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Biological Oxidations and Antioxidant Activity of Natural Products

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1. Introduction

Oxygen is the most prevalent element in the earth's crust. It exists in air as a diatomic molecule, O₂. Except for a small number of anaerobic bacteria, all living organisms use O₂ for energy production and it is essential for life as we know it. Energy production by organisms from food material requires "oxidation", which implies the loss of electrons. However the potential of O₂ to oxidize also makes it toxic. Oxidation can inactivate important enzymes, and anaerobes that do not have antioxidant mechanisms do not survive in an O₂ environment (Magder, 2006).

Life under aerobic conditions is characterized by continuous production of free radicals, which is counterbalanced by the activity of antioxidant enzymes and non-enzyme defences. Under physiological conditions oxidising agents and antioxidant defences are in balance. Living cells can either produce or take in anti-oxidative defense molecules which include enzymes such as catalase, superoxide dismutase, glutathione peroxidase, and non-enzymatic antioxidants such as glutathione, and vitamins C and E. However, if the production of free radicals exceeds the antioxidant capacity of a living system, these reactive oxygen and nitrogen species can react with lipids, proteins, and DNA causing structural and/or functional damage to the cell's enzymes and genetic material (Barreiros et al., 2006). The predominance of oxidants, and their consequent damage is called oxidative stress. Oxidants are generated in normal metabolism, in mitochondria, in peroxisomes, as cytosolic enzymes such as xanthine oxidase which is present in the cytosol of many tissues, and also can be found in the blood circulation bound to glycosaminoglycan sites in the arterial wall (Magder, 2006).

According to Sanchez et al. (2003), the body's mechanisms against the excess of reactive oxygen species (ROS), and oxidative stress may be classified as follows: (I) preventive mechanism; proteins which have a coordinated nucleus of iron or copper with the capacity to

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bind (albumin, myoglobin, metallothionein, ceruloplasmin, ferritin, transferrin,), which prevents the overproduction of HO^\bullet , (II) repairing mechanism; enzymes which repair or eliminate damaged biomolecules by ROS, like glutathione peroxidase, glutathione reductase, and methionine-sulphoxide reductase, and (III) scavenger mechanism; enzymes with capacity to scavenge excess ROS like superoxide dismutase, glutathione peroxidase, catalase, other metalloenzymes, and chemical entities with scavenging capacity like polyunsaturated fatty acids, vitamins C and E, uric acid, bilirubin, carotenoids, and flavonoids.

The term reactive oxygen species (ROS) includes radicals or chemical species that take part in radical type reactions (i.e. gain or loss of electrons) but are not true radicals in that they do not have unpaired electrons. Examples of non-radical ROS include hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), ozone (O_3) and singlet oxygen ($^1\text{O}_2$). Examples of radical ROS include super oxide anion radicals ($\text{O}_2^{\bullet-}$) and hydroxyl radical species ($^\bullet\text{OH}$). Besides oxygen-based radicals, there are also reactive nitrogen species such as nitric oxide (NO) and nitrogen dioxide (NO_2). An important product of the two radicals $\text{O}_2^{\bullet-}$ and NO is peroxynitrite (ONOO^-), this reaction occurs at a diffusion limited rate (Halliwell & Gutteridge, 2007; Magder, 2006). ROS may be generated through endogenous processes like mitochondrial respiration, the activation of polymorphonuclear leukocytes, arachidonic acid metabolism, enzymatic functions, and iron or copper mediated catalysis, among others. The human organism produces these ROS as a functional part of the harmonic balance between several physiological processes (Gupta & Verma, 2010).

Oxidative stress, caused by an imbalance between ROS and the anti-oxidative defense systems is considered to be a major etiological or pathogenic agent of cardiovascular and neurodegenerative diseases, cancers, Alzheimer's, diabetes and aging. Because they inhibit or delay the oxidative process by blocking both the initiation and propagation of oxidizing chain reactions, antioxidants for the treatment of cellular degenerations are beginning to be considered (Jang et al., 2010). Oxidative stress and its effects on human health have become a serious issue. Under stress, our bodies end up having more reactive oxygen species than antioxidant species, an imbalance that leads to cell damage (Krishnaiah et al., 2011). Cell degradation eventually leads to partial or total functional loss of physiological systems in the body. Currently, the incidence of free radical imbalance at the onset and during the evolution of more than 100 diseases (cardiovascular, neurological, endocrine, respiratory, immune and self-immune, ischaemia, gastric disorders, tumor progression and carcinogenesis, among others) has been demonstrated (Gupta & Verma, 2010).

Oxidation is essential to most living organisms for the production of energy and biological processes such as metabolic regulation, metabolic energy control, and activation/inactivation of biomolecules, signal transduction, cell exchange, endothelium-related vascular functions and gene expression. Reactive oxygen species are produced *in vivo* during oxidation. Free radical-scavenging is one of the known mechanisms by which antioxidants inhibit lipid peroxidation (Blokina et al., 2003). According to Jadhav et al. (1996), lipid oxidation is the reaction of oxygen with unsaturated fatty acids. In the initial stages free radicals form molecules susceptible to attacks from atmospheric oxygen O_2 , by removal of allylic hydrogen from fatty acid molecular carbons. These free radicals act as propagators of the reaction, and are converted to peroxides and hydro-peroxides (also radicals), which are the primary products of lipid oxidation. In the end, the new radicals combine to form stable and secondary products of oxidation by splitting and rearrangement, to form volatile and non-volatile epoxide compounds.

In recent years, substantial evidence has accumulated and indicated key roles for reactive oxygen species and other oxidants in causing numerous disorders and diseases. The evidence has brought the attention of scientists and the general public to an appreciation of antioxidants for prevention and treatment of diseases, and maintenance of human health (Halliwell & Gutteridge, 2007).

Antioxidants stabilize or deactivate free radicals, often before they attack targets in biological cells. Although almost all organisms possess antioxidant defense and repair systems to protect against oxidative damage, they cannot prevent the damage entirely.

Interest in naturally occurring antioxidants has considerably increased for use in food, cosmetic and pharmaceutical products, replacing synthetic antioxidants which are often restricted due to carcinogenic effects (Djeridane et al., 2006; Wannes et al., 2010). Aromatic and medicinal plants source natural antioxidants like polyphenols and essential oils which are secondary metabolites.

The purpose of this chapter is to review antioxidant classes, and methods for *in vitro* assessment of the antioxidant activity of natural products. We do not pretend to do a comprehensive review of the literature, but rather to present introductory information on the subject. In this chapter there is a list of some Brazilian medicinal plants with antioxidant activity.

2. Natural products and antioxidant activity

In this section we present the main classes (natural and synthetic) of antioxidant compounds, as well as important methods for assessment of *in vitro* antioxidant activity of natural products and plant extracts. Antioxidant activity is an important and fundamental function in life systems. Many other biological functions such as the anti-mutagenic, anti-carcinogenic, and anti-aging responses, originate from this property.

2.1 The main classes of antioxidant compounds

Antioxidants inhibit or delay the oxidation of other molecules by limiting either the initiation, or the propagation of oxidizing chain reactions. The natural antioxidants are phenolic compounds (tocopherols, flavonoids, phenolic acids), nitrogen compounds (alkaloids, chlorophyll derivatives, amino acids, and amines), carotenoids or ascorbic acid. Synthetic antioxidants are phenolic structures with various degrees of alkyl substitution (Velioglu et al., 1998).

In general, antioxidants are substances present in low concentrations (compared to the oxidizable substrate), which significantly delay or inhibit oxidation. The radicals formed from antioxidants do not propagate the lipid oxidative chain reaction mentioned above, but are neutralized by reaction with other radicals to form stable products, or recycled by other antioxidants. The chemical structures of natural and synthetic antioxidants most commonly used are shown in Table 1. Table 2 presents some Brazilian medicinal plants having antioxidant activity.

2.1.1 Synthetic antioxidants

Synthetic antioxidants such as butylated hydroxyanisole (BHA), and butylated hydroxytoluene (BHT), have been used as antioxidants since the beginning of this century.

Antioxidant	Chemical structure	Synthetic or Natural?
Ascorbic acid		Synthetic or natural
Butylated hydroxyanisole (BHA)		Synthetic
Butylated hydroxytoluene (BHT)		Synthetic
Gallic acid		Natural
Propyl gallate		Synthetic
Quercetin		Natural
Tertiary butylhydroquinone (TBHQ)		Synthetic
α-Tocoferol		Natural
Trolox®		Synthetic

Table 1. Chemical structures of natural and synthetic antioxidants commonly used.
(Adapted from Alves et al., 2010).

Plant name	Chemical content	Reference
<i>Acacia podallyriifolia</i>	Phenolic compounds	Andrade et al., 2007
<i>Anacardium occidentale</i>	Hydroalcoholic extract	Broinizi et al., 2008
<i>Anadenanthera peregrina</i>	Ethanol extract and partitions	Mensor et al., 2001
<i>Anaxagorea dolichocarpa</i>	Ethanol extract and partitions	Almeida et al., 2011
<i>Apuleia leiocarpa</i>	Ethanol extract and partitions	Mensor et al., 2001
<i>Baccharis articulata</i>	Flavonoids	Borgo et al., 2010
<i>Baccharis trimera</i>	Methanol extract	Morais et al., 2009
<i>Brillantaisia palisatii</i>	Ethanol extract and partitions	Mensor et al., 2001
<i>Brosimum guianense</i>	Ethanol extract and partitions	Mensor et al., 2001
<i>Camelia sinensis</i>	Methanol extract	Morais et al., 2009
<i>Cenostigma macrophyllum</i>	Phenolic compounds	Sousa et al., 2007
<i>Copernicia cerifera</i>	Phenolic compounds	Sousa et al., 2007
<i>Croton argyropphylloides</i>	Essential oil	Morais et al., 2006
<i>Croton nepetaefolius</i>	Essential oil	Morais et al., 2006
<i>Croton zenhtneri</i>	Essential oil	Morais et al., 2006
<i>Cymbopogon citratus</i>	Methanol extract	Morais et al., 2009
<i>Duguetia chrysocarpa</i>	Ethanol extract and partitions	Almeida et al., 2011
<i>Encholirium spectabile</i>	Ethanol extract	Carvalho et al., 2010
<i>Hyptis elegans</i>	Ethanol extract and partitions	Mensor et al., 2001
<i>Hyptis tetracephala</i>	Ethanol extract and partitions	Mensor et al., 2001
<i>Jacaranda puberula</i>	Ethanol extract	Santos et al., 2010
<i>Lantana camara</i>	Ethanol extract and partitions	Mensor et al., 2001
<i>Lantana trifolia</i>	Ethanol extract and partitions	Mensor et al., 2001
<i>Laurencia dendroidea</i>	Sesquiterpenes	Gressler et al., 2011
<i>Lippia alba</i>	Methanol extract	Morais et al., 2009
<i>Lonchocarpus filipes</i>	Flavonoids	Santos et al., 2009
<i>Marsypianthes chamaedrys</i>	Ethanol extract and partitions	Mensor et al., 2001
<i>Matricaria recutita</i>	Methanol extract	Morais et al., 2009
<i>Mentha arvensis</i>	Methanol extract	Morais et al., 2009
<i>Palicourea rigida</i>	Flavonoids	Rosa et al., 2010
<i>Platypodium elegans</i>	Ethanol extract and partitions	Mensor et al., 2001
<i>Pseudopiptadenia contorta</i>	Ethanol extract and partitions	Mensor et al., 2001
<i>Punica granatum</i>	Etheric, alcoholic and aqueous extracts	Jardini & Mancini-Filho, 2007
<i>Pyrus malus</i>	Methanol extract	Morais et al., 2009
<i>Qualea grandiflora</i>	Phenolic compounds	Sousa et al., 2007
<i>Raphiodon echinus</i>	Ethanol extract and partitions	Mensor et al., 2001
<i>Terminalia brasiliensis</i>	Phenolic compounds	Sousa et al., 2007
<i>Terminalia fagifolia</i>	Phenolic compounds	Sousa et al., 2007
<i>Turnera ulmifolia</i>	Phenolic compounds	Nascimento et al., 2006
<i>Verbena litoralis</i>	Ethanol extract and partitions	Mensor et al., 2001
<i>Vitex cymosa</i>	Ethanol extract and partitions	Mensor et al., 2001
<i>Vitex polygama</i>	Ethanol extract and partitions	Mensor et al., 2001

Table 2. Some Brazilian plants having antioxidant activity.

They are commonly used to preserve food. Restrictions on the use of these compounds are being imposed because of their carcinogenicity. Thus, the interest in natural antioxidants has increased considerably (Velioglu et al., 1998). The replacement of synthetic with natural antioxidants (because of implications for human health) may be advantageous.

Although synthetic antioxidants, such as BHA, BHT and propyl gallate, have been commonly added to food products to retard lipid oxidation, the demand for natural antioxidants has increased because of the negative perception consumers have about the long-term safety of synthetic antioxidants. Yet, regular consumption of fruit and vegetables containing natural antioxidants is correlated with decreased risks for diseases such as cancer and cardiovascular diseases (Jang et al., 2010).

In the food industry, synthetic antioxidants such as ascorbic acid and BHT have been widely used as additives to preserve and stabilize foods and animal feed products for freshness, nutritive value, flavour, and colour. Yet, at least one study has shown BHT to be potentially toxic, especially in high doses, making it important to consider health risks associated with long-term dietary intake of BHT (Oliveira et al., 2009a).

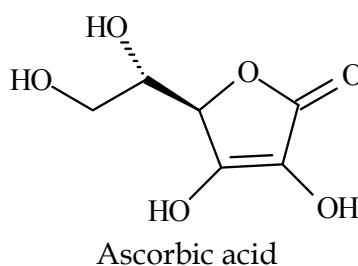
In recent years, while the toxicity of synthetic chemical antioxidants has been criticized, studies have begun to investigate the potential of plant products to serve as antioxidants for protection against free radicals. Phenolics, flavonoids, tannins, proanthocyanidins, and various plant and herbal extracts have been reported to be radical scavengers that inhibit lipid peroxidation.

Synthetic antioxidants Trolox®, and TBHQ (tertiary butylhydroquinone) are widely used. TBHQ is a derivative of hydroquinone, substituted with a *tert*-butyl group. It is a highly effective antioxidant used in foods as a preservative for unsaturated vegetable oils and many edible animal fats. The *tert*-butyl substituents in TBHQ, BHA and BHT function mainly to increase the lipid solubility.

2.1.2 Natural antioxidants

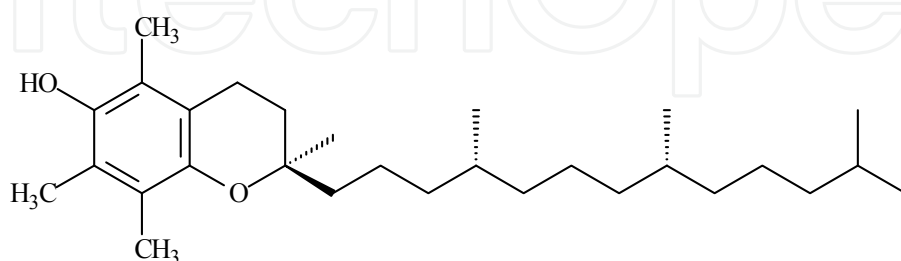
2.1.2.1 Ascorbic acid

Ascorbic acid (vitamin C) is widely known for its antioxidant activity and is therefore used in cosmetics and degenerative disease treatments. Vitamin C has many physiological functions, among them a highly antioxidant power to recycle vitamin E in membrane and lipoprotein lipid peroxidation. Paradoxically, however, it should also be noted that, *in vitro*, vitamin C is also capable of pro-oxidant activity. It has long been known that the combination of ascorbate and ferrous ions generates hydroxyl radicals, which induces lipid peroxidation (Haslam, 1996). Vitamin C is a potent antioxidant for hydrophilic radicals, but poor against lipophilic radicals.

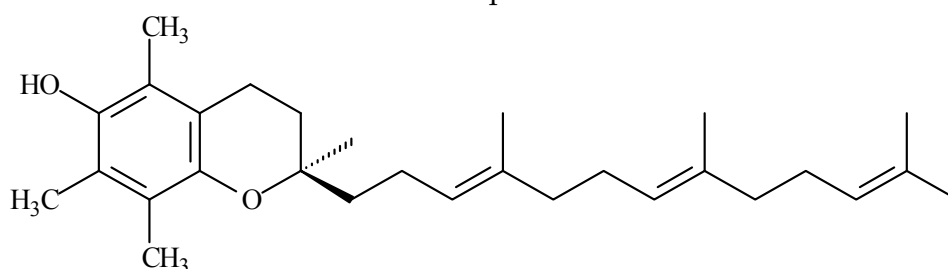


2.1.2.2 Tocopherols

Tocopherols and tocotrienols are widely distributed in nature. Vitamin E is the common name given to a group of lipid-soluble compounds of which α -tocopherol is the most familiar. It is found in lipoproteins and membranes, and acts to block the chain reaction of lipid peroxidation by scavenging intermediate peroxy radicals being generated. The highly steric (hindered) α -tocopheryl radical is much less reactive in attacking fatty acid side chains and converts back to its parent phenol thru ascorbic acid, thus breaking the chain reaction (Haslam, 1996).



α -Tocopherol

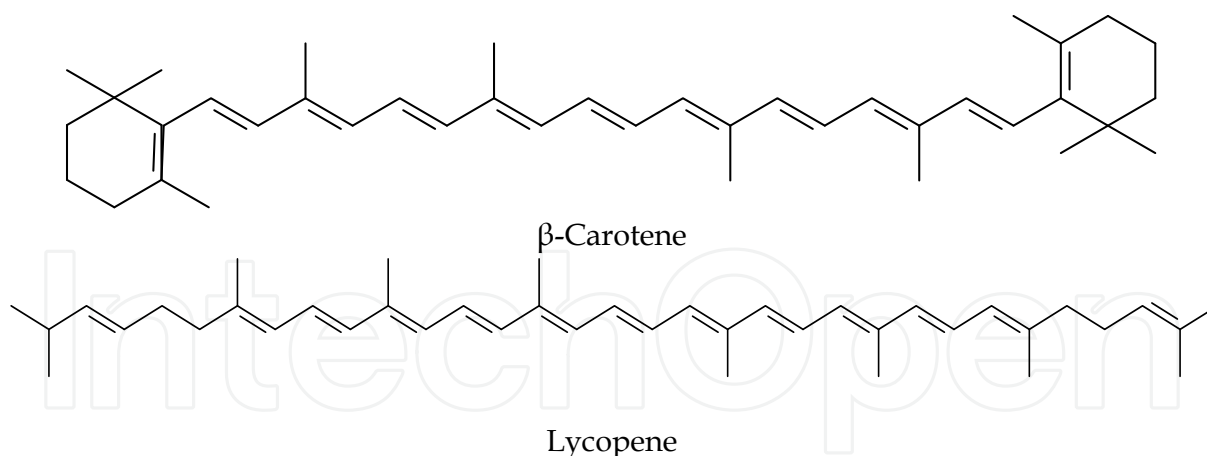


Tocotrienol analogue

2.1.2.3 Carotenoids

Carotenoids protect lipids against peroxidative damage by inactivating singlet oxygen (without degradation) reacting with hydroxyl, superoxide, and peroxy radicals. Relative to phenolics and other antioxidants, carotenoids are not particularly good quenchers of peroxy radicals, but they are exceptional at quenching singlet oxygen, at which most other phenolics and antioxidants are relatively ineffective. The antioxidant activity of carotenoids is due to the ability to delocalize unpaired electrons through their structure of conjugated double bonds. Three proposed mechanisms for free radical reactions involving carotenoids are reported in the literature. Much of our present knowledge comes from epidemiological studies and indicates that the incidence of some forms of cancer and cardiovascular disease appear to be lower in populations with large relative intakes of antioxidant nutrients such as vitamins C, and E, and the various carotenoids (Haslam, 1996).

The β -carotene is the most abundant of the carotenoids and widely used in therapies. It is almost completely insoluble in water but readily soluble in hydrophobic environments, and slightly polar solvents. β -carotene is highly reactive with electrophiles and oxidants. While many studies have shown β -carotene inhibition of lipid auto-oxidation in biological tissues and food, few details of the kinetics or mechanism of these reactions have been revealed (Alves et al., 2010). Lycopene is also well known for its antioxidant activity.



2.1.2.4 Phenolic compounds

Phenolic compounds are commonly found in both edible and non-edible plants, and they have been reported to have multiple biological effects, including antioxidant activity. Crude extracts of fruits, herbs, vegetables, cereals, and other plant materials rich in phenolics are increasingly being used in the food industry because they retard oxidative degradation of lipids and improve the quality and nutritional value of food. The antioxidant constituents of plants are also raising interest among scientists, food manufacturers, and consumers as the trend of the future is toward functional food with specific health effects for the maintenance of health, protection from coronary heart disease, and cancer (Kähkönen et al., 1999).

Phenolic compounds are considered secondary metabolites and are synthesized by plants during normal development, and in response to infections, wounding, UV radiation, and insects. These phytochemical compounds derived from phenylalanine and tyrosine occur ubiquitously in plants and are very diversified (Naczek & Shahidi, 2004).

Phenolic plant compounds fall into several categories; simple phenolics, phenolic acids (derivatives of cinnamic and benzoic acids), coumarins, flavonoids, stilbenes, tannins, lignans and lignins (Figure 1). Chief among these are the flavonoids which have potent antioxidant activities.

2.1.2.5 Flavonoids

Flavonoids are naturally occurring in plants and are thought to have positive effects on human health. Studies on flavonoidic derivatives have shown a wide range of antibacterial, antiviral, anti-inflammatory, anticancer, and anti-allergic activities (Di Carlo et al., 1999; Montoro et al., 2005). With their biological activity, flavonoids are important components of the human diet, although they are generally considered as non-nutrients. Sources of flavonoids are foods, beverages, different herbal drugs, and related phytomedicines (Montoro et al., 2005).

Flavonoids are an important class of phenolic compounds, and have potent antioxidant activity. The antioxidant property of flavonoids was the first mechanism of action studied with regard to their protective effect against cardiovascular diseases. Flavonoids have been shown to be highly effective scavengers of most oxidizing molecules, including singlet oxygen, and various free radicals (Bravo, 1998) implicated in several diseases.

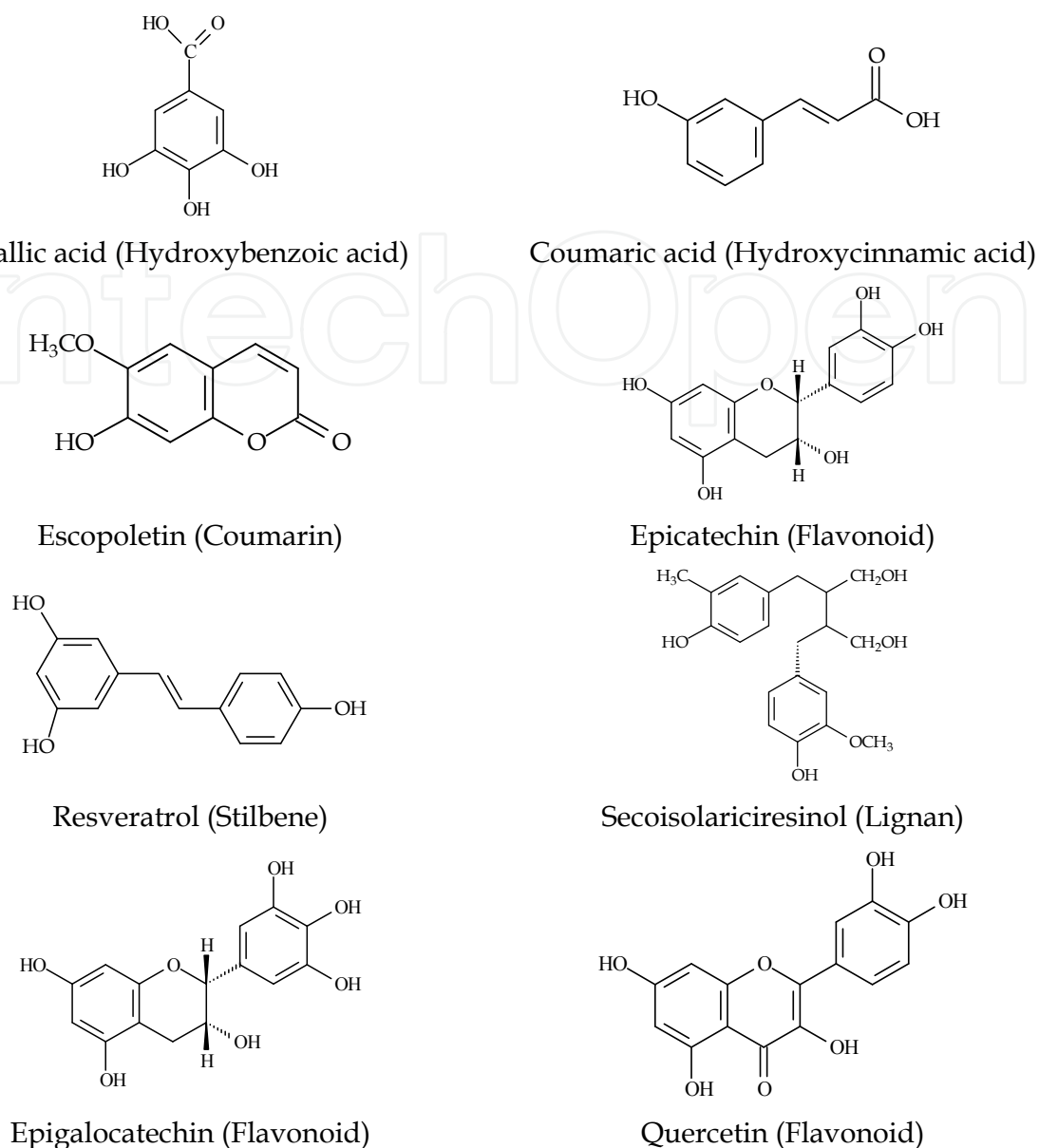


Fig. 1. Chemical structures of some phenolic compounds.

The antioxidant mechanism involves suppressing reactive oxygen formation, by inhibiting enzymes, chelating trace elements involved in free-radical production, scavenging reactive species, and up-regulating and protecting antioxidant defences (van Acker et al., 1996).

More than 4000 flavonoids have been identified, and the number is still growing. Flavonoids can be further divided into chalcones, anthocyanins, flavones, isoflavones, flavanones, flavononols and flavanols (Ignat et al., 2011). The chemical structures of the main classes of flavonoids are shown in Figure 2.

Anthocyanins are probably the largest group of phenolic compounds in the human diet, and their strong antioxidant activities suggest their importance in maintaining health (Velioglu et al., 1998). When consumed regularly, by humans, these flavonoids have been associated with a reduction in the incidence of diseases, such as cancer and heart disease.

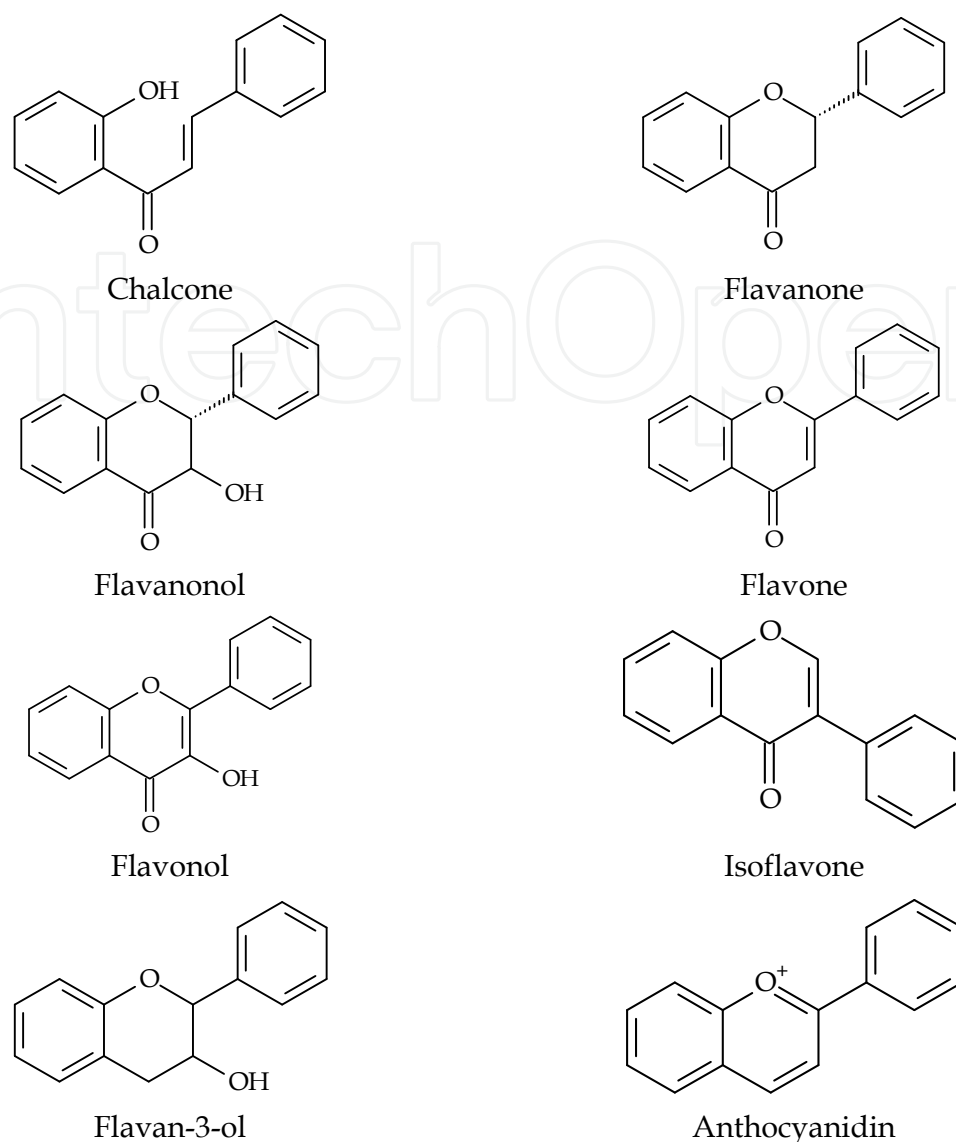


Fig. 2. Basic structures of the main classes of flavonoids (Adapted from Coutinho et al., 2009).

2.1.2.6 Essential oils

Essential oils also called volatile or ethereal oils are aromatic compounds, oily liquids obtained from different plant parts, and widely used as food flavours. Essential oils are complex mixtures comprised of many single compounds. Chemically they are derived from terpenes, and their oxygenated compounds. Essential oils have been useful in food preservation, aromatherapy and the fragrance industry (Bakkali et al., 2008).

In nature, essential oils have an important role in protecting plants. They serve as antibacterial agents, antivirals, antifungals, and insecticides, and also against the action of herbivores. They sometimes attract insects to help the spread of pollen or repel other unwanted insects. They are liquid, volatile, transparent, rarely coloured, soluble in organic solvents, and have lower densities than that of water. Synthesized by all organs of the plant, such as buds, flowers, leaves, stems, seeds, fruits, roots and bark, they are stored in secretor cells, cavities, channels, epidermal cells, and glandular trichomes (Bakkali et al., 2008).

Terpenoids form a large and structurally diverse family of natural products derived from isoprenoid units C_5 . These compounds have carbon skeletons being multiples of n (C_5), and are classified as hemiterpenes (C_5), monoterpenes (C_{10}), sesquiterpenes (C_{15}), diterpenes (C_{20}), sesterpenes (C_{25}), triterpenes (C_{30}) and tetraterpenes (C_{40}) (Dewick, 2002). Monoterpenes are primary components of essential oils, and the effects of many medicinal herbs have been attributed to them. Among various monoterpenes that have antioxidant activity are carvacrol, thymol, γ -terpinene and terpinolene, linalool, and isopulegol, among others (Figure 3).

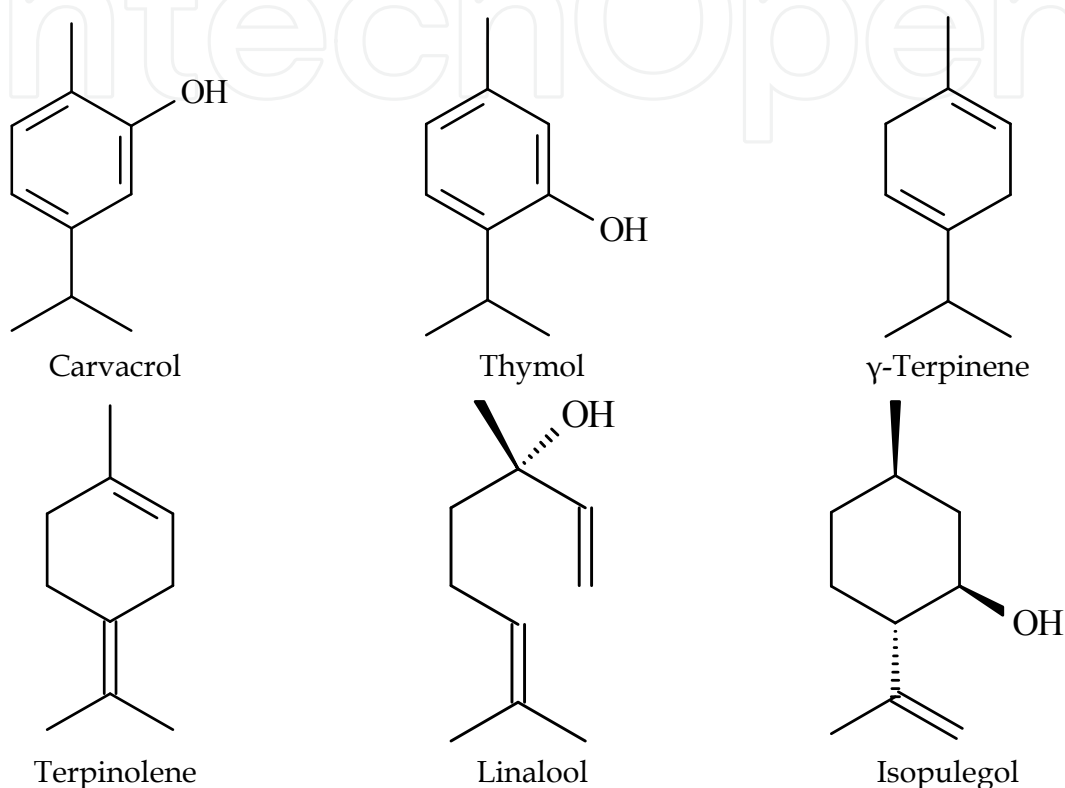


Fig. 3. Chemical structures of monoterpenes with antioxidant activity.

2.2 Methods of antioxidant activity assessment for natural products

Studies on free radicals and the development of new methods for evaluation of antioxidant activity (AA) have increased considerably in recent years. The noted deleterious effect of free radicals on cells in relation to certain diseases has encouraged the search for new substances that can prevent or minimize oxidative damage. Due to the different types of free radicals and their different forms of action in living organisms, it is unlikely that a single, simple and accurate universal method by which antioxidant activity can be measured will ever be developed. However, the search for faster and more efficient testing has generated a large number of methods to assess the activity of natural antioxidants, and they use a variety of systems to generate free radicals (Alves et al., 2010).

Several techniques have been used to determine the antioxidant activity *in vitro* in order to allow rapid screening of substances and/or mixtures of potential interest in the prevention of chronic degenerative diseases. These studies are extremely important, since substances that have low antioxidant activity *in vitro*, will probably show little activity *in vivo*. What follow are some methods for antioxidant activity evaluation and their main applications.

2.2.1 DPPH assay

DPPH reactivity is one popular method of screening for free radical-scavenging ability in compounds, and has been used extensively for antioxidants in fruits and vegetables. This method was first described by Blois in 1958, and was later modified slightly by numerous researchers. DPPH is a stable free radical that reacts with compounds that can donate a hydrogen atom. The method is based on scavenging of DPPH through the addition of a radical species or antioxidant that decolourises the DPPH solution (Figure 4). The degree of colour change is proportional to the concentration and potency of the antioxidants. Antioxidant activity is then measured by the decrease in absorption at 517 nm. A large decrease in the absorbance of the reaction mixture indicates significant free radical scavenging activity of the compound under test (Krishnaiah et al., 2011). This method is considered, from a methodological point of view, one of the easiest, most accurate and productive for evaluation of antioxidant activity in fruit juices, plant extracts and pure substances like flavonoids and terpenoids (Alves et al., 2010). The method is influenced by the solvent and the pH of the reactions. The antioxidants BHA, BHT and Trolox® can be used as references in the experiments.

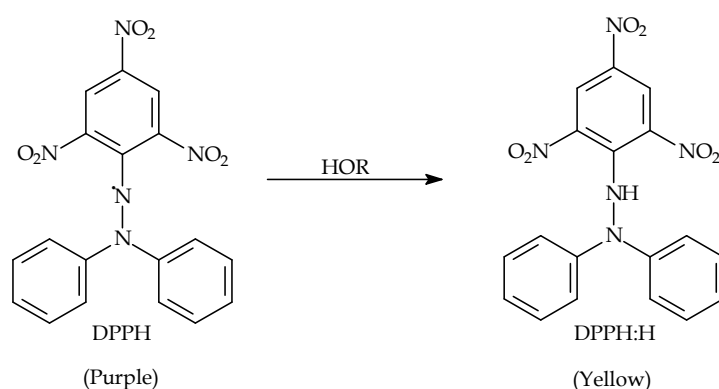


Fig. 4. Radical and non-radical forms of DPPH.

The electron donation ability of natural products can be measured by 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) purple-coloured solution bleaching. The anti-radical activity (three replicates per treatment) is expressed as IC_{50} ($\mu\text{g/ml}$), the concentration required to cause a 50% DPPH inhibition. The presence of the phenolic hydroxyls appears essential for scavenger properties.

2.2.2 β -carotene bleaching test

The β -carotene/linoleic acid oxidation method evaluates the inhibitory activity of free radicals generated during the peroxidation of linoleic acid. The method is based on spectrophotometric discoloration measurements or (oxidation) of β -carotene-induced oxidative degradation products of linoleic acid. This method is suitable for plant samples.

The β -carotene bleaching method is based on the loss of β -carotene's yellow colour due to its reaction with radicals formed by linoleic acid oxidation when in an emulsion. The rate of the β -carotene bleaching can be slowed in the presence of antioxidants (Kulisic et al., 2004). The reaction can be monitored by spectrophotometer, β -carotene loss of staining at 470 nm, with

intervals of 15 min for a total time of 2 h. The results are expressed as IC_{50} ($\mu\text{g/ml}$), the concentration required to cause a 50% β -carotene bleaching inhibition. Tests are realized in triplicate. The results can be compared with synthetic standards such as BHA, BHT and Trolox®, or natural, such as gallic acid and quercetin (Alves et al., 2010).

2.2.3 ABTS method

The 2,2'-azinobis(3-ethylbenzthiazoline-6-sulphonic acid), commonly called ABTS, radical scavenging method was developed by Rice-Evans and Miller and was then modified by Re et al. in 1990. The modification is based on the activation of metmyoglobin with hydrogen peroxide in the presence of ABTS⁺ to produce a radical cation. The improved method generates a blue/green ABTS⁺ chromophore via the reaction of ABTS and potassium persulfate. It is now widely used. Along with the DPPH method, the ABTS radical scavenging method is one of the most extensively used antioxidant assays for plant samples. The ABTS radical cation is generated by the oxidation of ABTS with potassium persulfate, its reduction in the presence of hydrogen-donating antioxidants is measured spectrophotometrically at 734 nm. Decolourisation assays measure the total antioxidant capacity in both lipophilic and hydrophilic substances. The effects of oxidant concentration and inhibition duration, of the radical cation's absorption are taken into account when the antioxidant activity is determined. Trolox is used as a positive control. The activity is expressed in terms of Trolox-equivalent antioxidant capacity for the extract or substance (TEAC/mg) (Krishnaiah et al., 2011).

2.2.4 ORAC assay

The peroxy radical is an oxidant commonly found in biological substrates. It is less reactive than OH^\bullet having a half-life from seconds to nanoseconds (Alves et al., 2010). The ORAC (oxygen radical absorbance capacity) assay uses beta-phycoerythrin (PE) as an oxidizable protein substrate, and 2,2'-azobis(2-amidinopropane)dihydrochloride (AAPH), as a peroxy radical generator, or a Cu^{2+} - H_2O_2 system as a hydroxyl radical generator. To date, it is the only method that takes the free radical reaction to completion, and uses an area-under-the curve (AUC) technique for quantification, combining both the inhibition percentage and the length of inhibition time for free radical action into a single quantity. The assay has been widely used in many recent studies of plants (Krishnaiah et al., 2011). Trolox is used as a standard antioxidant.

2.2.5 Reducing power assay

In this assay, the yellow colour of the test solution changes to green depending on the reducing power of the test specimen. The presence of the reductants in the solution causes the reduction of the Fe^{3+} /ferricyanide complex to the ferrous form. Therefore, Fe^{2+} can be monitored by absorbance measurement at 700 nm.

In the reducing power method, the sample is mixed in 1 ml of methanol with a phosphate buffer (5 ml, 0.2 M, pH 6.6) and potassium ferricyanide (5 ml, 1%). The mixture is incubated at 50 °C for 20 min. Next, 5 ml of trichloroacetic acid (10%) are added to the reaction mixture, which is then centrifuged at 3000 RPM for 10 min. The upper layer of the solution (5 ml) is mixed with distilled water (5 ml), and ferric chloride (1 ml, 1%), and the absorbance

is measured at 700 nm. A stronger absorbance indicates increased reducing power (Krishnaiah et al., 2011).

2.2.6 NBT assay or the superoxide anion scavenging activity assay

Xanthine oxidase (XO) is the enzyme responsible for conversion of xanthine into uric acid, resulting in the production of hydrogen peroxide and superoxide (Figure 5). It is considered a major biological source of reactive oxygen species. It is possible that inhibition of this enzymatic process by compounds that exhibit antioxidant properties may have therapeutic use (Alves et al., 2010).

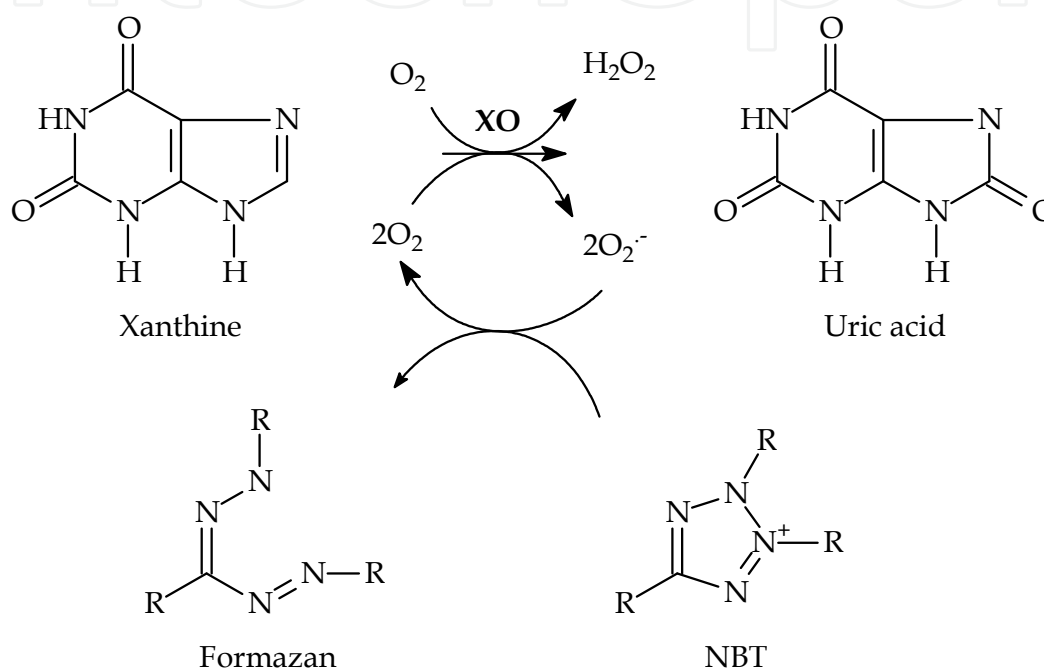


Fig. 5. Formation of formazan from NBT (Adapted from Alves et al., 2010).

The scavenging potential for superoxide radicals is analysed with a hypoxanthine/xanthine oxidase-generating system coupled with nitroblue tetrazolium (NBT) reduction (measured spectrophotometrically). The reaction mixture contains 125 μ l of buffer (50 mM KH₂PO₄/KOH, pH 7.4), 20 μ l of a 15 mM Na₂EDTA solution in buffer, 30 μ l of a 3 mM hypoxanthine solution in buffer, 50 μ l of a 0.6 mM NBT solution in buffer, 50 μ l of xanthine oxidase in buffer (1 unit per 10 ml buffer), and 25 μ l of the plant extract in buffer (a diluted, sonicated solution of 10 μ g per 250 μ l buffer). Microplates (96 wells) are read at 450 nm 2.5 min after the addition of the xanthine oxidase using a series 7500 Microplate Reader. Superoxide scavenger activity is expressed as percent inhibition compared to the blank, in which buffer is used in place of the extract. When using this system, any inhibition by tannins in the plant extracts must be due to their antioxidant activity and any action upon the enzyme must be excluded as a possibility (Krishnaiah et al., 2011).

2.2.7 Chelating effect on ferrous ions

Chelating activity of samples can be determined by the ferrozine assay. Ferrozine quantitatively forms complexes with Fe²⁺. In the presence of other chelating agents, the

complex formation is disrupted with a resulting decrease in the red colour of the complex. Measurement of the rate of colour reduction allows estimation of the chelating activity of the coexistent chelator (Yamaguchi et al., 2000; Wannes et al., 2010).

2.2.8 Determination of phenol content by Folin-Ciocalteu method

Folin-Ciocalteu phenol reagent consists of a mixture of the hetero-poly phosphomolybdic and phosphotungstic acids in which the molybdenum and tungsten are in the 6⁺ state. On reduction with certain reducing agents, molybdenum blue and tungsten blue are formed, in which the mean oxidation state of the metals is between 5 and 6. It is known that Folin-Ciocalteu reagent reacts not only with phenols but also with a variety of other compounds. The total phenolic content measured by the Folin-Ciocalteu procedure does not give a full picture of the quantity, or quality of the phenolic constituents in the extracts. In addition, there may also be interference arising from other chemical components present in the extract, such as sugars or ascorbic acid (Singleton & Rossi, 1965). Gallic acid is used as a standard for the calibration curve. The total phenolic content is expressed as mg of gallic acid equivalent (GAE). Figure 6 shows the reaction of gallic acid with molybdenum, a component of the Folin-Ciocalteu reagent.

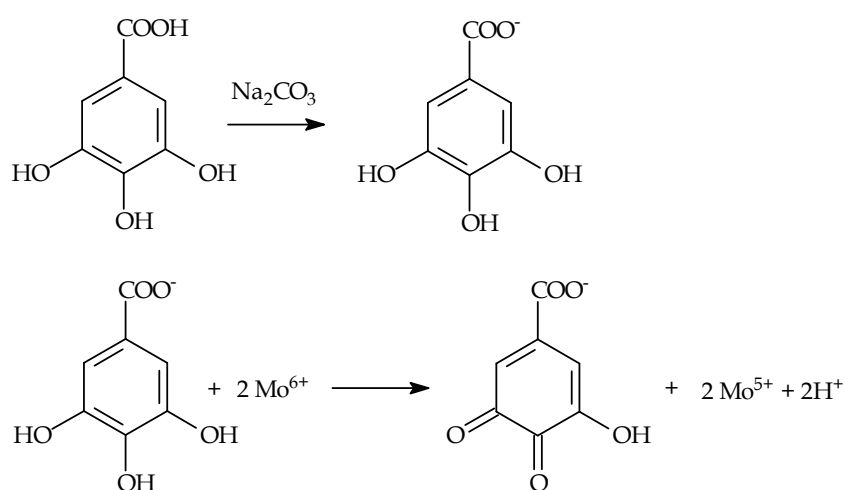


Fig. 6. Reaction of gallic acid with molybdenum, a component of the Folin-Ciocalteu reagent (Adapted from Oliveira et al., 2009b).

2.2.9 Total flavonoid content

Total flavonoid content is determined by using a colorimetric method described previously (Dewanto et al., 2002). Briefly, 0.30 mL of the EtOH and AcOEt extracts or (+)-catechin standard solution is mixed with 1.50 mL of distilled water in a test tube followed by addition of 90 μL of a 5% NaNO_2 solution. After 6 min, 180 μL of a 10% $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$ solution is added and allowed to stand for another 5 min before 0.6 mL of 1 M NaOH is added. The mixture is brought to 330 μL with distilled water and mixed well. The absorbance is measured immediately against the blank at 510 nm using a spectrophotometer in comparison with the standards prepared similarly with known (+)-catechin concentrations. The results are expressed as mg of catechin equivalents per gram of extract (mg CE/g) through a calibration curve with catechin.

3. Conclusion

Oxidative stress is involved in the development of various diseases and their symptoms, especially degenerative diseases. Scientific knowledge of the antioxidant activity of natural products, along with state of the art *in vitro* methods for evaluation has been increasing over time. *In vitro* testing has become an important tool in the search for bioactive substances, and for raw material selection studies as well. These tests have demonstrated the importance of diets rich in fruits and vegetables by confirming the presence of antioxidants that help fight free radicals, and which in moderate consumption are beneficial to human health.

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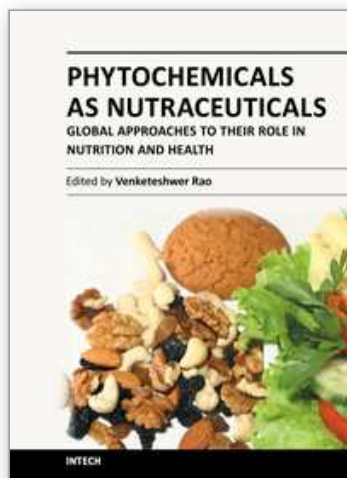
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