plants and soil microorganism (Hättenschwiler and Vitousek 2000).

Effect of Environmental Changes on Phenolics

Environmental changes such as elevated CO_2, warming, N deposition, and drought may be responsible in affecting phenolic production from plant tissues, subsequent degradation in soils, and SOM decomposition.

Elevated CO_2

Elevated CO_2 usually increases phenolic concentrations in plants. In field CO_2 enrichment experiments, phenolic compounds in plant tissues, such as leaves, needles, stems, and rhizomes, increase by 11–18% (Booker and Maier 2001). Elevated CO_2 can increase carbon supply and nutrient (e.g., nitrogen) stress in trees, resulting in decreased carbon demand. Such change is known to accelerate the accumulation of total nonstructural carbohydrates and the synthesis of carbon-based secondary or structural compounds (Penuelas and Estiarte 1998). The downstream processes are impacted by the changes in the concentration of phenolics in plant tissues. For example, (Siegenthaler et al. 2010) found that elevated CO_2 induces a production of phenolic-rich litters, resulting in declining SOM decomposition. The effects of elevated CO_2 on litter chemistry and decomposition rates in upland vegetation and demonstrated that elevated CO_2 increases lignin content in leaf litter significantly, but there is no significant effect on decomposition rate (Norby et al. 2001).

Warming

An increase in atmospheric CO_2 concentration is involved in accompanying rising temperature. The effect of warming on phenolic production and degradation can be measured with an emphasis on whole organic matter decomposition. Unlike the rather unidirectional influences of elevated CO_2, warming has various effects on the production of phenolics (Table 3.2). Increase in temperature has led to both an increase (Yi and Wetzstein 2010) and a decrease (Veteli et al. 2007) in phenolic production. Warmer conditions usually accelerate biochemical reactions and may result in lowering production of secondary metabolites because plant growth would be enhanced.

A lower phenolic content under warming conditions has been reported in a meta-analysis by Zvereva and Kozlov (2006). However, interactive or simultaneous effects of elevated CO_2 and warming in relation to phenolics production have not been reported (Raisanen et al. 2008) because two effects often negate each other (Zvereva and Kozlov 2006).

N Deposition

Nitrogen enrichment can be studied in terms of atmospheric nitrogen deposition and fertilizer additions. Phenolic concentrations remain unchanged after nitrogen enrichment (Nybakken et al. 2009). Often, nitrogen enrichment decreases phenol oxidase (Ellis et al. 2009), while hydrolases are often activated. Sinsabaugh (2010) reviewed that responses of phenol oxidase to nitrogen enrichment can differ by the

types of ecosystem determined, as it decreases its activity in the forest and increases it in grassland or agricultural system. These contrasting results may originate from the initial lignocellulose contents in litter. Bragazza et al. (2006) have reported that nitrogen deposition can accelerate carbon release from peat bogs by activating phenol oxidase.

Drought

Global climate change models often predict an increase in frequency and intensity of drought. Such changes can affect water availability in terrestrial ecosystems and water levels in wetlands. In wetlands, the effects of drought on nutrient cycling draw much attention due to their close association with water. Ellis et al. (2009) have shown resultant stimulated decomposition due to increased level of phenol oxidase in the drought. However, in another report it was found that the activity of phenol oxidase decreased in peatland due to stimulated drought (Toberman et al. 2010). Also, he suggested that initial water content in soils may be responsible for these contrasting responses and that a hyperbolic relation exists between water content and phenol oxidase.

Health-Promoting Activity of Phenolic Compounds

Dietary polyphenols are attracting great interest as they may play a role in lowering the incidence of certain degenerative diseases associated with consumption of plant-based foods. However, scientific evidence is not yet conclusive, and more human studies are needed (Torronen 2009). The biological effects of dietary phenolics may involve more complex mechanisms such as modulation of cell signaling pathways and impact on intestinal flora (Manach et al. 2009). Wine phenolics, being potent antioxidants, can efficiently reduce oxidizing species and inhibit the peroxidation of dietary lipids in the digestive tract. One of the major effects of dietary tannins is the reduction of nutrient absorption, resulting from their interaction with other food macromolecules and inhibition of digestive enzymes. Synthesis of salivary PRPs that show particularly high affinity toward tannins may be a defense mechanism developed by mammals to counteract these deleterious effects. However, complexation of tannins with food matrix components such as pectins may limit their interaction with PRPs. Interactions with food components or PRPs may also impact the intestinal absorption of tannins (Cai and Bennick 2006) and modify gut microflora metabolism (Bazzocco et al. 2008), provided they survive the digestive process.
Almost all phenolics possess several common biological and chemical properties. They are shown in Fig. 3.

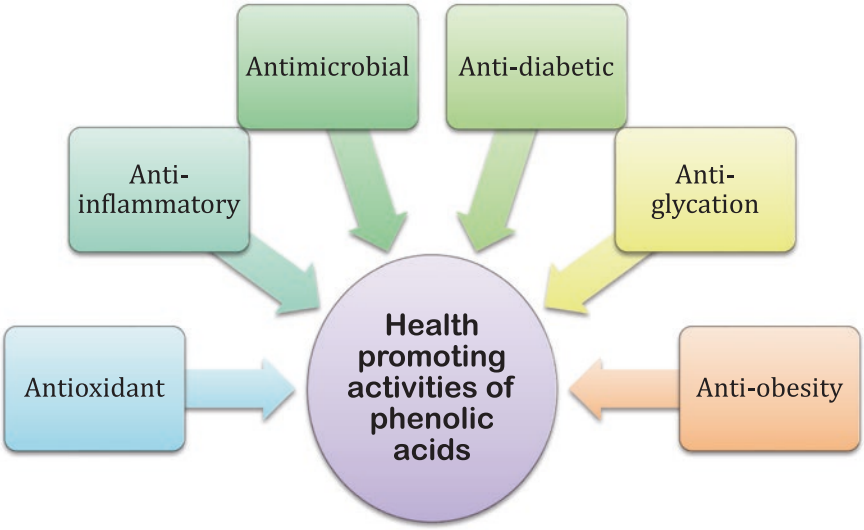

Fig. 3 Health-promoting activities of phenolic acids

Antioxidant Activity

It has been known for a long time that phenolic compounds occurring naturally in plants present a broad spectrum of health-promoting properties resulting from their biological activity. Certainly, antioxidant activity is involved in these properties. The most important element for every living organism is oxygen. Reactive oxygen species (ROS) may be toxic and mutagenic. The reactive oxygen forms include superoxide, singlet oxygen, hydrogen peroxide, and hydroxyl radical. Figure 4 presents the factors and mechanisms of oxidative stress.

Oxidative stress triggering damage in cell structures, including lipids, proteins, and DNA, occurs due to excessive production of ROS. This damage results in many disorders such as cancer, inflammation, cataract, hypertension, diabetes, cardiovascular disease, and Parkinson and Alzheimer diseases (Sies 2010). Reactive oxygen species can also negatively influence some immunological processes and aging, as well as pathophysiological mechanisms leading to skin inflammatory disorders (Cai et al. 2014). Oxidative stress can be understood as the disturbance of the homeostasis between reactive oxygen forms and the antioxidative defense system in the organism (Yoo et al. 2014).

Antioxidant activity of phenolic compounds is associated with the ring structure of the molecule, conjugated double bonds and the presence of functional groups in the ring. Various mechanisms of action are involved in order to make the antioxidant activity of phenolics possible – inhibition of the ROS formation and the ROS trapping and the extinction of singlet oxygen; and reduction of the metal ions chelated (catalysts for reactions responsible for the formation of ROS), interruption of

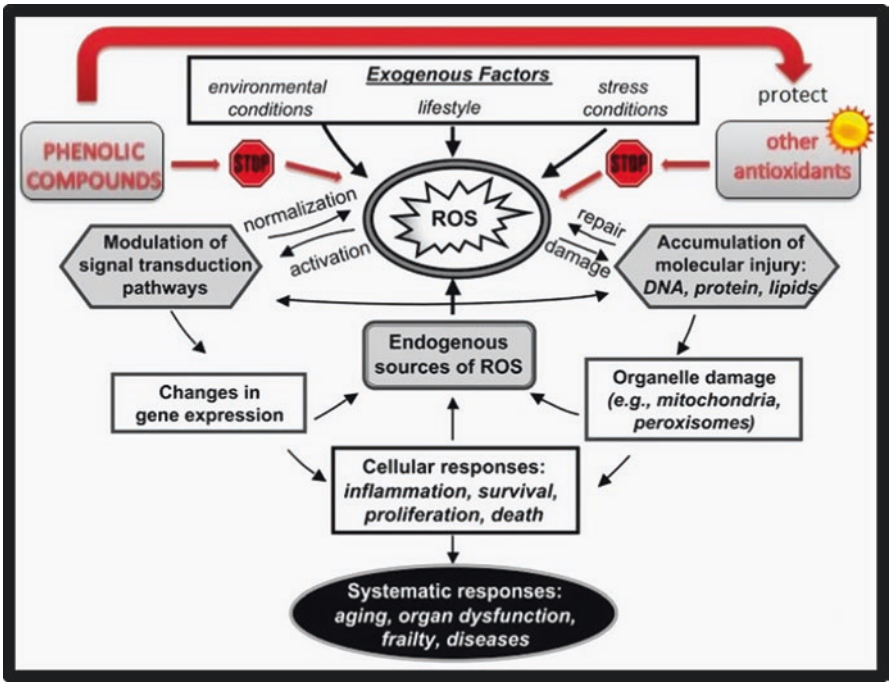

Fig. 4 The scheme of factors involved in the formation of free radicals and a cellular response to ROS. The red arrow and the text in red emphasize the importance of phenolic compounds, other antioxidants, and the relationship between them. The sun signifies protection of other antioxidants by phenolic compounds (Adapted from Sies 2010)

cascade of free radical reactions in lipid peroxidation which helps in protection of other compounds with antioxidant activity (Alov et al. 2015).

The skin is well equipped with two crucial means of defense against oxidative stress: antioxidant enzymes (catalase, glutathione peroxidase, and peroxide dismutase) and nonenzymatic molecules (vitamins, ubiquinone, glutathione) (Dudonne et al. 2011). Thus it is recommended to increase the number of natural antioxidants through the diet or external application. As an example of natural exogenous antioxidants, antioxidant vitamins (especially vitamins C and E), lipoic acid, coenzyme Q, melatonin, resveratrol, curcumin, and other polyphenols can be consumed (Sadowska-Bartosz and Bartosz 2014). These compounds are safe and more biologically active than synthetic antioxidants (Cai et al. 2014).

Anti-inflammatory Properties

Every day, every single organism is exposed to external factors which may cause various types of damage, irritation, or allergies. The body's defensive reaction against the negative effects of these factors is inflammation. During the complex

process of inflammation, there is a production of an excess of free radicals. ROS (reactive oxygen species) and RNS (reactive nitrogen species) formation is associated with the triggering of biological responses to activation of the transcription factor AP-1 and nuclear transcription factor kappa B (NF-κB) (Pastore et al. 2009). These factors help to regulate secretion of signaling molecules, such as pro-inflammatory cytokines and interleukins, which results in skin inflammation, showing the appearance of redness and swelling of the inflamed area. The crucial functions of polyphenols are inhibition of pro-inflammatory mediators, neutralization of free radicals, ROS, RNS, and thus inhibition of lipid peroxidation (Rhein and Fluhr 2010).

During inflammation, arachidonic acid is released from cell membrane phospholipids. Phospholipase A2 (PLA2) is an enzyme used in this reaction, which is stimulated by oxidative stress. Either the cyclooxygenase or lipoxygenase pathway is used to transform the released arachidonic acid. Polyphenols may be responsible in inhibition of both reactions, most frequently due to the interruption of substrate binding to the enzyme by disrupting the hydrogen bonding system or due to ions chelated in the active center of the enzyme (Arct et al. 2003).

Antimicrobial Actions

Phenolic compounds possess significant antimicrobial activities (Czemplik et al. 2011). There are many infections or diseases along with the dermal kind that are treated with a broad-spectrum antibiotic activity. It may lead to the negative influence of antibiotics on natural microflora of the skin and lead to resistance of many bacterial strains (Pinho et al. 2014). Thus, polyphenol activity has special significance in the case of strains resistant to antibiotics, e.g., pneumococci resistant to β-lactam and macrolides, enterococci resistant to glycopeptide antibiotics and vancomycin, *Pseudomonas aeruginosa* with its defense mechanism against phagocytic activity of polymorphonuclear leukocytes, and *Staphylococcus aureus* resistant to methicillin (Anani et al. 2015). The most frequent causes of hospital infections of skin wounds like ulcers, burns, or bedsores which lead to many healing problems includes bacteria of some genera such as *Enterococcus*, *Pseudomonas*, and *Staphylococcus* (Langley et al. 2005).

Recently over 90% of staphylococci, pneumococci, and enterococci isolated from serious infections were found to be resistant to antibiotics; thus the demand for antibacterial products is still rising. These products may be used for multi-strain bacterial infections, without causing a simultaneous toxic effect on human tissues (Czemplik et al. 2011). The antibacterial properties of phenolics may result from the mechanism of their action on cell membranes (Wu et al. 2013).

Anti-diabetic Properties

Diabetes mellitus has been grouped into two types, namely, type 1 and type 2, and is one of the most rapidly increasing disorders with approximately 425 million diabetic patients worldwide. Polyphenols, especially flavonoids, phenolic acids, and tannins, have the important property of inhibiting α-glucosidase and α-amylase, which are key enzymes and responsible for the digestion of dietary carbohydrates to glucose (Moo-Huchin et al. 2015). McDougall et al. (2005) studied the potential inhibitory activity of strawberries, raspberries, blueberries, and blackcurrants on α-glucosidase and α-amylase enzymes. The polyphenols present in them had an inhibitory effect on both enzymes which resulted in reduced blood glucose levels (Lin et al. 2016).

According to Wilson et al. (2008), increased cranberry juice intake could promote positive health benefits. The highest α-amylase and α-glucosidase inhibitory activities among herb, fruit, and fungal-enriched cheeses were observed in cranberry-enriched cheese by in vitro studies (Apostolidis et al. 2007). Combining cranberry with oregano which contains high rosmarinic acid content enhanced the antioxidant activity and total phenol content proving a possible candidate with antidiabetic activity (Apostolidis et al. 2006).

Antiglycation Properties

Glycation is a nonenzymatic condensation reaction between reducing sugars and amino groups of proteins that undergo rearrangements to stable ketoamines, which leads to the formation of advanced glycation end products (AGEs) (Ahmed 2005). It is a spontaneous reaction and depends on the degree and duration of hyperglycemia, half-life of the protein, and permeability of the tissue to free glucose (Berrou et al. 2009). Increased glycation and buildup of tissue AGEs have been implicated in diabetic complications as they can alter enzyme activity, decrease ligand binding, modify protein half-life, and alter immunogenicity (Kostolanska et al. 2009). Hyperglycemia is usually considered as a clinical hallmark of diabetes which results into the formation of AGEs. Therefore, glycation also plays an important therapeutic target for the treatment of diabetic complication. Several natural compounds have been reported to possess antiglycating properties (Ali et al. 2014).

Polyphenolic compound on the other hand is a diverse class of plant secondary metabolites (Hasna 2009) characterized by a polyphenol structure, which means that they have several hydroxyl groups on two or more benzene rings. Several plant species such as fruits, spices, and vegetables have been reported to possess polyphenols which confer on them the ability to remove free radicals formed from glycation and its end products from the system (Cinta et al. 2010). Culinary spices not only provide high concentrations of bioactive compounds but also tend to provide few calories which is significant in type 2 diabetes, often associated with abdominal

obesity (Schroder 2007). Most frequently, alligator pepper, ginger, and nutmeg are the spices of interest.

Alligator pepper (*Aframomum melegueta*), also known as Guinea pepper or grains of paradise, is a member of the Zingiberaceae family. The seeds are used as a spice in food flavors and have a wide range of ethnobotanical uses. They are used as a remedy for treating stomachache, diarrhea, and snakebite (Umukoro and Ashorobi 2007). *Zingiber officinale* (family: Zingiberaceae), also known as "ginger," is one of the most commonly used spices in many parts of the world. It has been cultivated for thousands of years for medicinal purposes and as a spice. It is used extensively in traditional medicine to treat headaches, nausea, febrile conditions, colds, arthritis, rheumatic disorders, and muscular discomfort (Khaki et al. 2009). *Myristica fragrans* (Myristicaceae) with a common name, nutmeg, is an aromatic tree which is cultivated in many tropical countries. Its dried kernel has been claimed to possess medicinal properties (digestive, carminative, and expectorant) in Indian medicine (Nadkarni 1998). It also possesses hypolipidemic, antithrombotic, antiplatelet aggregation, antifungal, aphrodisiac, and anti-inflammatory activities (Evans 1996).

Shin et al. (2015) found that *Silybum marianum* flower extract inhibited the glycation between BSA and glucose in vitro and was further confirmed in human explants. Silibinin was identified to be the compound contributing to the antiglycating property. Similarly, rosmarinic acid, a typical polyphenol from Lamiaceae plant, acted as the major active component of *Melissa officinalis* in suppressing glycation associated disorders (Yui et al. 2017). Phenol-enriched maple syrup extract reduced the formation of AGEs by 40% in the bovine serum albumin (BSA)-fructose assay and by 30% in the BSA-methylglyoxal (MGO) assay (Liu et al. 2017). In other report pure phenolic compounds like gallic acid, ferulic acid, and cinnamic acid were shown to possess significant antiglycating properties (Banan and Ali 2016).

Other Activities

Anthocyanins are considered as modulators of adipose tissue metabolism. Tsuda (2008) found that dietary cyanidin 3-glucoside-rich purple corn color (PCC), an anthocyanin, can ameliorate high-fiber diet-induced insulin resistance in mice. The enzymes included in the fatty acid and triacylglycerol synthesis were suppressed and downregulated by dietary PCC, thereby contributing to the suppression of triacylglycerol accumulation.

Recently, phenolics, particularly flavonoids, have been intensively investigated because of these broad potential pharmacological activities (Huang and Ferraro 1882). Diets rich in phenolics may appear to protect against cardiovascular diseases, neurodegenerative disorders, and some forms of cancer.

Several phenolics have been recognized as active chemopreventive agents. Epigallocatechin 3-gallate (EGCG) from green tea and theaflavins from black tea

exert strong inhibitory effects on diverse cellular events associated with multistage carcinogenesis. Curcumin, a yellow ingredient from turmeric (*Curcuma longa*), has also been extensively investigated for its cancer-preventive potential (Lin 2004).

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