



# Natural Antioxidants: Assays and Extraction Methods/Solvents Used for Their Isolation

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## Abstract

The present chapter aims at providing a brief knowledge regarding plant-based secondary metabolites which act as natural antioxidants (NAs) (viz., flavonoids, phenolic acids, stilbenes, coumarins, lignans, tannins, lignins, alkaloids, sulfur compounds, and essential oils) their mode of action, methods and solvents used for their isolation, and various techniques (in vitro and in vivo) used for the assessment of their antioxidant potential. Apart from this, the chapter also provides a brief knowledge concerning oxidative stress (OS), free radicals (FRs), reactive nitrogen species (NOS), reactive oxygen species (ROS), biological roles of FRs/reactive species (RS), and various routes of their production. The oxidative stress is a situation, where the quantity of FRs/RS in the body of an organism surpasses the homeostatic equilibrium of indigenous antioxidants and FRs/RS. This oxidative stress condition is the cause of more than hundred types of ailment in living beings. The natural antioxidants retard or slow down the oxidation of other biomolecules/molecules and help to alleviate the oxidative stress condition. Further, these NAs are economic, eco-friendly, nontoxic as compared to synthetic antioxidants, and easily accessible to common people. These NAs can be attained from any part of the plant, viz., roots, rhizomes, stems, bark, leaves, flowers, and fruits. The plant-based natural antioxidants are also well known for their wide variety of therapeutic potential.

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## 1.1 Introduction

Secondary metabolites (SMs) of plant origin have been used from ancient times as therapeutic agents for the cure of many diseases, flavoring agent, food preservatives, insect repellent, etc. People in tribal areas of countries like India, China, Bangladesh, Bhutan, Nepal, Pakistan, Egypt, etc. largely used these SMs as medicines, which formed the basis of the conventional medical systems (Ravishankar and Shukla 2007; Ekor 2014; Sharma et al. 2019). On the other hand, nowadays most of the synthetic therapeutic agents used for the cure of various kinds of illnesses have many side effects and cause many serious health issues. Due to these reasons in the present scenario, strategies have been made to replace synthetic chemicals with SMs of plant origin because the latter have less or no side effects and are cost-effective, eco-friendly, biodegradable, and nontoxic in nature.

Presently, the interest of researchers on natural compounds (mainly polyphenolics) which exhibit antioxidant properties and beneficial to human beings as food additive or as specific preventive pharmaceuticals is growing at a fast pace (Pinelo et al. 2005; Petlevski et al. 2013; Bettaieb et al. 2010). As a result, natural antioxidants have become a vital part of preservation technology and modern health care. The various crude extracts isolated from different parts of plants such as roots, stem, bark, leaves, and flowers are rich in polyphenolics and are progressively used in the food and cosmetic industry as these check the oxidative breakdown of lipids and help to improve the quality of food and cosmetic products. Due to this, polyphenolics and other antioxidant constituents isolated from plants have made scientists, manufacturers (food and cosmetic products), and consumers aware, since the drift of the future is toward functional food and superior cosmetic products with definite health effects in order to maintain good health (Sen et al. 2010; Nunes et al. 2012; Nile et al. 2017; Bhardwaj et al. 2019).

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## 1.2 Medicinal Plants and Antioxidant Potential

Apart from their own endogenous self-sufficient antioxidant defense system, human beings also take antioxidants from external sources through diet in order to maintain the internal balance between reactive species (FRs, free radicals; ROS, reactive oxygen species; and RNS – reactive nitrogen species) and antioxidants during different adverse conditions (stress, hypertension, smoking, heavy physical workout, and various diseased conditions) that encourage the production of the former in the human body. The external sources (mainly plant based) have varying amounts of

antioxidants, which are proficient to prevent or retard the oxidation of various bioactive compounds in the body (Sen et al. 2010; Nunes et al. 2012).

Aromatic medicinal, herbal, and other higher plants contain various classes of SMs (phenolic acid, flavonoids, tannins, phytosterol, terpenoids, saponins, etc.). These classes of SMs have been well known for their antioxidant potential (Oluwaseun and Ganiyu 2008; Sen et al. 2010; Patial et al. 2019). The extracts isolated from herbal and aromatic medicinal plants are taken as decoctions or infusions, owing to their remedial actions. Further, the bioactive compounds present in these plants are a part of the physiological metabolism of living flora due to which they are supposed to have a superior compatibility with human body (Sen et al. 2010; Nile et al. 2017).

The different medicinal and herbal plants might serve not merely as flavoring agents but also as eco-friendly food antioxidants and nutrient supplements. These natural antioxidants also help to prevent the deterioration of various foodstuff products. The use of SMs (phytochemicals) as a drug (antioxidant) to treat various disorders which result in oxidative stress conditions and to scavenge the FRs and reactive species has now clinically been proved as less toxic and more efficient as compared to the existing synthetic drugs and antioxidants (Wannes et al. 2010; Sen et al. 2010; Petlevski et al. 2013). Many synthetic antioxidants (phenolics with alkyl substituents) such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), tertiary butyl hydroquinone (TBHQ), and propyl gallate have been used from the beginning of this century as additives to protect and stabilize various food products for flavor, freshness, nutritive value, and color. However, various studies on the use of synthetic antioxidants have revealed that these are toxic in nature (cause cancer) and are not fit for long-term consumption (Wanasundara and Shahidi 1998; Bouayed and Bohn 2010; Nunes et al. 2012; Muniyandi et al. 2017). Hence, it is an aggressive need of present time to use SMs/phytochemicals of plant origin as drug/antioxidants to cure and prevent oxidative stress and related diseases. Till date, a large number of plant species have been analyzed as sources of potent and safe bioactive SMs which are mainly responsible for the antioxidant potential of studied plant species (Nile et al. 2017; Muniyandi et al. 2017).

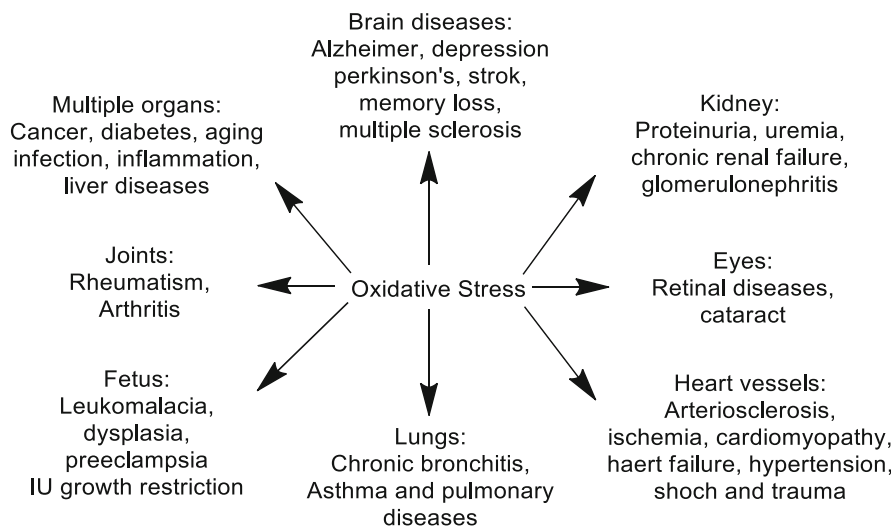
Till date, approximately 27,000 alkaloids; 2000 lactins and peptides; 700 non-protein amino acids; 150 glucosinolates; 100 amines; 60 cyanogenic glycosides; 3000 monoterpenes; 5000 sesquiterpenes; 2500 diterpenes; 5000 triterpenes, steroids, and saponins; 500 tetraterpenes; 2000 phenylpropanoids (coumarins and lignans); 4000 flavonoids, anthocyanins, and tannins; 800 polyketides; 1500 polyacetylenes; and 600 carbohydrates and organic acids have been characterized and identified from various plant species (Wink 2015). All these classes of SMs act as natural antioxidants, but the polyphenolic compounds are the major ones (Ignat et al. 2011; Dzialo et al. 2016; Nunes et al. 2012; Ganesan and Xu 2017 and references therein).

### 1.3 Oxidative Stress

“Oxidative stress” is the term used to represent the state of any disturbance in the equilibrium of reactive species (FRs, ROS, and RNS) and antioxidants in favor of the former as a result of various factors, viz., aging, trauma, inflammation, pollution, poor diet, cold, infections, increase in oxidative metabolism, strenuous physical activity, radiation, toxicity, and drug actions (Sies 1985; Ullah et al. 2016). In this state, generally there is excess production and/or inadequate elimination of highly reactive molecules like FRs, ROS, and RNS from the cell (Johansen et al. 2005; Tian et al. 2007). Oxidative stress conditions may lead to the offensive functioning of healthy cell by damaging the biomolecules (like RNA, DNA, proteins, carbohydrates, and lipids) and ultimately lead to the death of the cell. Oxidative stress conditions are supposed to play a crucial role in the aging process and have been the vital reason for the onset of over 100 kinds of diseases (Fig. 1.1) (Agarwal and Prabakaran 2005; Dufor et al. 2007; Sharma et al. 2010; Sen et al. 2010; Rahal et al. 2014; Ullah et al. 2016).

#### 1.3.1 Free Radicals (FRs), Reactive Oxygen Species (ROS), and Reactive Nitrogen Species (RNS)

A moiety or molecules that have one or more than one unpaired electron in its outermost molecular/atomic orbital are known as FRs. The FRs are short-lived and very reactive in nature and can exist independently. These FRs pass their unpaired electrons to living cells and result in the oxidation of cell components and molecules,



**Fig. 1.1** Various diseases associated with oxidative stress condition

which leads to the abnormal functioning of the cells and finally to death. On the other hand, the non-radical reactive species (specially RNS and ROS) do not have odd electrons but are capable to lead the free radical reactions easily in living beings, i.e., oxidation of various biomolecules (Halliwell and Gutteridge 1999; Halliwell and Gutteridge 2007). The reactive species generated from nitrogen and oxygen are described as the main classes of radical/reactive species produced in living beings. These radical/reactive species act as key intermediates in various natural processes going on in the body of living beings such as cytotoxicity, neurotransmission and control of vascular tone, etc. (Sharma et al. 2010; Sen et al. 2010; Erzsebet et al. 2016; Ullah et al. 2016 and references therein). The various radical/reactive species are classified as follows (Erzsebet et al. 2016; Halliwell and Gutteridge 2007):

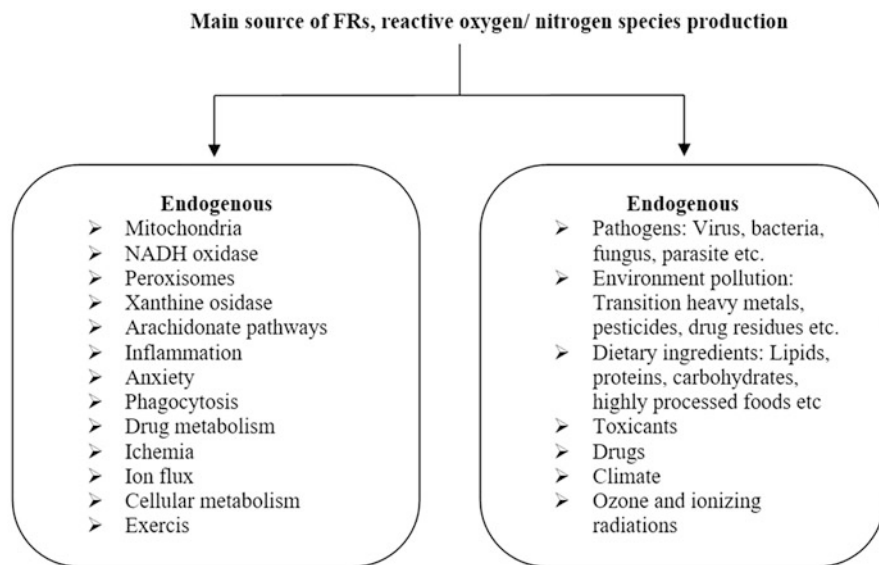
- Free radical species: hydroperoxyl radical ( $\text{HOO}^\bullet$ ), superoxide radical ( $\text{O}_2^{\bullet-}$ ), hydroxyl radical ( $\text{HO}^\bullet$ ), lipid radical ( $\text{L}^\bullet$ ), lipid peroxy radical ( $\text{LOO}^\bullet$ ), peroxy radical lipid alkoxyl radical ( $\text{LO}^\bullet$ ), ( $\text{ROO}^\bullet$ ), nitrogen dioxide ( $\text{NO}_2^\bullet$ ), nitric oxide ( $\text{NO}^\bullet$ ), protein radical ( $\text{P}^\bullet$ ), and thiyl radical ( $\text{RS}^\bullet$ )
- Non-free radical reactive species: ozone ( $\text{O}_3$ ), hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), singlet oxygen ( $^1\text{O}_2$ ), dinitrogen trioxide ( $\text{N}_2\text{O}_3$ ), lipid hydroperoxide (LOOH), hypochlorous acid (HOCl), nitrous oxide ( $\text{N}_2\text{O}$ ), peroxy nitrite ( $\text{ONOO}^-$ ), peroxy nitrous acid ( $\text{ONOOH}$ ), nitryl chloride ( $\text{NO}_2\text{Cl}$ ), and nitrous acid ( $\text{HNO}_2$ )

### 1.3.2 Biological Roles of Free Radical/Reactive Species

FRs and other reactive species (ROS and RNS) are known to be essential evil, as they play crucial role in the beginning and evolution of life on the earth. Inside the cell, they play substantial part in activating the different signaling pathways like mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinase (ERK) pathways that modify gene expression, as well as initiate cell death in association with superoxide dismutase. Further, these are also able to react with nucleic acids, proteins, membrane lipids, various enzymes, and other small molecules, leading to cellular damage. For example, RNS produced by neurons and macrophages act as neurotransmitters and mediators of immunity, respectively. These are also known for thrombosis, angiogenesis, vascular tone, and leukocyte adhesion. Similarly, ROS and RNS are known to play a vital role in single transduction, gene transcription, and regulation of other metabolisms in cell (Halliwell and Gutteridge 2007; Ullah et al. 2016; Erzsebet et al. 2016 and references therein).

### 1.3.3 Production Route of FRs, RNS, and ROS

FRs, RNS, and ROS are generated in the cells and it's surrounded by both exogenous and endogenous substances. These can be generated by different reactions (non-enzymatic) of organic molecules with oxygen, from reactions that are initiated by various ionizing radiations, also from oxidative phosphorylation, and many other



**Fig. 1.2** Different routes/sources of FRs/reactive species generation in living beings

metabolic reactions that occur in the mitochondrion. FRs/reactive species are continuously generating in the cell and environment (Rahal et al. 2014; Sen et al. 2010; Ullah et al. 2016 and references therein). The different routes/sources of FRs/reactive species generation are illustrated in Fig. 1.2.

## 1.4 Antioxidants

Antioxidants are the substances that inhibit or delay the oxidation of a substrate, even when present in minute concentration. These play an important role in protecting the body tissue against the damage caused by oxidative stress conditions. Antioxidants form a barrier against the various reactive species (FRs, ROS, RNS, etc.) which help to delay the onset of various degenerative oxidation processes within the body (Jacob 1995; Sen et al. 2010). In order to protect the cells/organs from the damage caused by FRs/reactive species, the bodies of living beings have developed an extremely complicated and complex antioxidant defense system. This antioxidant defense system has an array of components, which may be endogenous (in vivo synthesized) or exogenous (consumed) in origin (Table 1.1) (Droge 2002; Willcox et al. 2004). These components act interactively and synergistically to offset the ill effects caused by FRs/reactive species or oxidative stress conditions. Hence, these components may be regarded as biomarkers of oxidative stress condition (Sen et al. 2010; Ullah et al. 2016). The SMs isolated from natural sources with FRs, ROS, and RNS scavenging properties might have immense significance as

**Table 1.1** Antioxidant defense systems of human beings

Antioxidant defense system	
Endogenous antioxidants	Exogenous antioxidants
	Prime dietary antioxidants from plants and other source
Bilirubin	Flavonols: kaempferol*, quercetin*, myricetin*, etc.
Uric acid	Flavanols: catechin* and proanthocyanidins*
NADH and NADPH	Flavones: apigenin* and luteolin*
Ubiquinone (coenzyme Q 10)	Isoflavones: glycitein* and genistein*
Thiols: glutathione, lipoic acid, and N-acetyl cysteine	Anthocyanidins: pelargonidin* and cyanidin*
Proteins that bind metal: albumin (copper), metallothionein (copper), ceruloplasmin (copper), myoglobin (iron), ferritin (iron), and transferrin (iron)	Flavanones: eriodictyol*, naringenin*, hesperetin*, etc. *and their glucosides
Enzymes: catalase, superoxide dismutase, and glutathione peroxidase	Phenolic acids: caffeic acid, chlorogenic acid, gallic acid, etc.
	Carotenoids: $\beta$ -carotene, lycopene, zeaxanthin, lutein, etc.
	Vitamins: vitamins E and C
	Trace elements: selenium and zinc

\*Glucosides of Flavonoids and phenolic acids

therapeutic agents against several diseases initiated due to oxidative stress conditions (Sen et al. 2010; Muniyandi et al. 2017).

The noble antioxidants must have the following properties (Sen 2003; Sharma et al. 2010):

- They must be effectual at low concentration.
- They must be sufficiently soluble in oxidizable medium/product.
- They must be biodegradable, harmless, and non-nuisance meaning eco-friendly at the effective dose even after prolonged storage.
- Their disintegration product should also be biodegradable, harmless, and non-nuisance.
- They must be tasteless and odorless and should not transmit color to the product.
- They must be effectual and stable over a broad range of pH, temperature, and environmental conditions.
- They must be neutral and chemically uncreative with other components present within the body.

#### 1.4.1 Mode of Action of Antioxidants

Antioxidants neutralize or deactivate the FRs/reactive species frequently before they attack targets in biological cells. Antioxidants act as singlet oxygen quencher, radical

scavenger, electron donor, hydrogen donor, peroxide decomposer, metal-chelating agents, and enzyme inhibitor. In intracellular and extracellular environment, both enzymatic and nonenzymatic antioxidants are present which help to neutralize/detoxify/deactivate various FRs and reactive species (ROS and RNS) (Kumar 2011; Niki 2011, 2016 and references therein).

Overall, the antioxidants act by various routes such as the following (Kumar 2011):

- They involve in a variety of chain terminating reactions, e.g.,  $\alpha$ -tocopherol, and trap the various FRs in lipid phase.
- They react with various ROS and RNS due to which the concentration of these reactive species decreases in the intracellular/extracellular environment, e.g., glutathione.
- They scavenge different kinds of initiating radicals, e.g., superoxide dismutase, that bind to superoxide free radicals and act in the lipid phase.
- They form chelating complex with different transition metal catalysts. These chelating complexes help to eliminate numerous transition metals which are well-known prooxidants, e.g., lactoferrin, ferritin, and transferrin, that keep the oxidative stress in check, whereas albumin and ceruloplasmin keep the concentration of copper in check.

All the organisms have well-developed and complex antioxidant defense/repair systems to check the damage triggered by oxidative stress, but they are unable to prevent the damage completely. Also, nowadays production of FRs, RNS, and ROS in the human body has been increased due to wrong feeding habits, pollution, stress (mental or physical), stressful environment conditions, etc. Owing to this, the interest of researchers from various fields has significantly increased in naturally occurring antioxidants for their use in pharmaceutical, food, and cosmetic products. Further, these naturally occurring antioxidants are safer and have no side effects as compared to synthetic ones, which have mutagenic effects, carcinogenic effects, and many other side effects (Nunes et al. 2012; Muniyandi et al. 2017 and references therein). Furthermore, the synthetic drug taken to cure the various illnesses is capable of generating free radical, which becomes the basis of oxidative stress and ultimately leads to tissue damage. For example, a variety of nonsteroidal anti-inflammatory medications are broadly used for the cure of inflammation, rheumatism, fever, pain, and cardiovascular disease, but their chronic administration results in the generation of FRs and reactive species in human body which may lead to gastrointestinal hemorrhage or perforation, gastric erosions and gastric or duodenal ulceration, etc. (Kamboj 2000; Sen et al. 2010).

The current investigations have shown that the antioxidants isolated from natural sources that have FRs/RNS/ROS scavenging potential might have immense reputation as therapeutic agents against a variety of ailments initiated because of oxidative stress. The phytochemicals isolated from various extracts and essential oils of plant origin were found to be effective scavengers of FRs/RNS/ROS and inhibitors of lipid peroxidation (Muniyandi et al. 2017; Sen et al. 2010 and references therein).



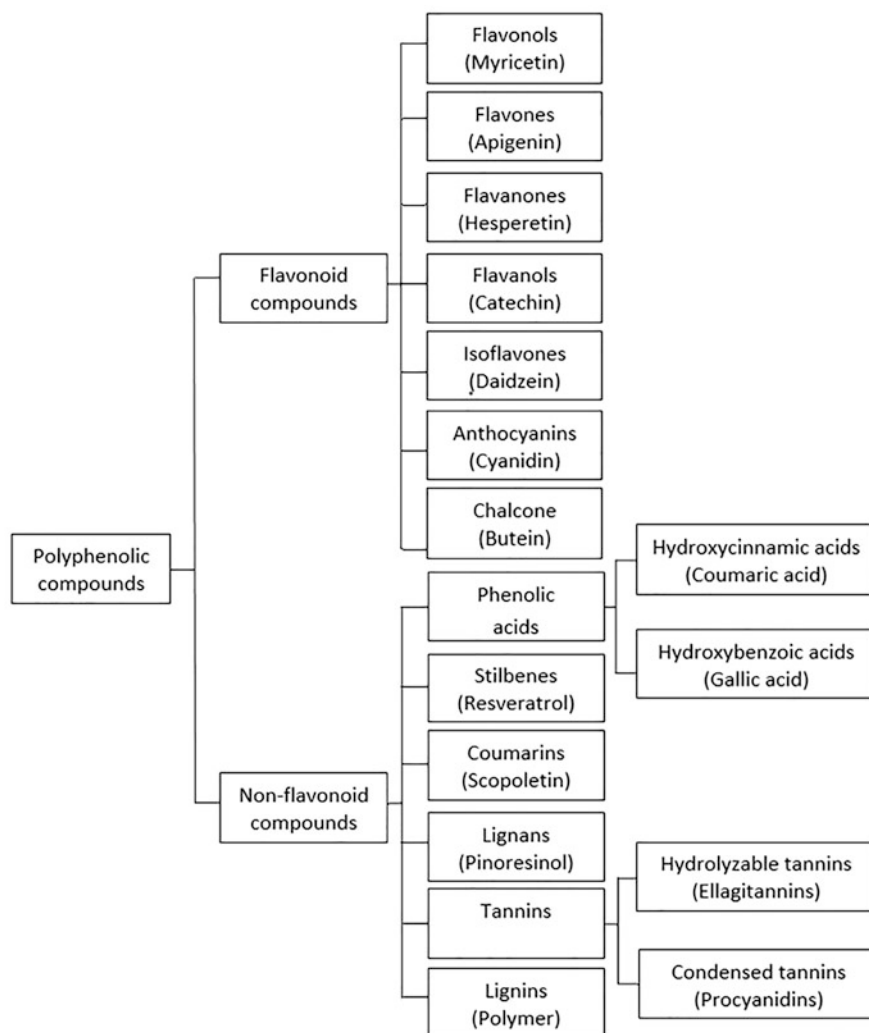
## 1.4.2 Polyphenols as Natural Antioxidants

Polyphenolic compounds are usually found in every part of edible as well as nonedible plants. Plants synthesized polyphenolic compounds during their normal development and also in response to different infections, wounding conditions, insects, and UV radiation. In plants polyphenolic compounds are mainly synthesized through shikimate (directly provide phenylpropanoids like coumarins and hydroxycinnamic acid) and polyketide or acetate (provide simple phenols and many quinines) pathways. These are mainly derived from tyrosine and phenylalanine which occur commonly in all plants (Naczka and Shahidi 2004). Polyphenolic compounds help to protect the plants from pathogens, oxidative stress, UV radiation, harsh climatic conditions, and grazing (Naczka and Shahidi 2006). Whereas, in the human body polyphenolic compounds act as antioxidant and show various biological properties like anticancer, anti-inflammatory, diabetic, cardioprotective, neuroprotective, osteoprotective, antiasthmatic, antiaging, antiseptic, antihypertensive, cerebrovascular protection, hepatoprotective, cholesterol lowering, antibacterial, antifungal, and antiviral (Nunes et al. 2012; Dzialo et al. 2016; Ganesan and Xu 2017). All these facts are also strongly supported by various epidemiological studies and associated meta-analyses done by various researchers from all over the world (He and Sun 2016; Liu et al. 2014; Grosso et al. 2017; Ganesan and Xu 2017).

Antioxidant potential of polyphenolic compounds is attributed to the presence of reactive functional groups, conjugated double bonds, and their annular structure. The polyphenolics show their antioxidant potential through diverse mechanisms of action such as FRs/RNS/ROS trapping, inhibition of FRs/RNS/ROS formation, scavenging of singlet oxygen, by reduction of chelated metal ions (that assist in the generation of FRs/RNS/ROS) by terminating/interrupting the free radical reactions involving lipid peroxidation, and by shielding/protecting the other molecules having antioxidant activity (Dzialo et al. 2016 and references therein).

At present, the curiosity of scholars in natural constituents (polyphenolics) which exhibit antioxidant activity and supplied to living beings as food preservative or as specific protective pharmaceuticals is escalating gradually. Owing to this, natural antioxidants have become vibrant part of preservation technology and modern health care. The variety of crude extracts isolated from various parts of plants like roots, tuber, bark, stem, cladode, leaves, flowers, and fruits are rich in polyphenolics and are gradually used in food, cosmetic, and pharmaceutical industry as they check the oxidative breakdown of lipids and help to improve the quality of food, cosmetic, and pharmaceutical products. As a result of this, polyphenolics and other natural antioxidants obtained from plants and other natural sources are raising awareness among scientists, manufacturers (food, cosmetic, and pharmaceutical products), and consumers since the drift of the future is toward functional food, superior cosmetics, and pharmaceutical products with definite health-promoting effects in order to uphold good health (Nunes et al. 2012 and references therein).

Nowadays, about 8000 polyphenolic compounds are identified from the plants, which primarily act as antioxidants (Harborne et al. 1999). According to the most



**Fig. 1.3** Classification of naturally occurring polyphenolic compounds

common classification, these polyphenolics are divided into two main categories: one is flavonoids and other is non-flavonoid compounds. The flavonoid compounds are further divided into seven groups, viz., flavonols, flavones, flavanones, flavanols, isoflavones, anthocyanins, and chalcones, while coumarins, stilbenes, lignans, lignins, tannins (condensed and hydrolysable tannins), and phenolic acids (hydroxybenzoic and hydroxycinnamic acids) are the categories of non-flavonoid compounds (Fig. 1.3). Among these classes, phenolic acids, flavonoids, and tannins are the key ones, which have strong antioxidant potential (Ignat et al. 2011; Nunes et al. 2012; Dzialo et al. 2016 and references therein).

### 1.4.3 Flavonoids

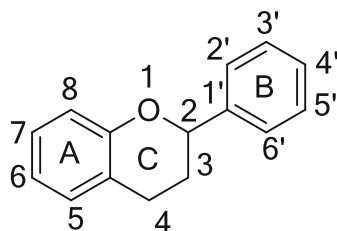
Flavonoids are the category of SMs that have changeable phenolic structures and are widely found in plants. The first flavonoid was isolated in 1930 from oranges. Then, it was supposed to be a component of a new group of vitamins and was named as vitamin P. Afterward, it was established that the new component was a flavonoid (rutin). Till date, over 4000 flavonoids have been recognized (Kumar and Pandey 2013 and references therein).

Flavonoids have low molecular weight and occur as glycosides, aglycones, and methylated derivatives. The primary structure of flavonoids is aglycone (Fig. 1.4) and is composed of 15 carbon atoms with C6–C3–C6 arrangement. Basically, the structure is composed of two aromatic rings (A and B) joined through a three-carbon bridge, generally as a heterocyclic ring (denoted by letter C). Ring A is derived through malonate/acetate pathway, although the ring B is synthesized via shikimate pathway using phenylalanine. The key subclasses of flavonoids (flavones, flavanols, isoflavones, flavonols, flavanones, anthocyanins, and chalcones) resulted from the differences in the substitution pattern of the heterocyclic ring C. Out of all the classes of flavonoids, flavonols and flavones are broadly distributed and structurally most diverse. The substitutions on the rings A and B resulted in different constituents within all classes of flavonoids. The different substitutions might involve alkylation, acylation, oxygenation, sulfonation, and glycosylation, (Ignat et al. 2011; Kumar and Pandey 2013; Dzialo et al. 2016 and references therein).

The six-member heterocyclic ring C coupled with the benzene ring A is either  $\alpha$ -pyrone (in case of flavanones and flavonols) or its dihydro derivative (in case of flavanones and flavanols). The position of benzene ring B at heterocyclic ring C segregates the flavonoid class into isoflavonoids (at carbon 3) and flavonoids (at carbon 2). Flavonoids usually have hydroxyl (OH) group at positions 5', 4', 3', 7, 5, and 3. Among the glycoside derivative of flavonoids, the glycosidic bond is usually present at positions 7 or 3 and the linked carbohydrate moiety can be D-glucose, L-rhamnose, galactose, arabinose, or glucose rhamnose (Kumar and Pandey 2013 and references therein).

Flavonoids are generally found as glycosides (significantly as O-glycoside as compared to C-glycosides) dissolved in the vacuolar fluid of the plant cell. In various plant species, anthocyanins accumulate in the vesicles formed within the vacuole. Flavonoids are found very frequently in the dicotyledonous plants and are the only metabolites that have pharmacological potential. Flavonoids are mainly found in

**Fig. 1.4** Basic flavonoid aglycone structure



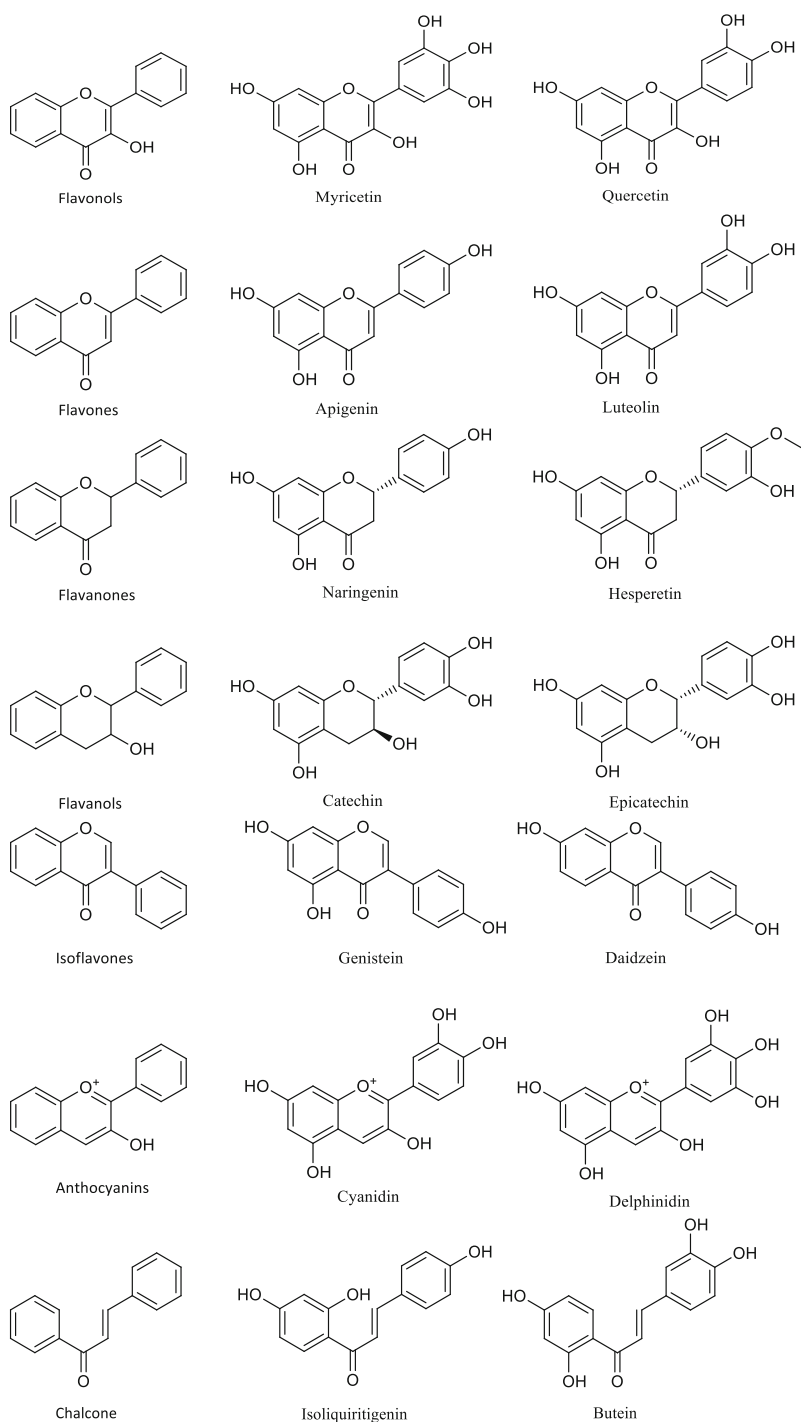
medicinal and aromatic plants, fruits, vegetables, legumes, seeds, certain grains, wine, coffee, cocoa, green tea, and herbal essences (Dzialo et al. 2016 and references therein).

Flavonoids have high redox potential, owing to which they act as singlet oxygen quenchers, hydrogen donors, and reducing agents. Apart from this, they also act as metal chelator. These properties of flavonoids make them powerful antioxidants. The antioxidant potential of flavonoids is varied according to the extent of structural conjugation, number/position of the hydroxyl functional groups, and the existence of electron-withdrawing and electron-donating functional groups in the ring structure. Flavonoids help to protect the plants from the injury produced by oxidative stress, pathogens, UV radiations, fungal infections, and herbivores. Flavonoids, when taken frequently by humans, help to reduce the chance of onset of a variety of degenerative diseases related to the heart, kidney, brain, etc. (Kumar and Pandey 2013; Dzialo et al. 2016 and references therein). Figure 1.5 represented the chemical structures of the key classes of flavonoids.

**Flavonols** Flavonols have the 3-hydroxyflavone backbone and extensively occur in plant kingdom. These are mainly found in various fruits and vegetables. Their content widely depends upon the different environmental conditions such as climate, storage, cooking, and growing conditions. Flavonols are the most active class of the flavonoid group and show a broad range of biological activities. Thus, the positive effects of fruit and vegetable diet against different diseases have been generally ascribed to flavonoids and more exclusively to flavonols. Many flavonols are commercialized as nutritional supplements either in pure form or as a mixture of flavonoids or extracts. Quercetin, myricetin, morin, rhamnetin, kaempferol, fisetin, and azaleatin are some naturally occurring flavonols, whereas rutin, robinin, astragalin, azalein, and myricitrin are some glycosides of flavonols that occur naturally (Ignat et al. 2011 and reference therein).

**Flavones** Flavones are naturally occurring group of oxygen heterocycles having 2-phenyl-1-benzopyran-4-one or 2-phenylchromen-4-one backbone (Marais et al. 2006). These are mainly found in cereals, fruits, vegetables, and herbs. Flavones have inimitable potential to modulate various enzyme systems, due to which they show a vast variety of biological activities such as antioxidant, anticancer, antibacterial, anti-allergic, antiviral, anti-osteoporotic, etc. Apigenin, luteolin, tangeritin, chrysin, wogonin, acacetin, and zapotin are the examples of some naturally occurring flavones (Singh et al. 2014 and references therein).

**Flavanols** Flavanols are also regarded as flavan-3-ols and have 2-phenyl-3,4-dihydro-2*H*-chromen-3-ol or 2-phenyl-3,4-dihydro-2*H*-benzopyran-3-ol skeleton (Marais et al. 2006). Flavanols are frequently present in polymerized form (oligomers (dimers to pentamers) and polymers – six or more units) within various plant-based food products. These are commonly found in fruits (mostly in peels and seeds), tea, cereals, and cocoa, but are absent in legumes and vegetables, with the exception of broad beans and lentils. Epicatechin, catechin, epigallocatechin,



**Fig. 1.5** The chemical structures of different key classes of flavonoids

gallo catechin, and their galloyl substituted derivatives, fisetinidol, mesquitol and guiboustinidol, are some examples of naturally occurring flavanols (Pascual-Teresa et al. 2010 and reference therein).

**Flavanones** Flavanones have a saturated three-carbon chain with a ketone functional group at C4. Flavanones differ from flavones by a C2-C3 single bond (Marais et al. 2006). These are extensively present in around 42 families of higher plants particularly in Leguminosae, Compositae, Lamiaceae, and Rutaceae. These are isolated from all the parts of plants such as seeds, fruits, flowers, cladode, leaves, bark, stem, peels, roots, tubers, rhizomes, etc. Flavanones are frequently glycosylated by a variety of disaccharide at position C7. The citrus fruit contains high concentration of flavanones, and their concentration is higher in peel than the fleshy part of citrus fruit. Besides citrus plants, these also occurred in certain aromatic plants. Till date, approximately 350 flavanone aglycones and 100 glycosides of flavanone are recognized in nature. Naringenin, hesperetin, eriodictyol, butin, sakuranetin, and sterubin are some major flavanone aglycones, whereas hesperidin, naringin, poncirin, and sakuranin are some key flavanone glycosides obtained from a variety of higher plant species (Ignat et al. 2011; Khan and Zill-E-Huma 2014 and reference therein).

**Isoflavones** Isoflavones have an aromatic ring at C3 position as compared to their corresponding flavones, which have an aromatic ring at C2 position. Isoflavones have also structural resemblance to estrogens as they hold hydroxyl groups at positions C7 and C4 like estradiol (Klejdus et al. 2007). These are present in over 300 plant species belonging to 59 families. Plants belonging to family Leguminosae and subfamily Papilionideae are the major source of isoflavones in the nature. These are mainly found in the seeds and roots. Isoflavones are also produced by some bacteria and fungi. Isoflavones play significant role in the process of symbiosis among rhizobium-legume and also play key role in defense mechanisms against plant pathogens. Among the members of family Leguminosae, soybeans are the foremost sources of isoflavones in nature. Isoflavones are present as conjugates of glucose in the plants. These gluco-conjugates are biologically inactive and are hydrolyzed to their active forms (aglycones) by the action of bacteria residing in the human intestine. Isoflavones are well acknowledged for their health-promoting effects like estrogenic or antiestrogenic activity, anti-inflammatory effect, antioxidant activity, antiproliferation of cancer cells, prevention of osteoporosis, reduction in cardiovascular disease, regulation of the immune system, alteration in cellular signaling, and alleviation of postmenopausal syndrome. Daidzein, genistein, biochanin A, glycitein, luteone, wighteone, and formononetin are the examples of some naturally occurring isoflavone aglycones. Out of these, daidzein and genistein are the most significant biologically active isoflavones in humans (Reinli and Block 1996; Ignat et al. 2011; Preedy 2013 and references therein).

**Anthocyanins** Anthocyanins are polymethoxy and polyhydroxy derivatives of 2-phenylbenzopyrylium, which form the core of anthocyanins known as

anthocyanidin. This anthocyanidin core is also linked to many glycosidic moieties (glucose, fructose, galactose, rhamnose, arabinose, and xylose) at the positions C7, C5, or C3. The anthocyanidin core consists of an aromatic ring A fused with heterocyclic ring C that contains oxygen, which further united to a third aromatic ring B by a C-C single bond (Konczak and Zhang 2004; Smeriglio et al. 2014). Anthocyanins are water-soluble vacuolar pigments with blue, red, or purple appearance at different pH. These are synthesized by the phenylpropanoid pathway and are present in all plant parts (seeds, fruits, flowers, leaves, cladode, stems, bark, and roots). The isolated anthocyanins are very unstable in nature, and their stability depends upon various factors such as chemical structure, light, oxygen, pH, solvents, storage temperature, concentration, and the presence of metallic ions and enzymes (Ignat et al. 2011 and references therein).

Anthocyanins play a significant role in attracting animals for seed dispersal and pollination and in absorbing UV radiation from light, thereby protecting the plants from damage caused by UV radiations (Castañeda-Ovando et al. 2009). Anthocyanins are well recognized for their antioxidant potential. Apart from this, they also show a wide range of biological activities from cytoprotective, anti-inflammatory, and anti-obesity to neuroprotective, antimicrobial, and lipidomic potential (Smeriglio et al. 2016). Pelargonidin, cyanidin, peonidin, delphinidin, petunidin, malvidin, aurantidin, europinidin, and rosinidin are the anthocyanidins that occur most commonly in plants. The glycoside derivatives of cyanidin, pelargonidin, and delphinidin are the most frequent pigments found in nature, which form 80%, 69%, and 50% of leaves, fruits, and flower's total pigment content, respectively (Smeriglio et al. 2016 and references therein).

**Chalcones** Chalcones are the naturally occurring compounds made up of 1,3-diphenylprop-2-en-1-one backbone that has two aromatic rings linked through a  $\alpha$ ,  $\beta$ -unsaturated three-carbon carbonyl system. These are present in large amount in edible plants and act as metabolic pioneers for the formation of innumerable flavonoids and isoflavonoids. The rich sources of chalcones are hops, citrus fruits, vegetables (tomatoes, shallot, potatoes, etc.), spices (licorice, cardamom), bean sprouts, and beer. Plants rich in chalcones are employed in various traditional herbal medicine systems (Yerragunta et al. 2013; Chavan et al. 2016). Presently, chalcones are significantly attracting the attention of the scientific world due to their wide range of therapeutic potential, viz., antioxidative, analgesic, antibacterial, immunomodulatory, antimalarial, anti-inflammatory, antiviral, antileishmanial, antiplatelet, antihyperglycemic, anticancer, cytotoxic, and inhibition of aldose reductase and tyrosinase activities (Sankappa et al. 2015). Butein, isoliquiritigenin, derricin, lonchocarpin, heliannone A, kukulkanin B, lichochoalcone A, morachalcone, and dorsmannin are the examples of some naturally occurring chalcones (Yerragunta et al. 2013; Singh et al. 2014; Chavan et al. 2016 and references therein).

#### 1.4.4 Non-flavonoid Polyphenolics

The naturally occurring non-flavonoid polyphenolic constituents are primarily classified into stilbenes, coumarins, tannins, lignans, lignins, and phenolic acids (hydroxycinnamic and hydroxybenzoic acids). Among these, phenolic acid, coumarins, and tannins are the significant ones. Figure 1.6 represents the examples of a few compounds of various classes of non-flavonoid polyphenolics (Rentzsch et al. 2009).

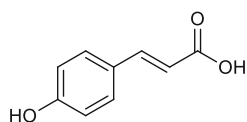
**Phenolic Acids** These are broadly distributed in plant kingdom and comprise nearly one-third of the total dietetic phenols. These occur both in free and bound state in the plants, and in the bound state these are linked with various other natural molecules (flavonoids, sterols, fatty acids, and cell wall polymers) via ester, acetal, or ether bonds. Phenolic acids are mostly found in coffee, tea, tobacco leaves, wine, cinnamon, blueberries, blackberries, cherries, fruits (kiwis, apples, plums, grapes, etc.), vegetables (onion, potatoes, cabbage, radish, spinach, broccoli, etc.), bean, olive oil, and grains. Phenolic acids comprise of two subcategories, the hydroxycinnamic (C6-C3) and hydroxybenzoic (C6-C1) acids (Fig. 1.6) (Bravo 1998). The hydroxybenzoic acids are generally present in low concentration in edible plants, with some exceptions like red fruits, black radish, and onions which have high content of hydroxybenzoic acids. Protocatechuic, gallic, vanillic, syringic, and p-hydroxybenzoic acids are some of the key hydroxybenzoic acids commonly found in plants. The hydroxybenzoic acids of plant origin mostly occur in their glycosidic form. The hydroxycinnamic acids and their derivatives are the most prevalent phenolic acids occurring in plants. Caffeic, ferulic, chlorogenic, sinapic, o-coumaric, m-coumaric, and p-coumaric acids are the major hydroxycinnamic acids found most frequently in plants (Ignat et al. 2011; Kumar et al. 2015 and references therein).

The phenolic acids are produced in the plant species as the protective measure against UV light, insects, bacteria, and viruses. Further, some plant species also synthesized the phenolic compounds in order to retard the growth of other plant contestants (allelopathy). Phenolic acids have high redox potential due to which they show high antioxidant potential. Apart from this, phenolic acids also show antitumor, antimicrobial, cytotoxic, chemopreventive, apoptotic, and neuroprotective activities (Saibabu et al. 2015 and references therein).

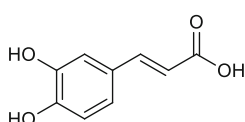
**Coumarins** Coumarins represent one of the key classes of non-flavonoid polyphenolics having 2H-1-benzopyran-2-one framework found naturally in plants. In coumarin benzene ring is fused with  $\alpha$ -pyrone ring due to which these are also known as benzopyrones. Coumarins are further subdivided into different groups ranging from simple coumarins to polycyclic coumarins like furocoumarins and pyranocoumarins. Coumarins are mainly present in the integument of flowers, fruits, seeds, leaves, stems, and roots, but their higher concentration is usually found in flowers and fruits. Till date about 1300 different coumarins have been isolated from



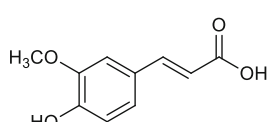
## Phenolic acids - hydroxycinnamic acid



Coumaric acid

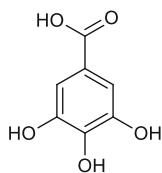


Caffeic acid

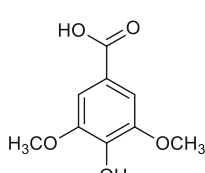


Ferulic acid

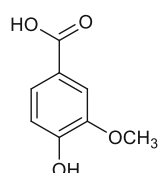
## Phenolic acids - hydroxybenzoic acid



Gallic acid

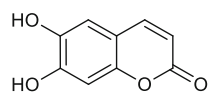


Syringic acid

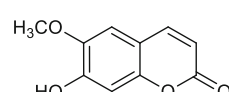


Vanillic acid

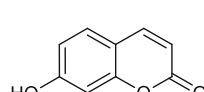
## Coumarin



Esculetin

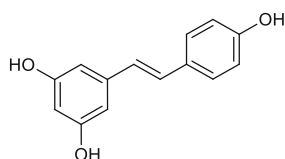


Scopoletin

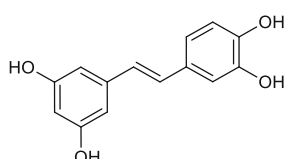


Umbelliferone

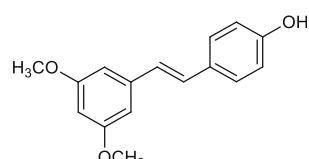
## Stilbenes



Resveratrol

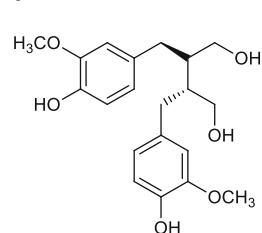


Piceatannol

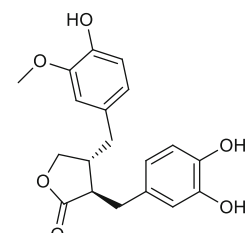


Pterostilbene

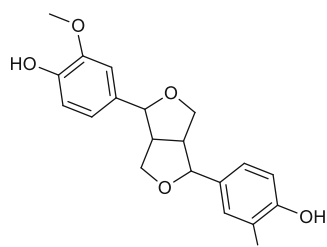
## Lignans



Secoisolariciresinol



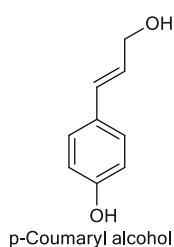
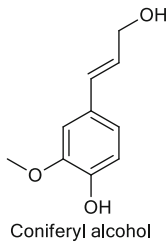
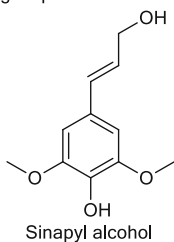
Matairesinol



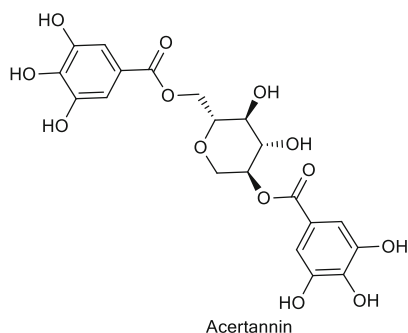
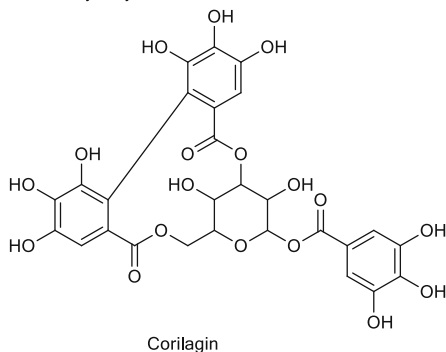
Pinoresinol

**Fig. 1.6** Chemical structures of a few compounds/precursors of various classes of non-flavonoid polyphenolics

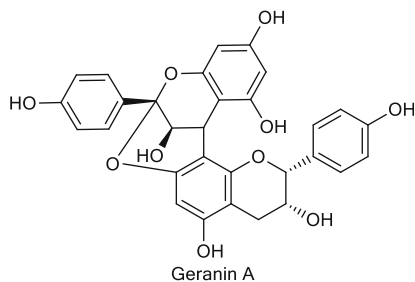
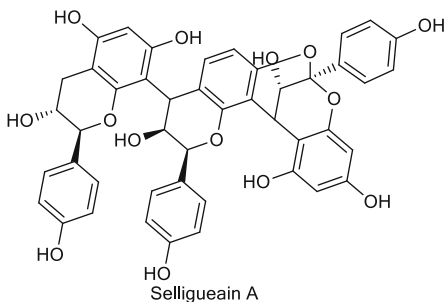
## Lignin precursors



## Tannins - hydrolyzable tannins



## Tannins - condensed tannins



**Fig. 1.6** (continued)

more than 100 plant species belonging to over 40 different families. Vegetables, fruits, seeds, nuts, coffee, tea, and wine are the rich sources of coumarins. Coumarins are well acknowledged for their pharmacological activities, viz., antioxidant, antihypertensive, anti-inflammatory, anticonvulsant, anticancer, anticoagulant, antitubercular, antiadipogenic, antihyperglycemic, antibacterial, antifungal, and antiviral. Esculetin, scopoletin, umbelliferone, ammosesinol, ostruthin, fraxidin, osthole, and novobiocin are the examples of some naturally occurring simple coumarins (Venugopala et al. 2013; Matos et al. 2015 and references therein).

**Stilbenes** Stilbenes are composed of two phenyl rings linked through a methylene bridge of two carbons. Stilbenes are found in stereoisomer form, and the stilbenes of natural origin exist in the trans form. Naturally, these are found both in free and glycosylated forms as polymeric, trimeric, and dimeric stilbenes. Stilbenes are present in very low concentration in the human diet. These are produced in plants as a response to a variety of stress conditions and infections caused by pathogens. Currently, stilbenes are isolated from over 70 plant species (berries, grapes, peanuts, etc.) (Bavaresco 2003). Resveratrol, pterostilbene, and piceatannol are the common naturally occurring stilbenes, which are well known for their antioxidant, anticancer, anti-inflammatory, antifungal, and phytoalexin potential (Ignat et al. 2011; Kumar et al. 2015 and references therein).

**Lignans** Naturally occurring lignans are derived from two phenylpropane (C6-C3) units by oxidative dimerization, and the final product is linked by two specific carbons (C2-C2'). Further reactions of lignans (cyclization, isomerization, etc.) lead to a broad range of structures. Lignans are mostly found in their free form, whereas their bonded forms (glycoside derivatives) occur only in a small amount. The naturally occurring lignans and their synthetic derivatives show potential applications in cancer chemotherapy (skin, breast, colon, and lung cancer) (Saleem et al. 2005). Many lignans, like secoisolariciresinol and matairesinol, are considered as phytoestrogens and are processed through the intestinal microflora into enterolactone and enterodiol. Thus, like isoflavones, lignans have both antiestrogenic and estrogenic activities. Apart from this, lignans also show various pharmacological activities such as antioxidant, antiviral, anticancer, anti-inflammatory, hepatoprotective, and immunosuppressive. Lignans are mostly found in the form of complicated biopolymers, due to which they lack a distinct primary carbon base, and every particular lignan has their own unique chemical structure. The key sources of lignans are the seeds like linseed, flax, cereals, legumes, grains, fruits, certain vegetables, and algae. Secoisolariciresinol, matairesinol, pinoresinol, and podophyllotoxin are some naturally occurring lignans in the plants (Ignat et al. 2011; Kumar et al. 2015 and references therein).

**Tannins** Generally, these have high molecular weight and are widely distributed in plants. In plants tannins mostly occur in two forms, hydrolyzable tannins (formed by self-polymerization of phenolic acids or with sugar) and condensed tannins (combination of flavonoids). Mostly the hydrolyzable tannins are derived from gallic acid. The gallic acid gets esterified to generate a core polyol followed by further esterification of galloyl groups which oxidatively cross-linked to produce further complex hydrolyzable tannins (Hagerman 2002). Condensed tannins also known as proanthocyanidins are the polymeric flavonoids. In spite of well-established or understood biosynthetic pathways of flavonoid synthesis, the steps leading to condensation or polymerization of flavonoids to produce condensed tannins are yet to be elucidated. The frequently examined condensed tannins are synthesized from flavan-3-ols like (+)-catechin and (–)-epicatechin. In biological systems, the tannins act as potential protein precipitating agents, metal ion chelators, and biological

antioxidants. Further, like other polyphenols, tannins also show various biological activities like antimicrobial, anticarcinogenic, anti-inflammatory, antioxidant, and cardiovascular system-protective. Tannins showed strong antioxidant potential, which is mainly attributed to their potential to scavenge free radical, to form chelate with transition metals, and to inhibit lipid peroxidation and activity of pro-oxidative enzymes. Tannins are widely found in both angiosperms and gymnosperms. These are known to occur in around 180 and 44 families of dicotyledons and monocotyledons, respectively. Hydrolyzable tannins are frequently found in leaf, cladode, bud, fruits, seed, root, and stem, whereas condensed tannins are mostly found in wood and bark of the plants (Mole 1993; Chung et al. 1998; Smeriglio et al. 2017 and references therein).

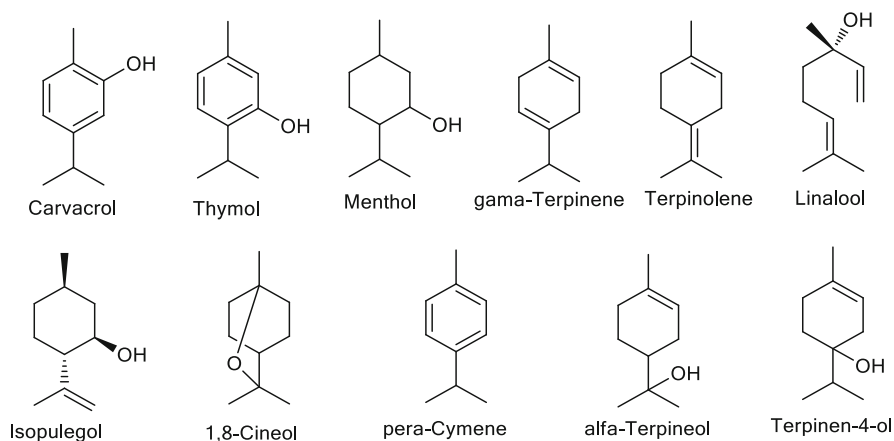
**Lignin** Lignin is a complex organic polymer comprised of different phenylpropane units linked together. These phenylpropane units originated from three aromatic alcohol precursors (monolignols) sinapyl, coniferyl, and p-coumaryl alcohols. Lignin is the key component present in the structural materials of support tissues (cell walls, wood, and bark) of vascular plants and some algae. Lignin is the second largest polymer on the planet after cellulose obtained from the biomass. The woody plants contain up to 15–25% of lignin of their dry weight. In woody plants, lignin provides mechanical support for binding plant fibers together, structure and strength to the cell walls, protection against biochemical stresses, and help in the flow of plant fluid (water) and nutrients. Lignins and their derivatives also show many pharmacological activities such as antiviral, antitumor, antidiabetics, immunomodulator, and obesity control (Laurichesse and Averous 2014; Vinardell and Mitjans 2017 and references therein).

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## 1.5 Essential Oils as Natural Antioxidants

Essential oils are the natural, aromatic, oily, volatile, transparent, rarely colored liquids with strong odor isolated from different plant parts. Essential oils are complex mixtures of volatile terpenes and their oxygenated derivatives, hydrocarbons, aromatic compounds, simple alcohol, ketones, and ether. These are widely used as flavoring agents in food and pharmaceutical industries. Till date, about 3000 essential oils are isolated, out of which only 300 are commercially significant particularly in food, pharmaceutical, agronomic, cosmetic, sanitary, and perfume industries (Silva et al. 2003; Bakkali et al. 2008; Amorati et al. 2013 and references therein).

Essential oil plays a vital role in plants, where they act as insecticides, antibacterial, antifungal, and antiviral agents. They also protect plants against the action of different herbivores. Sometimes, they also help to attract the various insects, which further help to spread the pollen or to repel harmful insects. Essential oils are synthesized by all the plant parts, viz., buds, leaves, flowers, cladode, fruits, stems, roots, bark, and seeds. In the plants, these are stored in epidermal cells,

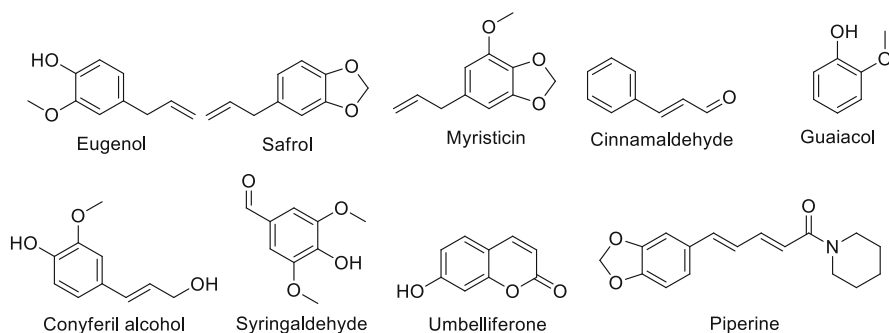


**Fig. 1.7** Different volatile monoterpenes that have antioxidant potential

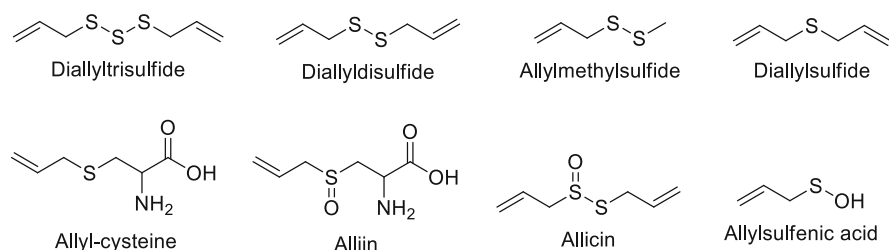
secretory cells, glandular trichomes, channels, and cavities. (Bakkali et al. 2008; Nunes et al. 2012 and references therein).

Terpenoids are structurally diverse group of compounds derived from multiples of  $n$  isoprene units ( $C_5$ ). These are classified as follows: hemiterpenes ( $C_5$ ), only one isoprene moiety; monoterpenes ( $C_{10}$ ), two isoprene moieties; sesquiterpenes ( $C_{15}$ ), three isoprene moieties; diterpenes ( $C_{20}$ ), four isoprene moieties; sesterpenes ( $C_{25}$ ), five isoprene moieties; triterpenes ( $C_{30}$ ), six isoprene moieties; and tetraterpenes ( $C_{40}$ ) – eight isoprene moieties. Mono-, sesqui-, and diterpenes and their oxygenated derivative are basic components of the essential oils. Essential oils are well recognized for their pharmacological potential such as sedative, expectorant, diuretic, antispasmodic, antiviral, anti-inflammatory, antipyretics, insecticidal, antimicrobial, fungicidal, etc. (Bakkali et al. 2008; Amorati et al. 2013 and references therein).

Essential oils also act as natural antioxidants and this is one emerging field with growing interest, particularly in cosmetics, food science, aroma science, and complementary medicine. Antioxidant potential plays a central role in a variety of essential oil's biological activity that is described through the contribution of oxidative stress. Components of essential oils particularly terpenes and phenols help to delay or stop the oxidative reaction going on in the organic matter (Amorati and Foti 2012). Further, the phenolic content of essential oil is very limited, due to which their antioxidant potential of essential oils is mainly attributed to the existence of mono-, sesqui-, and diterpenes and their oxygenated derivatives. Carvacrol, thymol, menthol,  $\gamma$ -terpinene, linalool, terpinolene, 1,8-cineole, isopulegol,  $p$ -cymene,  $\alpha$ -terpineol, and terpinen-4-ol are the different monoterpenes, which show antioxidant activity (Fig. 1.7) (Nunes et al. 2012, Amorati et al. 2013 and references therein). Some volatile phenylpropanoids (viz., eugenol, saffrole, myristicin, cinnamaldehyde, guaiacol, coniferil alcohol, syringaldehyde, umbelliferone, etc.) and alkaloids (piperine) present in various essential oils obtained from some



**Fig. 1.8** Different volatile phenylpropanoids and alkaloid that have antioxidant potential



**Fig. 1.9** Different volatile sulfur-containing natural antioxidants present in the essential oil of *Allium* species

particular plants also act as natural antioxidants (Fig. 1.8) (Brewer 2011; Amorati and Foti 2012; Amorati et al. 2013 and references therein).

## 1.6 Sulfur-Containing Natural Antioxidants

The essential oils/extracts obtained from various species of genus *Allium* (scallions *Allium fistulosum*, garlic *Allium sativum*, leeks *Allium ampeloprasum*, onions *Allium cepa*, shallots *Allium cepa* var. *aggregatum*, etc.) were rich source of different sulfur-containing compounds mainly volatile in nature. The composition of sulfur-containing compounds in different extracts and essential oils mainly depends upon the methods of isolation. The essential oil isolated from garlic by hydro and steam distillation mainly contained diallyl disulfide (25%) and diallyl trisulfide (50%) (Banerjee et al. 2003; Brewer 2011; Amorati et al. 2013 and references therein). It also contained diallyl sulfide, methyl allyl disulfide, and methyl allyl trisulfide in small amount. All these sulfur-containing compounds act as natural antioxidant. Some compounds such as alliin, allicin, and allyl sulfenic acid are not present in *Allium* species but are formed during their processing (upon homogenation) and are also known to possess high antioxidant potential (Brewer 2011; Amorati et al. 2013

and references therein). Figure 1.9 represents various sulfur-containing natural antioxidants present in different *Allium* species.

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## 1.7 Miscellaneous Natural Antioxidants

Apart from above key classes of SMs which act as natural antioxidant, there also exists another class of SMs which are also known for their antioxidant potential such as alkaloids, carotenoids, and vitamins. Alkaloids mainly having tetrahydro-beta-carboline, piperidine, benzyloisoquinoline, and boldine moieties in their structure also act as potent source of natural antioxidant (Fig. 1.10). These alkaloids are mainly present in spices, plants, fruits, and different food products (Cassels et al. 1995; Herraiz and Galisteo 2002; Brewer 2011; Muthna et al. 2013). Carotenoids (viz., beta-carotene, lycopene, zeaxanthin, etc., Fig. 1.10) represent another key class of natural antioxidants and are mainly obtained from vegetables and fruits such as carrots, potatoes, apricots, and papayas (Anwar et al. 2018). Further, various vitamins (vitamins A, C, and E, Fig. 1.10), minerals (Se selenium, Mg magnesium, Zn zinc, Cu copper, etc.), proteins, and peptides of plant origin also act as significant source of natural antioxidant (Sarmadi and Ismail 2010; Anwar et al. 2018)

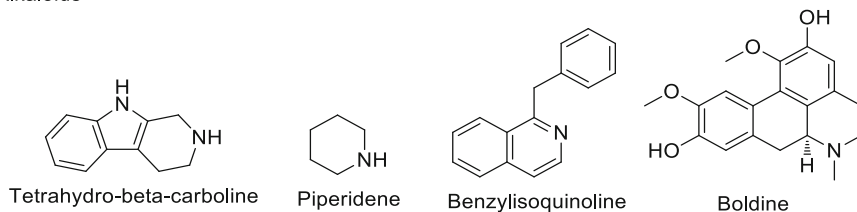
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## 1.8 Extraction Methods Used for Isolation of Natural Antioxidants

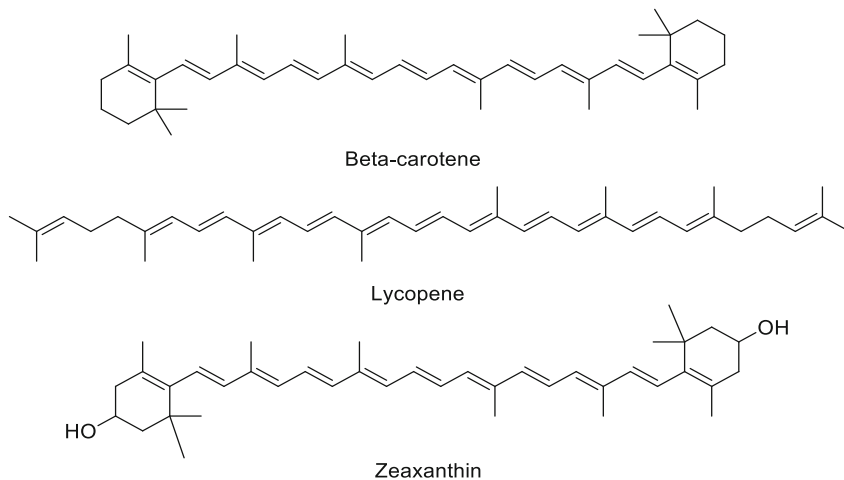
Natural antioxidants belong to various classes of SMs, and these can be obtained from plants by using both conventional and modern extraction methods. Modern extraction methods are more economical and eco-friendly in nature as compared to the conventional extraction methods, and due to this the latter ones are more and more often being replaced by the former ones (Pisoschi et al. 2016). The percentage extractive yield largely depends upon the extraction method and solvent used. Further, it also depends upon the time of extraction, feed-to-solvent ratio, temperature, and repeated extraction cycle number. Solubility of SMs largely depends upon extraction temperature and time. Higher extraction temperature significantly increases the solubility and mass transfer of SMs and simultaneously decreases the surface tension/viscosity of extraction solvents that consequently enhance the extraction rate of SMs (Brunner 2005; Mojzer et al. 2016). In order to remove unwanted compounds such as fats and fatty esters, waxes, chlorophylls, and other long-chain hydrocarbons, the plant material is first extracted with nonpolar solvents such as pet ether, cyclohexane, hexane, benzene, dichloromethane, chloroform, etc. (Mojzer et al. 2016).

Natural antioxidants can be extracted from dried, frozen, and fresh plant samples like roots, tuber, rhizomes, stems, peel, bark, leaves, cladode, flowers, and fruits. Prior to extraction, the plant material should be pre-treated by grinding, milling, homogenizing, and drying. The choice of drying method significantly influences the content of natural antioxidants. Mostly shade air-drying and freeze-drying methods

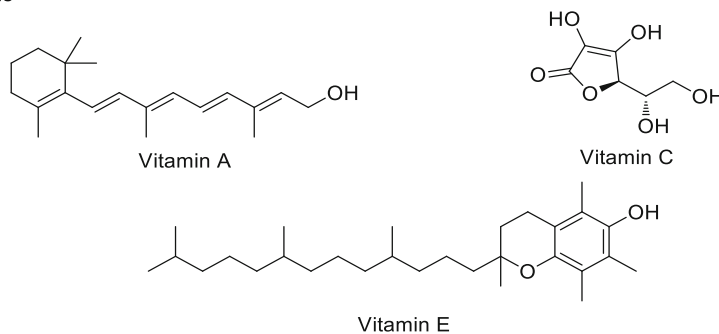
## Alkaloids



## Carotenoids



## Vitamins



**Fig. 1.10** Miscellaneous classes of natural antioxidants obtained from plants

are used for drying of plant material prior to the isolation of natural antioxidants (Abascal et al. 2005; Ajila et al. 2011; Mojzer et al. 2016).

Regardless of various disadvantages, conventional liquid-solid and liquid-liquid isolation methods are still frequently used extraction techniques. These conventional techniques have been widely used from past many years mainly due to their wide-ranging applicability, efficiency, and ease of use (Qiu et al. 2010; Mojzer et al.



2016). Soxhlet, maceration, infusion, percolation, decoction, cold pressing, etc. are the various conventional extraction methods used to isolate SMs/natural antioxidants (Azmir et al. 2013; Pisoschi et al. 2016). Out of these conventional extraction procedures, maceration and Soxhlet are most widely used for the isolation of natural antioxidants owing to their high efficiency and wide-ranging applicability as compared to other conventional methods (Mojzer et al. 2016; Sharma and Cannoo 2017). For the isolation of volatile natural antioxidants, the most widely used extraction techniques are hydro-distillation and steam distillation.

Nowadays, several modern extraction methods like ultrasound-assisted extraction, pressurized liquid extraction, ultrasound-microwave-assisted extraction, microwave-assisted extraction, negative pressure cavitation extraction, supercritical fluid extraction, and matrix solid-phase dispersion, etc. are also employed for the isolation of natural antioxidants. These modern methods use less solvent volume, reduce the extraction time, and improve the percentage extractive yield. These procedures are more sensitive, selective, and reproducible in nature as compared to conventional techniques. Among the modern extraction methods, supercritical fluid extraction, microwave-assisted extraction, and ultrasound-assisted extraction are usually used extraction approaches for the isolation of natural antioxidants (Gupta et al. 2012; Brusottia et al. 2014; Pisoschi et al. 2016; Cikos et al. 2018).

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## 1.9 Extraction Solvents Used for Isolation of Natural Antioxidants

A variety of nonpolar, medium polar, and polar solvents (hexane, dichloromethane, chloroform, ether, ethyl acetate, acetone, butanol, ethanol, methanol, water, and combination of polar solvents) has been used for the isolation of natural antioxidants by various researchers all over the world. Among these solvents, ethanol, methanol, and water are most frequently used solvents. These solvents have high dielectric constant and good polarity. Owing to this, these are used most favorably to extract polar natural antioxidants like phenolic acids, flavonoids, tannins, etc. according to the fact “like dissolves like” (Alam et al. 2013; Sharma and Cannoo 2016a, b; Patial et al. 2019). As the ethanol is organic and nontoxic in nature, it is largely used for the extraction of natural antioxidants as compared to methanol which is toxic in nature. Further, the removal of water from the extract after extraction is a big problem due to its high boiling points and it needs some special methods of freeze-drying. On the other hand, the nonpolar and medium polar solvents have been used only in some specific cases. In addition, their availability also confines their use for the isolation of natural antioxidants (Alam et al. 2013; Sharma and Cannoo 2016a, b; Patial et al. 2019).

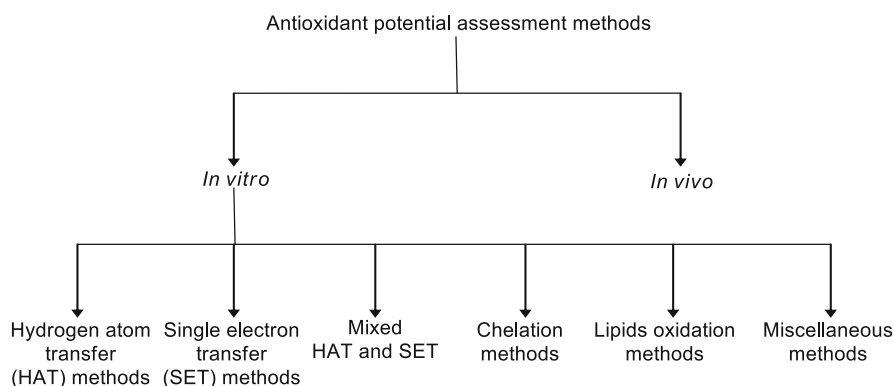
## 1.10 Methods of Evaluation for Antioxidant Potential of Natural Antioxidants

The different naturally occurring antioxidants have diverse mechanisms of action. These may serve as decomposers of various peroxides, terminators of chain initiation reaction, hydrogen abstractors, binders in transition metal ion catalysts, and scavengers of various free radicals. As a result, it is very difficult to assess an absolute antioxidant potential profile of any NOAs/plant extract by a single antioxidant assay (Niki 2011, 2016; Sharma and Cannoo 2016a, b). Thus, in order to have an absolute antioxidant potential profile of plant extract, it's better to evaluate antioxidant potential by employing a number of antioxidant assays (Sharma and Cannoo 2016a, b; Sharma and Cannoo 2017).

Currently more than 35 types of antioxidant assays are in use for the assessment of antioxidant potential of various plant extracts, essential oils, and different food-stuff. Out of these 30 assays, various assays are used in one and other form with some modifications. These antioxidant assays can be broadly classified into two groups *in vitro* and *in vivo* assays (Fig. 1.11) (Huang et al. 2005; Alam et al. 2013; Pisoschi et al. 2016).

### 1.10.1 In Vitro Assay

Presently more than 25 types of *in vitro* methods are in use. Usually *in vitro* antioxidant methods are straightforward and relatively easy to perform as compared to *in vivo* methods. These *in vitro* methods can be further classified in different groups depending upon their mode of action such as single electron transfer (SET) methods, hydrogen atom transfer (HAT) methods, mixed methods (have both HAT and SET mechanism), chelation methods, lipid oxidation methods, and miscellaneous methods (Fig. 1.11). Table 1.2 presents the division of different *in vitro*



**Fig. 1.11** Classification of different antioxidant assessment methods

**Table 1.2** Various in vitro assays commonly employed to assess the antioxidant activity (Huang et al. 2005; Prior et al. 2005; Alam et al. 2013; Pisoschi et al. 2016)

Sr. no.	Underlying mechanism	Antioxidant assay
1.	Hydrogen atom transfer (HAT) methods	Total radical trapping antioxidant parameter (TRAP) assay
		Oxygen radical absorbance capacity (ORAC) assay
		Beta-carotene/crocin bleaching assay
		Inhibited oxygen uptake (IOU) assay
		Inhibition of induced low-density lipoprotein peroxidation assay
2.	Single electron transfer (SET) methods	N,N-Dimethyl-p-phenylenediamine (DMPD) assay
		Ferric reducing antioxidant power (FRAP) assay
		Cupric reducing antioxidant capacity (CUPRAC) assay
		Potassium ferricyanide reducing power (PFRAP) assay
		Total phenolic content assay by Folin-Ciocalteu assay
		Total antioxidant capacity (TAC)/ phosphomolybdenum assay
3.	Mixed methods (have both HAT and SET mechanism)	2,2-Diphenyl-1-picrylhydrazyl (DPPH) scavenging assay
		2,2'-Azinobis-(3-ethyl-benzothiazoline)-6-sulfonic acid (ABTS) assay
		Trolox equivalent antioxidant capacity (TEAC)
4.	Chelation methods	Tetramethylmurexide (TMM) assay
		Ferrozine/metal chelating assay
5.	Lipid oxidation methods	Thiobarbituric acid (TBA) assay
		Anisidine assay
		Conjugated diene assay
		Peroxide value assessment assay
6.	Miscellaneous methods	Hydrogen peroxide scavenging (H <sub>2</sub> O <sub>2</sub> ) assay
		Peroxynitrite (ONOO) radical scavenging assay
		Nitric oxide (NO) scavenging assay
		Hydroxyl radical scavenging assay
		Hydroxyl radical averting capacity (HORAC) assay
		Superoxide radical scavenging (SOD) assay
Xanthine oxidase assay		

antioxidant assays into different groups depending upon the mechanism of action (Prior et al. 2005; Alam et al. 2013; Pisoschi et al. 2016).

### 1.10.2 In Vivo Assay

All the in vivo methods are performed usually by the administration of natural antioxidant containing samples to the testing animals such as mice, rats, and rabbits at a definite schedule dose as illustrated in the respective method. After a definite span of time, the testing animals are generally sacrificed, and tissues, blood, and blood serum are used for the method. Due to this, in vivo methods are more difficult to perform as compared to in vitro methods. Presently about ten types of in vivo methods are in use, viz., ferric reducing ability of plasma, glutathione peroxidase (GSHPx) estimation, reduced glutathione (GSH) estimation, glutathione reductase (GR) assay, glutathione-S-transferase (GSt), low-density lipoprotein (LDL) assay, catalase (CAT) method, superoxide dismutase (SOD) method, lipid peroxidation (LPO) assay, and gamma-glutamyl transpeptidase activity (GGT) assay (Alam et al. 2013).

Among the various in vitro assays, the seven most frequently used methods in order of increasing frequency are potassium ferricyanide reducing power (PFRAP) < nitric oxide (NO) scavenging < metal chelating < beta-carotene < superoxide radical scavenging (SOD) < hydroxyl radical scavenging assay < DPPH scavenging assay. The DPPH assay is the most frequently used among the all assay as it is the simplest, easy to perform, and takes less time for completion. Whereas in case of various in vivo assays, the six most frequently used methods (to assess the antioxidant potential of natural antioxidant rich samples) in order of increasing frequency are glutathione reductase (GR) < reduced glutathione (GSH) < glutathione peroxidase (GSHPx) < catalase (CAT) < superoxide dismutase (SOD) < lipid peroxidation (LPO) assay (Alam et al. 2013).

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## 1.11 Conclusion

Nowadays, the scientific knowledge and awareness about naturally occurring antioxidants, their methods of isolation, and their antioxidant potential assessment techniques (in vivo and in vitro) have been increasing significantly. This chapter provides significant information regarding oxidative stress, free radicals (FRs), and reactive oxygen/nitrogen species, their biological roles, and various routes of their production. The chapter also provides brief knowledge about the importance of various classes of natural antioxidants and their mode of action along with the accessibility of different extraction methods/solvents to from their natural resources. At the end, the chapter presents the detailed information about the various in vitro and in vivo methods currently used for the evaluation of antioxidant potential of plant extracts, essential oils, vegetables, fruits, and other dietary sources. Presently, in vitro methods of antioxidant potential evaluation have become important tools for

the investigation of new bioactive SMs (natural antioxidants). These tests assist to authenticate the importance of essential oils, plant extracts, vegetables, fruits, and other dietary products as effective antioxidant by verifying the presence of natural antioxidants in them. These natural antioxidants aid to fight against the injuries caused by oxidative stress conditions. Nevertheless, the increasing demand for new bioactive SMs (natural antioxidants) from natural sources boosts the invariable search for new and novel methods to extract, isolate, and evaluate (biological potential) new potent SMs, which never ends. The knowledge provided in the present chapter will be helpful to the beginner in this field.

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