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Oxidants and Antioxidants in Complementary and Alternative Medicine: A Review

Tamamlayıcı ve Alternatif Tıpta Oksidan ve Antioksidanlar: Bir Derleme

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SUMMARY
Reactive oxygen species and antioxidants have attracted immense interest of researchers because of their implied role in the protection of biological systems. Though all living organisms possess their own antioxidant defense systems. No one can disregard the importance of dietary or exogenous antioxidants in the prevention of a variety of diseases. There is, therefore, a need to discover new and effective radical scavengers from natural sources that have low or no side effects for use in preventive and/or curative medicine. Natural antioxidant molecules can be considered as precursors for synthesis of a variety of more biologically active derivatives. Several studies have revealed that antioxidant compounds play an important role in inhibition of hydrolytic and oxidative enzymes, anti-inflammatory action and various other biological or pharmacological activities in addition to free radical scavenging. The present study was aimed to compile the rols of oxidants and antioxidants in complementary and alternative medicine.
Key words: Reactive oxygen species, oxidants, antioxidants, vitamin E, vitamin C, melatonin, carotenoids, polyphenols, phenolic acids, flavonoids, lignans, stilbenes, phytophenols, gallic acid, antioxidant activity, complementary and alternative medicine.

ÖZET
Reaktif oksijen türevleri ve antioksidanlar, biyolojik sistemlerin korunmasındaki üstü kapalı rollerinden dolayı araştırmacıların yoğun ilgisini çekmişlerdir. Gerçi tüm yaşayan organizmalar kendi antioksidan savunma sistemlerine sahiptir. Çeşitli hastalıkların önlenmesinde diyet kaynaklı veya eksojen antioksidanların önemini kimse göz ardı edemez. Bu yüzden, önleyici ve/veya küratif tıpta kullanım için yan etkisi düşük veya yan etkisi olmayan doğal kaynaklardan elde edilen yeni ve etkin radikal süpürücülerin keşfedilmesine ihtiyaç vardır. Doğal antioksidan moleküller, çeşitli biyolojik olarak daha aktif türevlerin sentezi için öncüler olarak dikkate alınmaldır. Birçok çalışma, antioksidan bileşiklerin hidrolitik ve oksidatif enzimlerin inhibisyonunda, antienflamatuar etkide ve serbest radikal süpürücülere ilaveten diğer biyolojik ve farmakolojik etkilerde önemli bir rol oynadığını ortaya koymuştur. Şimdiki çalışma tamamlayıcı ve alternative tıpta oksidan ve antioksidanlar rolünü derlemeyi amaçladı.
Anahtar kelimeler: Reaktif oksijen türevleri, oksidanlar, antioksidanlar, E vitamin, C vitamin, melatonin, karotenoidler, polifenoller, fenolik asitler, flavonoidler, lignanlar, stilbenler, fitofenoller, gallik asit, antioksidan etki, tamamlayıcı ve alternatif tıp.

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INTRODUCTION

Oxygen is a basic element for life to carry out biological functions such as catabolism of carbohydrates, proteins and lipids in order to generate energy for growth and other activities [1]. However, an equivalent role of oxygen as a toxic agent for living tissues has also been discovered. Oxygen, though not hazardous by itself, is involved in the synthesis of various kinds of "reactive oxygen species" (ROS) or free radicals [1]. These ROS or free radicals formed during metabolic reactions or through the action of ionizing radiation can interact with biomolecules and ultimately lead to an onset of degenerative diseases such as cancers, cardiovascular diseases (CVD) and other clinical disorders [2, 3]. To defend against the destructive action of these ROS, antioxidant defense system comprising of a group of compounds and enzymes has been created by nature to remove free radicals before causing any tissue damage [3, 4]. Some antioxidants are created in the body while others are sequestered from the diet or through nutritional supplementation [1]. Various plants and their phytoconstitutents are abundant sources of antioxidants. There is a large number of naturally occurring and synthetic antioxidants belonging to diverse categories such as carotenoids, phenols, polyphenolics, gallic acid derivatives, tannins, catechins etc. Examples include eugenol, guaiacol, vanillin, isovanillin, umbelliferone, sesamol, thymol, menthol, phylic acid, lípoic acid, quercetin, carnosol, rutin, butylated hydroxyanisole, butylated hydroxy toluene etc [1]. Vitamin C, E and minerals like zinc, copper, selenium are the most common and effective class of antioxidants with the protective action against various health related disorders.

Reactive Oxygen Species and Free Radicals

Reactive oxygen species or an oxidant is a collective term that includes all reactive forms of oxygen, including both radical and non-radical species that participate in the initiation and/or propagation of chain reaction. Primarily, at physiological levels, ROS play beneficial or even crucial roles as regulatory mediators in signaling or defense processes, including the erythropoietin production, promotion of apoptosis, angiogenesis, endothelium-dependent vasorelaxation, and destruction of bacteria and other foreign substances by macrophages [1]. However, compromised homeostatic pathways lead to elevated ROS levels (Figure 1) that may result in the damage of cellular components (i.e., proteins, lipids, DNA).

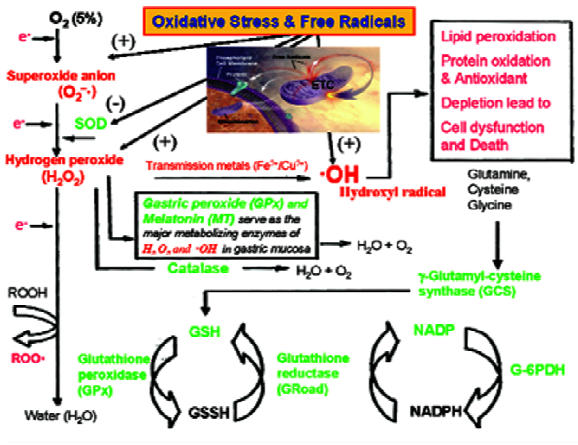


Figure 1: Compromised homeostatic pathways leading to elevated ROS levels [2]

A growing body of evidence implicates oxidative stress in both aging and a wide spectrum of human diseases including inflammatory disorders [3, 4]. ROS may be produced in a regulated manner in the form of free radicals during cellular metabolism, but they can also arise in an unregulated manner by metabolic dysfunctions and by exogenous stresses [1].

Free radicals represent a class of highly reactive intermediate chemical entities whose reactivity is derived from the presence of unpaired electron in their structure, which are capable of independent existence for a very brief interval of time [5]. Free radicals and other reactive species are derived either from normal essential metabolic processes or from external sources, such as exposure to direct sun-light, ozone, X-rays, cigarette smoking, industrial chemicals, and air pollutants etc.

Types and Sources of ROS

A number of free radicals or ROS are constantly produced in living system as a result of normal metabolic processes. These are formed by either of two ways by electron transfer reactions: (i) enzymatic reactions involving xanthine oxidase (XO), NADPH oxidases and lipoxygenases; (ii) non-enzymatic sequence of reactions such as the catalytic action of free transition metals (for example iron and copper), toxic action of certain chemicals for instance doxorubicin, attack of electrons leaked from the mitochondrial electron transport chain and effect of radiation including UV light and radon (Ra) gas. Different types of reactive species generated in biologic system are comprised of both free radical and non-radical species [5, 6].

Table 1: Types of reactive species (ROS and RNS)

Reactive species	Types	Symbol
Radicals	Superoxide	$O_2^{\bullet-}$
	Hydroxyl	$OH^{\bullet}$
	Alkyl	$R^{\bullet}$
	Alkoxyl	$LO^{\bullet}$ / $RO^{\bullet}$
	Peroxyl	$LOO^{\bullet}$ / $ROO^{\bullet}$
	Hydroperoxyl	$HO_2^{\bullet}$
	Thiyl radical	$R\text{-}S^{\bullet}$
	Nitric oxide	$NO^{\bullet}$
Non-radicals	Hydrogen peroxide	H_2O_2
	Hypochlorous acid	HOCl
	Hypobromous acid	HOBr
	Ozone	O_3
	Singlet oxygen	$^1O_2$
	Peroxynitrite	$ONOO^-$
	Lipid peroxide	LOOH
	Nitrous acid	HNO_2

The major reactive species have been listed in Table 1. A similar term, reactive nitrogen species (RNS), is also becoming widely used (Table 1).

Some of these species are much less 'reactive' than others, e.g. $O_2^{\bullet-}$ and $NO^{\bullet}$ react directly with few molecules in the human body, whereas hydroxyl ($OH^{\bullet}$) can react with anything and when generated *in vivo*, it will react at its site of formation.

Alkyl, alkoxyl, alkperoxyl radicals and lipid peroxide ($R^{\bullet}$, $RO^{\bullet}$, $ROO^{\bullet}$, and LOOH) are the end products of free radicals attack on fatty acid chains and lipid molecules. Owing to the presence of unsaturation, these biomolecules are more susceptible towards free radical attack.

Superoxide anion ($O_2^{\bullet-}$), though the reduced form of oxygen, itself is not damaging, but it plays an important role in the formation of powerful radicals like H_2O_2 and nitric oxide radicals, and also acts as an oxidant or reductant for transition metals [7]. Superoxide radicals' production takes place in cells at mitochondria, endoplasmic reticulum, and cell cytoplasm membranes. In mitochondria, two enzymatic sites have been clearly identified in addition to other enzymes of the electron transfer chain, as major sources for one-electron reduction of oxygen: ubiquinone-cytochrome C reductase, which involves auto-oxidation of the ubisemiquinone, NADH dehydrogenase, and semi-flavin cofactor [5]. $O_2^{\bullet-}$ is produced inside the membrane of the endoplasmic reticulum by the oxy complex of cytochrome P-450 and the action of NADH-cytochrome P-450 reductase [8]. In plasma, NADPH oxidase is involved in the generation of $O_2^{\bullet-}$ radicals by transferring one electron to molecular oxygen via the enzyme electron transfer chain reaction [9].

Xanthine oxidase has also been proposed to be a significant source of $O_2^{\bullet-}$ generation in reperfused tissues; uses molecular oxygen as its acceptor, and produces superoxide anion [9].

Hydroxyl radical ($OH^{\bullet}$) is an extremely reactive radical species that have the capability to react with every bio-molecule. These radicals are generated by the number of sources via Fenton reaction. Hydrogen peroxide (H_2O_2), oxygen radical ($O_2^{\bullet-}$), and transition metals (Fe^{+2} and Cu^{+}) are commonly involved in the generation of $OH^{\bullet}$. It is worth noting that, at variance with superoxide anion ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2), there is no direct generation of hydroxyl radical ($OH^{\bullet}$) in the cell. Both, superoxide anion and hydrogen peroxide are required to form the highly reactive hydroxyl radical [5].

Hypochlorous acid is not a free radical but a potent chlorinating and oxidizing agent. It is generated by the action of myeloperoxidase enzyme present in the phagosomes of neutrorphils [10], in the presence of hydrogen peroxide on chloride ions.

$$H_2O_2 + Cl^- \xrightarrow{\text{myeloperoxidase}} HOCl + OH^-$$

Hypochlorous acid can permeate cell membranes and, in the presence of superoxide radicals and transitional metal ions, produces hydroxyl radicals [11].

$$HOCl + O_2^{\bullet-} \longrightarrow {}^{\bullet}OH + Cl^- + O_2$$

$$HOCl + Fe^{2+} \longrightarrow {}^{\bullet}OH + Cl^- + Fe^{3+}$$

Other than ROS, hydrogen centred radicals result from attack of the H atom ($H^{\bullet}$). Carbon-centred radicals arise from the attack of an oxidizing radical on an organic molecule ($CCl_3^{\bullet}$). Two more forms are the sulfur-centred radical produced in the oxidation of glutathione resulting in the thiyl radical ($R\text{-}S^{\bullet}$) and a nitrogen-centred radical resulting in phenyl diazine radical.

Hydrogen peroxide (H_2O_2) is an oxidizing agent and a main source of hydroxyl radicals as mentioned earlier in the text. It produces $^{\bullet}OH$ through Haber-Weiss and Fenton reactions in the presence of $O_2^{\bullet-}$ and Fe^{+2} respectively.

$O_2^{\bullet-} + H_2O_2 \longrightarrow O_2 + OH^{\bullet} + OH^-$ (Haber-Weiss reaction)

$H_2O_2 + Fe^{2+} \longrightarrow OH^{\bullet} + OH^- + Fe^{3+}$ (Fenton reaction)

Nitric oxide (NO) synthesized enzymatically from L-arginine by nitric oxide synthase [12-14] is common gaseous RNS. This free radical does not readily react with most biomolecules but easily reacts with other free radicals and producing mostly less reactive molecules. Thus, in fact, nitric oxide functions more as an antioxidant rather than an oxidant [15].

L-arginine + O_2 + NADPH $\longrightarrow$ L-citrulline + $NO^{\bullet}$ + $NADP^+$

Peroxynitrite ($ONOO^-$) is produced due to the release of significant quantities of $NO^{\bullet}$ and $O_2^{\bullet-}$ from activated macrophages and neutrorphils during the inflammatory response [16].

$O_2^{\bullet-} + NO^{\bullet} \longrightarrow ONOO^-$

Singlet oxygen ($^1O_2$) is an electronically charged excited and mutagenic form of oxygen. It is generated during exercise, by radiations, by the action of peroxidases or lipoxygenases, and by the reaction of hydrogen peroxide with peroxynitrite or hypochlorite, plus during the respiratory burst of phagocytes [17, 18]. In mammalian cells, singlet oxygen can be generated during oxidative stress and attack many cellular molecules such as amino acids, nucleic acid bases and membrane lipids. Sunlight contains high energy and short-wavelength ultraviolet photons (comprising the UVB spectra, 290-320 nm). Chronic exposure to sunlight is a significant causative factor in the development of skin cancer because of destructive interactions of these UV photons with many cellular biomolecules [19].

Adverse Effects of ROS

Because of the high reactivity, free radicals can easily interact with various biomolecules inclusive of DNA, lipids, proteins and carbohydrates. ROS lead to local injury and eventual organ dysfunction following reaction with the biomolecules. They also accelerate the aging and related degenerative processes. Moreover, ROS are also involved in the pathogenesis of various clinical conditions involving almost every organ system of our body (Figure 2).

The vulnerability of biological membranes to peroxidation is due to the presence of polyunsaturated fatty acids (PUFA). The presence of double bonds in PUFA weakens the C-H bond of the adjoining carbon atom (allylic carbons) and facilitates the hydrogen abstraction step, which in turn 'initiates the peroxidation reactions [21].

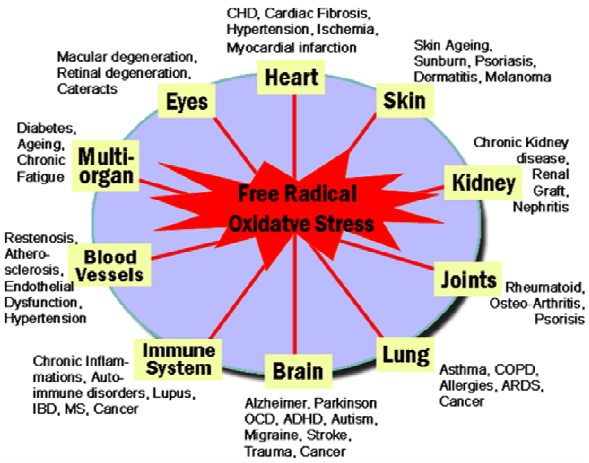


Figure 2: Clinical conditions reported to involve free radicals or oxidative stress [20]

Cell membranes contain a variety of PUFA such as and arachidonic, linoleic and linolenic acids, principally in the form of esters with phospholipids, triglycerides, or with cholesterol. Overall, an attack of one reactive free radical on PUFA molecule can convert multiple fatty acid side chains into lipid peroxides, damage membrane proteins, make the membrane leaky and eventually cause breakdown of the membrane [22, 23] (Figure 3).

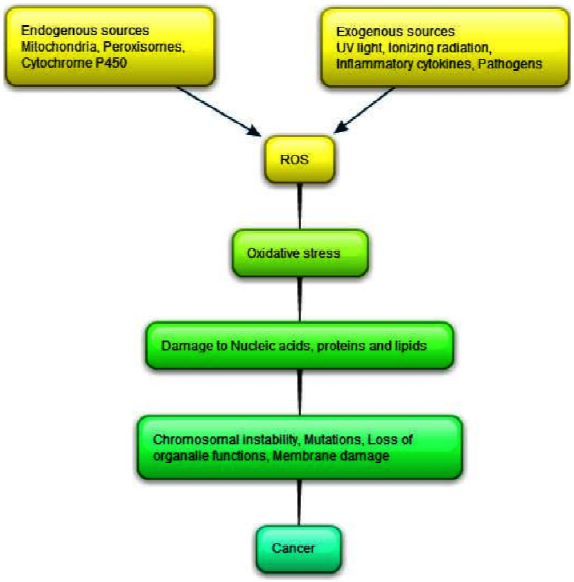


Figure 3: Sources and consequences of ROS generation in cancer [24]

Protein and nucleic acids are generally less susceptible to free radical attack than PUFAs and hence have less possibility part of involvement in the progression of chain reactions. This can happen only if radicals are allowed to accumulate, or if the damage is focused on a particular site of the protein [25, 26].

Deoxyribonucleic acid (DNA) is a sensitive target for free radicals-mediated damage in a living system. A free radical can damage the specific sites of DNA, leading to rupturing of strands, or it might delay the repair before replication occurs, leading to mutations [23]. It has been reported that ROS may be involved in cell death or sub-lethal injuries such as chromosomal aberrations, carcinogenesis and mutations by damaging DNA and the DNA repair processes [27, 28].

Mechanism of Action of ROS or Free Radicals

Oxidative process of free radical mediated reactions involves three processes: initiation, propagation, and termination. Initiation starts with the abstraction of a hydrogen atom form the biomolecule. For example, fatty acid (LH) can be converted into radicals. The free radicals such as hydroxyl ($OH^{\bullet}$), alkperoxyl ($ROO^{\bullet}$), and alkoxyl ($RO^{\bullet}$) are capable of oxidizing PUFAs. Extremely rapid addition of oxygen to the fatty acid radicals then generates peroxyl radicals ($LOO^{\bullet}$) that propagate the reaction by initiating a new chain of oxidation with the formation of lipid peroxide $LOOH^{\bullet}$). This chain reaction continues till antioxidant interrupts it through scavenging the radicals: the termination step [21, 22]. The mechanism of lipid peroxidation of unsaturated fatty acid as a model is presented in Figure 4.

Antioxidants

To protect cells and organs from the oxidative stress induced by ROS, living organisms have evolved with an extremely efficient and highly sophisticated protective system, called as "antioxidant defensive system" that involves a variety of endogenously and exogenously originated components. These components function interactively and synergistically to neutralize free radicals [30].

A widely acceptable definition of an antioxidant is "any substance which significantly delays or prevents oxidation of oxidizable substrates at low concentrations". The word oxidizable substrates cover crucial biomolecules such as DNA, lipids, proteins and carbohydrates, which are vital components of a biological system [15]. Oxidative stress occurs as a consequence of an increase in oxidative metabolism, which in turn produces a number of ROS. To prevent oxidative stress, antioxidants can play an important role in bestowing beneficial healthy effects [31]. Sufficient supplemental or dietary intake of proven antioxidants can significantly lower the risk of several chronic diseases of almost every organ system including cancers.

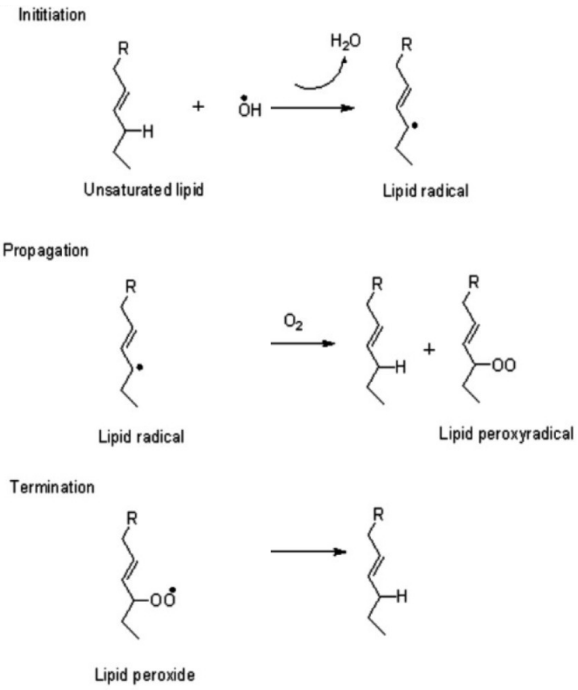


Figure 4: Mechanism of lipid peroxidation in unsaturated fatty acid molecule(s) [29]

Classification of Antioxidants

A number of antioxidants are collectively required for the removal of free radicals to protect the body from adverse effects of ROS. Certain enzymes as well as non-enzymatic cellular molecules are involved in the detoxification of ROS. Based on the nature of antioxidants, the human antioxidant system can be classified into two broader classes: enzymatic and non-enzymatic [1].

Enzymatic Antioxidants

The primary intracellular endogenous antioxidant defences are via the enzyme system. Enzymatic antioxidant defences include superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT) [1].

Superoxide dismutases (SODs) have been found in three isoforms. Manganese containing SOD (MnSOD) is a tetrameric protein that is localized in the mitochondrial matrix. It plays a critical role in scavenging $O_2^{\bullet-}$ engendered from the electron transport chain. Zinc and copper containing SOD (ZnCuSOD) is a dimer protein that is confined to a small area in the cell cytoplasm. It is thought to remove $O_2^{\bullet-}$ generated by endoplasmic reticulum and cytosolic oxidases. The extracellular SOD is found in the extracellular space as a tetrameric protein which catalyzes the dismutation of superoxide into H_2O_2 [32, 33].

$$2O_2^{\bullet-} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$$

Catalase (CAT) is located in peroxisomes and mitochondria. It is a large tetrameric protein that takes out H_2O_2 by catalyzing its conversion into water [34].

$$2\ H_2O_2 \xrightarrow{catalase} H_2O_2 + O_2$$

Glutathione peroxidases (GPxs) are a group of selenium-dependent enzymes. Four isoforms of GPx have been illustrated: cytosolic GPx1, plasma GPx, phospholipid-hydroperoxide PHGPx and gastrointestinal GPx-GI. All GPxs require glutathione (GSH) as a cofactor and secondary enzymes: glutathione reductase and glucose-6-phosphate dehydrogenase (G-6-PDH) to function. G-6-PDH produces NADPH to recycle the glutathione [35].

$$2GSH + H_2O_2 \xrightarrow{GPx} GSSG + 2\ H_2O$$

Non-Enzymatic Antioxidants

Non-enzymatic antioxidants may be further classified into two groups: endogenous and exogenous antioxidants. The transition metal binding proteins are major extracellular endogenous antioxidants found in human plasma. These include albumin, ceruloplasmin, hepatoglobin and transferring. They bind with transition metals and control the production of metal-catalyzed free radicals. Albumin and ceruloplasmin are the iron and copper ions sequesters respectively. Hepatoglobin binds with hemoglobin whereas ferritin and transferring bind with free iron [36, 33]. Bilirubin, ubiquinone, glutathione, lipoic and uric acids are non-protein endogenous antioxidants which inhibit the oxidation processes by scavenging ROS [37].

Many effective exogenous antioxidants are generally of dietary origin. The best known are vitamins such as ascorbic acid (vitamin C), carotenoids, vitamin E, quinines, lípoic acid and polyphenols. These molecules can hinder oxidative reactions by scavenging free radicals or ROS, while certain compounds may chelate redox active metals or inhibit particular oxidative enzymes. Vitamin E is a fat soluble, chain breaking free radical scavenger which reacts with lipid peroxyl radicals to yield a relatively stable lipid hydroperoxide and thus provide protection against membrane lipid peroxidation [38]. Conversely, vitamin C has multiple free radical scavenging properties, including the ability to regenerate α-tocopherol by reducing α-tocopheroyl radicals at the surfaces of membranes. It also helps in scavenging other free radicals and certain non-radicals such as HOCI [1].

Mechanism of Action of Antioxidants

Antioxidants can remove both free radical and non-radical species in a different manner depending upon the nature of ROS, required for neutralization. Two basic mechanisms have been proposed for the action of antioxidants: mechanism of removal of ROS initiators and a chain breaking mechanism.

Mechanism of Removal of Initiators of ROS

This process is mainly based on the inhibition of the enzymes involved in the generation of ROS. Xanthine oxidase (XO) is one of the major sources of superoxide anions production [39]. Lipid peroxides are generated from lipoxygenase during arachidonic metabolic pathway [40]. Antioxidants act by inhibiting these enzymes making them unavailable to harm the cellular system. Other main sources of free radicals are free transition metal ions which act as pro-oxidants by virtue of their attachment with carrier proteins, and are therefore toxic to the body. There are several metal binding proteins that chelate transition metals, which are capable of reacting with hydroperoxides to produce free radicals, lactoferrin and ferritin:

$$HOOH/LOOH + Fe^2 \longrightarrow LO^{\bullet} / HO^{\bullet} + OH + Fe^{3+}$$

These proteins function to keep iron-induced oxidant stress in control. Ceruloplasmin and albumin proteins are the copper and iron sequestrants respectively [31, 34].

Chain Breaking Mechanism of Action

Antioxidants scavenge free radicals by donating an electron to them while being oxidized themselves during the process. The chain reactions of reactive species can be arrested with the help of antioxidants, which can neutralize free radicals formed during the overall reaction. This mechanism is known as a

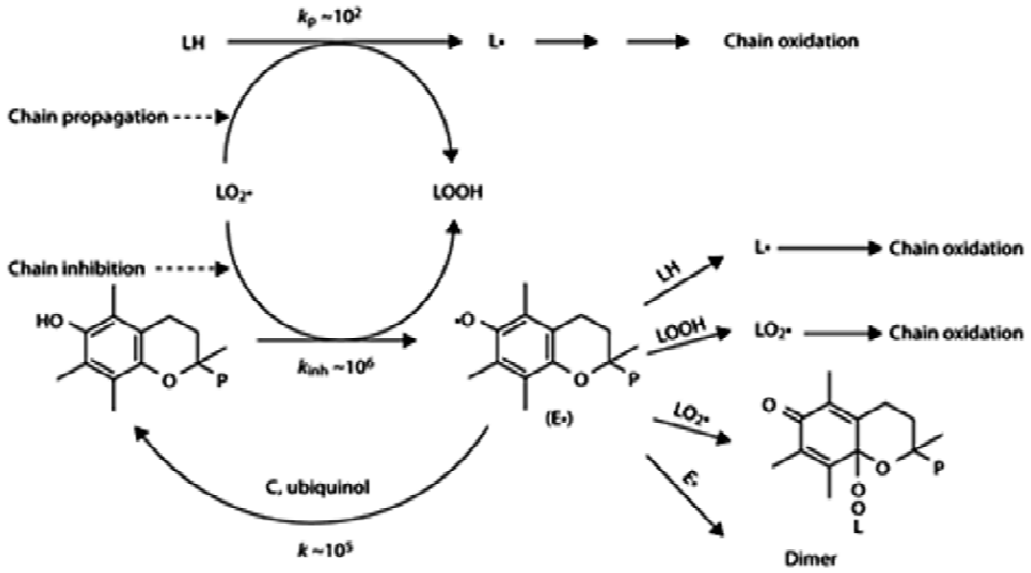


Figure 5: α-Tocopherol action as a radical scavenging antioxidant against lipid peroxidation [42]

"chain breaking antioxidation". Most of the polyphenolics work through the chain breaking (CB mechanism of action. Vitamins C and E, carotenes, flavonoids and coumarins are examples of chain breaking antioxidants (CBA).

Vitamin E (tocopherol, TH_2) is the most widely distributed lipid soluble antioxidant in nature. Its main function is to prevent the peroxidation of membrane phospholipids and to avoid cell membrane damage [38]. A detailed study of the effects of a benzene ring substituents on the rate of radical scavenging reaction showed that the reaction is accelerated by the presence of 4-methoxy and C-2 and C-6 methyl groups. The presence of C-1 hydroxyl group contributes to donating $H^{\bullet}$; whereas functional groups at C-4, C-2, and C-6 stabilize the resulting tocopheroyl radical [37, 41]. The reaction of vitamin E (TH_2) with peroxyl radical ($LOO^{\bullet}$) is summarized in Figure 5 where vitamin E scavenges lipid peroxyl radical ($LOO^{\bullet}$) before it attacks lipid (LH) to yield lipid hydroperoxide (LOOH) and a lipid radical ($L^{\bullet}$), which propagates the chain oxidation.

The resulting vitamin E radical ($E^{\bullet}$) may be (1) reduced by ascorbic acid (C) or ubiquinol to regenerate vitamin E; (2) attack lipid (LH) or lipid hydroperoxide (LOOH); (3) react with lipid peroxyl radical ($LOO^{\bullet}$) to yield an adduct, or (4) react with another vitamin E radical to yield a dimer, a non-radical stable product. The rate constants (k) are shown in $M^{-1}s^{-1}$; P is phytyl side chain.

Measurement of Antioxidant Activity

Free radicals have been implicated in a number of disorders in biological systems and certain other fields, i.e. deterioration of food, degradation of polymers, rubbers, plastics and cosmetics etc. To prevent the damaging effects of free radicals, a number of investigations have been made to discover antioxidants using various methodologies. A number of experimental models have been developed for the determination of antioxidant activities of different samples. These methods can be divided into two major categories [31, 43]:

1) Measuring the potential of any substance to donate an electron or α hydrogen atom to a specific reactive oxygen species or to any electron acceptor.

2) Measuring the ability to remove any source of oxidative initiation, e.g. chelation of transition metal ions, inhibition of enzymes and absorption of UV radiation.

Several factors influence the effectiveness of an antioxidant and require consideration of its physiochemical and pharmacological properties such as the site of action, bioavailability, its stability, its toxicity, besides type of reactive oxygen species that the antioxidant must react with. It is therefore necessary to select an appropriate method for evaluation of antioxidant potential. The following assays are commonly used for measurement of antioxidant activity:

Radicals Scavenging Assays

Stable free radical species are commonly used in an aqueous system to determine the antiradical activity of compounds. These radicals include 1, 1-diphenyl-2-picrylhydrazyl radical (DPPH) [44] and 2, 2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt ($ABTS^+$) [45].

DPPH assay: This assay measures reducing ability of antioxidants towards DPPH radical. DPPH radical is available commercially in deep blue colour. Antioxidant capacity can be measured by measuring the decrease in absorbance of methanolic solution of DPPH at 517 nm. Reaction is monitored by spectrophotometer [44]. The percentage of antioxidant capacity is measured by using the following equation:

% age of antioxidant capacity = (Ac – As/Ac) x 100

Where

Ac = Absorbance of negative control at 517 nm

As = Absorbance of sample at 517 nm

$ABTS^+$ assay: In this assay, $ABTS^+$ is oxidized by peroxyl radicals or other oxidants to its radical cation, $ABTS^{\bullet+}$, which is intensely coloured (dark green), and antioxidant capacity is measured as the ability of the test compounds to decrease the colour reacting directly with the $ABTS^{\bullet+}$ radical [45]. Results of test compounds are expressed relative to trolox. Decrease in absorbance by test compound and control is measured at 415 nm by using spectrophotometer.

Other radicals scavenging assays include superoxide anion scavenging assay [46] and hydroxyl radical scavenging assay [47]. In the majority of the studies, the phenazine methosulfate-NADH system [46] or the xanthine/XO system is used to produce superoxide anion radicals [48].

In several methods of detecting antioxidant activity, the ability of an antioxidant to prevent the oxidation of poly-unsaturated fatty acids (PUFA), such a linoleic acid is determined by exposing the PUFA to oxygen, light or free radical generators. The frequently used methods for measuring lipid peroxidation/peroxy radical scavenging ability include:

Conjugated diene assay: This assay is used for quantification of conjugated diene formed as a result of initial PUFA oxidation by measuring UV absorbance at 234 nm [49].

Lipid peroxide assay: In this method, oxidation of PUFA initiated by oxygen at allylic position of lipid results in an unstable mixture of lipid peroxides, which can be detected iodometrically [31].

Thiobarbituric acid reactive substances (TBARS) assay: In this assay model, lipid peroxides formation is measured by the detection of a stable product formed as a result of reaction of thiobarbituric acid with aldehydes, a decomposed product of lipid peroxides [50].

Enzyme Inhibition Assays

There are certain enzymes which produce reactive oxygen species during reactions. These enzymes include lipoxygenases, cycloxygenases and xanthine oxidase etc. Xanthine oxidase catalyzes the production of uric acid and superoxide anion using xanthine/hyproxanthine as substrate [51], whereas lipoxygenases and cycloxygenases produce lipid hydroperoxides as a result of arachidonic acid metabolism [40]. The inhibitors of these enzymes may decrease reactive oxygen species in biological systems [31].

Chelation of Transition Metals

The transition metals iron and copper are essential cofactors of several enzymes which are involved in oxygen metabolism. In biological systems, these metals are found with proteins and enzymes but when these are present in a free state, they can catalyze free radical reactions. The relative chelating capacity of samples can be studied spectrophotometrically by measuring the ability to release iron ions from an iron-EDTA complex and to chelate irons ions [52].

Chromatographic Procedures

Lipid hydroperoxides are the major end products obtained during oxidation of PUFA. Many developed methods measure either primary hydroperoxides or secondary aldehydic products of lipid oxidation. High performance liquid chromatography (HPLC) is one of the useful methods in the quantitative measurement of lipid hydroperoxides. These HPLC methods employ chemiluminescene detection for lipid hydroperoxides and thiobarbituric acid (TBA) assay [53].

Role of Antioxidants on Biological Activities

Substantial research work has been carried out to investigate the preventive role of antioxidants across different diseases. Each antioxidant has some significance and the best protection against oxidative stress comes from the presence of a wide assortment of interrelated antioxidants and their cofactors. The function of particular antioxidant primarily depends on what type of oxidative stress is imposed [1].

Lipid peroxidation can damage low-density lipoprotein (LDL) particles in several ways. *In vitro* studies have demonstrated that lipoxygenase, superoxide anion, peroxynitrite and myeloperoxidase can oxidize LDL [1] which can lead to heart diseases. Studies showed that antioxidants may provide protection against coronary heart diseases (CHD) [54, 55]. Vitamins have been shown to reduce the susceptibility of LDL to oxidation and are also known to be involved in elevating the levels of protection factors like HDL-cholesterol. Studies suggest that vitamin C may be helpful in reducing the risk of hypertension [56]. Further, a high intake of vitamin C appears to protect against gastric cancer, probably through scavenging free radicals formed in the gastric mucosa [57]. Further investigations on vitamin C have proved its preventive effects on inhibition of tumor growth [58].

Epidemiological investigations on vitamin E also suggested that it may be protective against the occurrence of Parkinson's disease. Some researchers have also shown that vitamin E intake can slow down the progression of Alzheimer's disease [59]. It has been reported that supplementation of vitamins C and E, combined with other antioxidants, can reduce symptoms of exercise induced oxidative stress [60].

Common Antioxidants

There are hundreds of antioxidants of natural and synthetic origin. Increasing interest in such compounds is due to their effective role against the destructive actions of free radicals. A number of studies have been carried out to identify different classes of antioxidants. A few important antioxidants have been discussed here.

Vitamin E

Vitamin E is the most common naturally occurring antioxidant. Its structure is closely related to phenolic benzochroman derivatives. It has a phytyl side chain attached to its chromanol nucleus. The ability of donating two electrons bestows for radical scavenging activity [42].

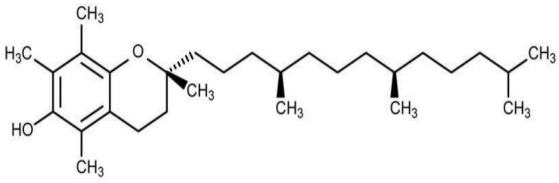


Figure 6: Structure of vitamin E (tocopherol)

Vitamin C

Ascorbic acid (vitamin C) is a water soluble electron donor vitamin. To act as an antioxidant, it donates two electrons from the C-2 and C-3 double bond carbons, which results in the formation of an intermediate free radical, semidehydroascorbic acid. The resulting ascorbate free radicals readily reduce to a neutral ascorbate molecule [61].

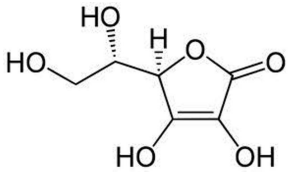


Figure 7: Structure of vitamin C (ascorbic acid)

Melatonin

Melatonin is another strong antioxidant that can cross cell membranes and the blood-brain barrier without any difficulty. In contrast to other antioxidants, this hormonal substance does not undergo redox cycling i.e. capability of a molecule to undergo repeated oxidation and reduction oxidation. It may allow other antioxidants (such as Ascorbic acid or vitamin C) to act as pro-oxidants and promote ROS formation. Melatonin, on oxidation, cannot be reduced to its earlier state because of the formation of several stable end-products upon reacting with ROS. Thus, it has been referred as a terminal (or suicidal) antioxidant [62].

Figure 8: Structure of melatonin

Carotenoids

Carotenoids are a large group of compounds with various structural features. The basic skeleton of carotenoids comprised of a polyisoprenoid C40 carbon chain with a number of conjugated double bonds. Owing to the presence of high conjugation, effective delocalization of electrons can occur along the entire length of the polyene chain. This distinctive character of carotenoids makes them

effective as singlet oxygen quenchers. β-Carotene is bicyclic in nature with β-ionone rings at both ends of the molecule [63]. Several epidemiological studies have revealed that the tomato intake, the main source of lycopene, has been associated with a lower risk of a variety of cancers [64].

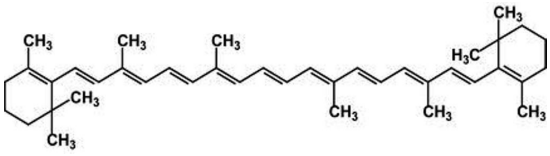


Figure 9: Structure of carotenoid

Polyphenols

Polyphenols are common constituents of foods of plant origin and major antioxidants of our diet. At present, there are more than 8000 known phenolic compounds including over 4000 identified as flavonoids [65-67]. The main dietary sources of polyphenols are fruits, vegetables, whole grains and beverages such as tea, coffee, wine etc. Fruits like apple, grape, pear, cherry, and various berries contain up to 200–300 mg polyphenols per 100 g fresh weight. Typically, a glass of red wine or a cup of tea or coffee contains about 100 mg polyphenols. Cereals, chocolate, and dry legumes also contribute to the polyphenol intake. The total dietary intake is about 1 g/d. It is much higher than that of all other known dietary antioxidants, about 10 times higher than that of vitamin C and 100 times higher than those of vitamin E and carotenoids [68].

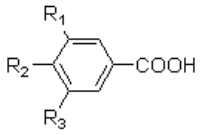
Polyphenols (also known as polyhydroxyphenols) are a structural class of mainly natural, but also synthetic or semisynthetic, organic chemicals characterized by the presence of large multiples of phenol structural units [69]. The name derives from the ancient Greek word polus, meaning "many" and the word phenol which refers to a chemical structure formed by attaching to an aromatic benzenoid (phenyl) ring, a hydroxyl ($^{-}$OH) group akin to that found in alcohols (hence the "-ol" suffix). The number and characteristics of these phenol structures underlie the unique physical, chemical, and biological (metabolic, toxic, therapeutic, etc.) properties of particular members of the class. They may be broadly classified as phenolic acids, flavonoids, stilbenes, lignans and tannins [69].

Phenolic acids

Substituted derivatives of hydroxybenzoic and hydroxycinnamic acids are the predominant phenolic acids present in plants. These derivatives differ in patterns of hydroxylations and methoxylations of their aromatic rings.

Hydroxybenzoic acids

Hydroxybenzoic acids have a general structure of the C6-C1 type derived directly from benzoic acid. They consist of gallic, p-hydroxybenzoic, protocatechuic, vanillic and syringic acids.



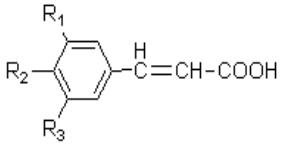
Acid	R_1	R_2	R_3
p-Hydroxybenzoic	H	OH	H
Protocatechuic	H	OH	OH
Vanillic	CH_3O	OH	H
Syringic	CH_3O	OH	CH_3O
Gallic	OH	OH	OH

Figure 10: Chemical structures of common benzoic acid derivatives

Hydroxybenzoic acids are commonly present in a bound form, constitute either complex structures like hydrolyzable tannins or simple molecules by combining with sugars or organic acids [67]. Gallic acid is a well-known plant acid which participates in the formation of hydrolysable gallotannins and combines with (-)-epicatechin to form epicatechin gallate which is present only in seeds. Its dimeric condensation product (hexahydroxydiphenic acid) and the related dilactone, ellagic acid are also common plant constituents; the former usually occurring bound as ellagitannins [66, 67].

Hydroxycinnamic acids

Hydroxycinnamic acids derived from cinnamic acid are important in plants due to their abundance and diversity [66]. They are rarely found in the free form but as combined forms of the basic molecules caffeic, chlorogenic, ferulic, p-coumaric, quinic, and sinapic acids (Figure 11). Bound forms of hydroxycinnamic acids are found to be esters of hydroxyacids such as tartaric acid, and their sugar derivatives in which one of the phenolic groups is linked with a sugar molecule. They may also be chemically linked with amines, flavonoids, lignin, suberin and cutin [67].



Acid	R_1	R_2	R_3
p-coumaric	H	OH	H
Caffeic	H	OH	OH
Sinapic	CH_3O	OH	CH_3O
Ferulic	CH_3O	OH	H

Figure 11: Chemical structures of common cinnamic acid derivatives

Additionally, the presence of a double bond in the lateral chain of the hydroxycinnamic acid leads to the possible existence of two isomeric forms cis and trans. Although native compounds are mainly of the trans form, interconversion of the two forms might occur in situ and be responsible for certain physiological responses in plants. This takes place very easily under the effect of light, in particular during extraction and purification prior to analysis of hydroxycinnamic acids. The cis form then appears more frequently, but the trans form nevertheless remains predominant [70].

Flavonoids

Flavonoids or bioflavonoids are a class of plant secondary metabolites. Over 5000 naturally occurring flavonoids have been characterized from various plants [65]. The diversity and complexity of the flavonoids depends on the variety of aglycones and the high number of glycosides possible, sometimes in acylated form, as well as the possibility of condensation into complex molecules such as condensed tannins. Flavonoids are usually present as glycosides with a sugar moiety linked through an OH group (known as O-glycosylflavonoids) or through carbon-carbon bonds (known as C-glycosylflavonoids) [69]. The attachment of hydroxyl groups and sugar will increase the hydrophilic properties of the flavonoid molecule while attachment of methyl esters or modified isopentyl units will increase the lipophilic character.

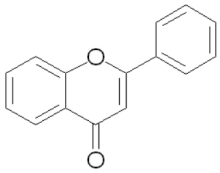


Figure 12: Chemical structure of the basic flavonoid skeleton

Flavonoid compounds possess the diphenylpropanes C15 (C6 + C3 + C6) basic skeleton (Figure 12). At the simplest level, the skeleton consists of two phenyl rings connected by a three-carbon bridge. Most flavonoids have a carbonyl function located at one end of the bridge. Commonly found flavonoids in plants comprise anthocyanidins, proanthocyanidins, chalcones, flavones, flavanols, flavonols, and flavanones (Figure 13).

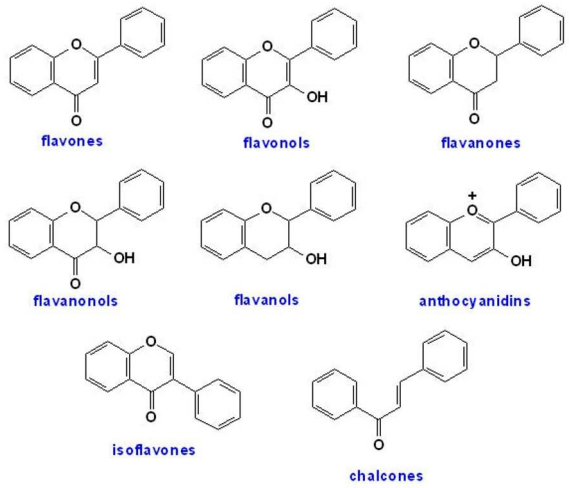


Figure 13: Basic flavonoid structures

Some of the most common flavonoids are catechin, a flavanol found in tea and several fruits; quercetin, a flavonol abundant in onion, tea, and apple; hesperetin, a flavanone present in citrus fruits; cyanidin, an anthocyanin giving its color to many red fruits (blackcurrant, raspberry, strawberry, etc.); daidzein, the main isoflavone in soybean; proanthocyanidins, common in many fruits, such as apple, grape, or cocoa and are responsible for their characteristic astringency or bitterness [68].

Stilbenes

Stilbene refers to one of the two isomers (cis or trans) 1,2-diphenylethene. Stilbenoids, hydroxylated derivatives of Stilbene belong to the family of

phenylpropanoids and share most of their biosynthesis pathway with chalcones. Stilbenoids are secondary products of heartwood formation in trees that can act as phytoalexins.

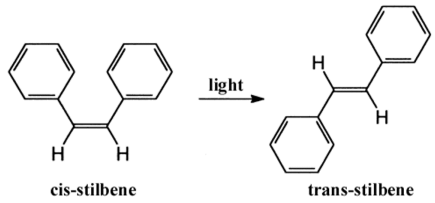


Figure 14: Cis-trans stilbene

An example of a stilbenoid is resveratrol, which is found in grapes and which has been suggested to have many health benefits [71].

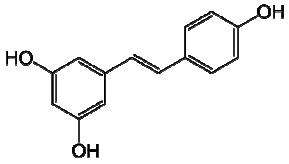


Figure 15: Structure of resveratrol

Lignans

The lignans are a group of chemical compounds found in plants. Lignans are one of the major classes of phytoestrogens, which are estrogen-like chemicals and also act as antioxidants. The other classes of phytoestrogens are the isoflavones and coumestans. Plant lignans are polyphenolic substances derived from phenylalanine via dimerization of substituted cinnamic alcohols, known as monolignols [72].

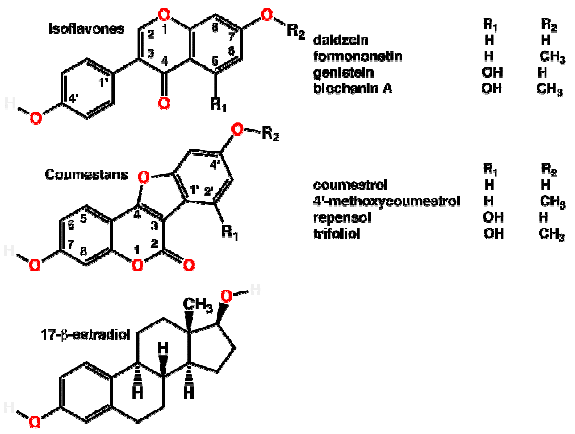


Figure 16: Classic lignans

Tannins

Tannins are water-soluble phenolic compounds having molecular weights between 500 and 3000 daltons. Structurally, tannins possess 12-16 phenolic groups and 5-7 aromatic rings per 1000 units of relative molecular mass [73]. This feature, together with their high molecular weight, clearly makes the tannins different both in structure and properties from the low molecular weight monomeric flavonoids and phenolic acids. As tannins possess a great number of hydroxyl groups, they also have the ability to bond reversibly with many natural macromolecules (e.g., polysaccharides, proteins, etc.) and various secondary metabolites (e.g., alkaloids). Depending on the chemical structure, tannins are conventionally subdivided into hydrolyzable and condensed tannins known also as proanthocyanidins.

Phenolics: structure - antioxidant activity relationship

The antioxidant activity of phenolic compounds is affected by their chemical structure. Structure-activity relationships have been used as a theoretical method for predicting antioxidant activity [74]. Phenolics can exert their antioxidant effect by inhibiting the activities of enzymes, by chelating metal ions, by interacting with other antioxidants such as ascorbate, and most importantly by scavenging free radicals. Flavonoids are capable of scavenging reactive oxygen species such as superoxide anion and hydroxyl radical, and the scavenging ability depends on their chemical structures [9, 13, 15, 16]. The reactivity of flavonoids toward hydroxyl radical is generally much higher than that towards superoxide anion though the hydroxyl radical is more reactive than the superoxide anion.

Antioxidant property of phenolic acids

The antioxidant activity of phenolic acids and their esters also depends on the number of hydroxyl groups in the molecule, and this would be strengthened by steric hindrance [75]. The electron withdrawing properties of the carboxylate group in benzoic acids has a negative influence on the H-donating abilities of the hydroxyl benzoates. Therefore, hydroxylated cinnamic acids are more effective antioxidants than their benzoic acid counterparts [73].

The monohydroxy benzoic acids show no antioxidant activity in the ortho and para positions in terms of hydrogen-donating capacity against radicals generated in the aqueous phase, but the m-hydroxyl acid does have an antioxidant activity. This is

consistent with the electron withdrawing potential of the single carboxyl functional group on the phenol ring affecting the o- and p-positions. The antioxidant activity of dihydroxylation in the ortho and meta positions to the carboxylated group is elevated compared with the meta, para disubstitution [73]. Incorporation of an ethylenic group between a phenyl ring carrying a hydroxyl group and the carboxylate group decreases the impact of the carboxylate group and has a highly favourable effect on the reducing properties of the OH group compared with cinnamic acid. Hence the monohydroxyl group in the cinnamic acids is more available as a hydrogen donor than the monohydroxyl groups in the benzoic acids [73].

Antioxidant property of flavonoids

The number and arrangement of phenolic hydroxyl groups on a benzene ring, the presence or absence of a conjugated double bond which makes an enol group, as well as the mutual spatial location of such polyhydroxylated benzene rings in the molecule of flavonoids and flavan derivatives often induce marked differences in the antioxidative effects. Hydroxylation of the B-ring is the major consideration for antioxidant activity [73] as the availability of the phenolic hydrogens can act as hydrogen-donating radical scavengers. Other important features include a carbonyl group at position 4 and a free hydroxyl group at position 3 and/or 5 that are required for maximum radical scavenging potential [73, 74]. Phenolics with a 2,3-double bond in conjugation with a 4-oxo function in the C ring are responsible for electron delocalization from the B ring. The antioxidant potency is related to structure in terms of electron delocalization of the aromatic nucleus where these compounds react with free radicals, therefore, the phenoxyl radicals are stabilized by the resonance effect of the aromatic nucleus.

Potential therapeutic effects of polyphenols

Phenolics have been reported to scavenge oxygen-derived free radicals as well as to inhibit lipid hydroperoxide formation catalyzed by metals, radiation and heme compounds [1]. Epidemiologic studies have shown a correlation between an increased consumption of phenolic antioxidants and a reduced risk of cardiovascular disease, neurodegenerative disease and certain types of cancer [1, 4, 5, 15]. In addition to antioxidant and antimicrobial function, phenolics are reported to possess numerous biological, pharmacological, and medicinal properties, including anticarcinogenic, immune-stimulating, antiallergenic, antiviral and estrogenic effects, as well as inhibition of various enzymes involved in carcinogenesis [1, 2].

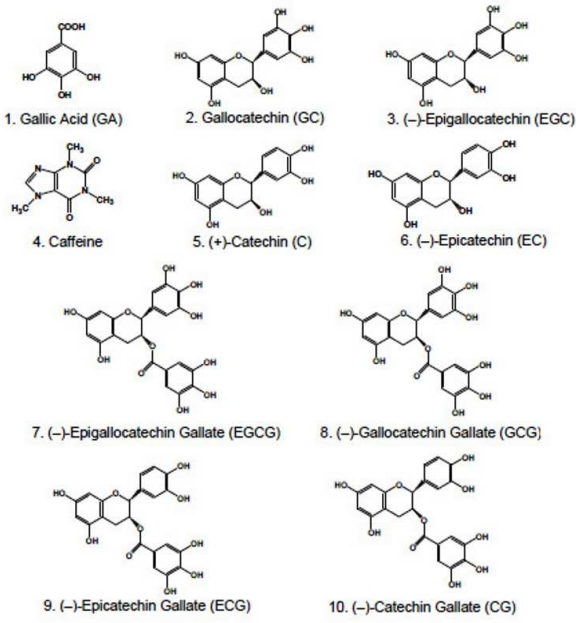


Figure 17: Structure formulae of gallic acid and its derivatives

Gallic acid (GA) and its gallate derivatives such as epicatechin, epicatechin gallate, and epigallocatechin gallate have also been reported with a number of biological and pharmacological activities including anti-inflammatory, antimutagenic, anticancer and antioxidant activity [11,74,75]. Because of pharmacological importance, the mammalian metabolism of gallic acid has been thoroughly studied in various studies [76-78]. *In vivo* experiments showed that the major GA metabolites are products of methylation (unconjugated and conjugated 4-*O*-methylgallic acid, 2-*O*-methylgallic acid), decarboxylation (unconjugated and conjugated pyrogallol, 4-*O*-methylpyrogallol), and dehydroxylation (resorcinol). Toxicological studies showed that the ‘‘no-observed-adverse effect-level’’ (NOAEL) of GA is at least 120 mg/kg/ day for F334 rats, and the level of gallates was reported to be as high as 1000 mg/kg for mice [79].

Similarly, tannins are reported to possess biologically multifunctional properties including antioxidant activity due to radical scavenging action and antibacterial, antiviral, anti-inflammatory, anti-HIV, antitumor activities, and inhibitory effects on

various enzymes [78]. Proanthocyanidins may protect LDLs against oxidation and inhibit platelet aggregation and therefore, prevent cardiovascular diseases [80].

As antioxidants, polyphenols may protect cell constituents against oxidative damage and, therefore, limit the risk of various degenerative diseases associated to oxidative stress. As compared to other antioxidants, research on their health effects started more recently. This late interest for polyphenols is largely explained by the complexity of their chemical structures. Numerous studies on animal models have shown that, when added to the diet, they limit the development of cancers, cardiovascular diseases, neurodegenerative diseases, diabetes, and osteoporosis. Some evidence suggests that the biological actions of these compounds are related to their antioxidant activity [62, 68] because of the presence of basic galloyl moiety.

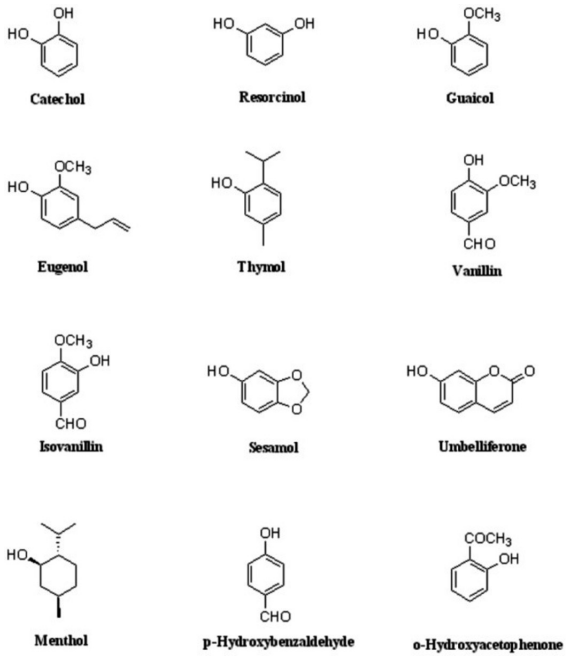


Figure 18: Structure formulae of key phytophenols

Other important phytophenols

Phytophenols of edible plants and related bioactive substances have recently drawn much attention as potential antioxidants. They are well known to have antioxidant, anti-inflammatory, antiviral, antiatherosclerortic and anticancer properties. These compounds are known to act by free radical scavenging and selectively interfering with various factors of abnormal proliferation of mammalian cells [80]. These include resorcinol, guaiacol, eugenol, thymol, vanillin, isovanillin, sesamol, umbelliferone, menthol, and catechol. These antioxidative phytophenol substances are very oxidizable due to their chemical properties [81]. These are known to constitute important components in the food substances in one's daily diet. Research has shown that daily intake of these phytophenols may assist in combating the harmful effects of free radicals in the biological system.

CONCLUSIONS

As the recent trend shows, efforts to counteract the damage caused by ROS or free radicals are gaining wider acceptance as a basis for novel therapeutic approaches. The concept of preventive medicine is experiencing increased significance in medically useful antioxidants. Discovery of new antioxidants with significant biological activity and ineffective toxicity is one of the things necessary. A novel approach proposed in this direction is the conjugation of gallic acid with above mentioned naturally occurring phytophenols that could lead to development of novel ester derivatives with synergistic and/or complimentary pharmacological activities as preventive and curative therapeutic agents in near future.

REFERENCES

1. Valko M, Leibfritz D, Moncol J, Cronin MT, Mazur M, Telser J. Free radicals and antioxidants in normal physiological functions and human disease. Int J Biochem Cell Biol. 2007; 39:44–84.
2. Czesnikiewicz-Guzik M, Konturek SJ, Loster B, Wisniewska G, Majewski S. Melatonin and its role in oxidative stress related diseases of oral cavity. J Physiol Pharmacol. 2007; 58(3):5–19.
3. Sedelnikova OA, Redon CE, Dickey JS, Nakamura AJ, Georgakilas AG, Bonner WM. Role of oxidatively induced DNA lesions in human pathogenesis. Mutat Res. 2010; 704:152–9.
4. Cui K, Luo X, Xu K, Murthy MRV. Role of oxidative stress in neurodegeneration: recent developments in assay methods for oxidative stress and nutraceutical antioxidants. Prog Neuropsychopharmacol Biol Psych. 2004; 28(5):771–99.
5. Halliwell B. Role of free radicals in the neurodegenerative diseases: therapeutic implications for antioxidant treatment. Drug Aging. 2001; 18(9):685–716.
6. Bakonyi T, Radak Z. High altitude and free radicals. J Sports Sci Med. 2004; 3:64–9.
7. Kumar S. Free radicals and antioxidants: human and food system. Adv Appl Sci Res. 2011; 2(1):129–35.
8. Henderson LM, Chappel JB. NADPH oxidase of neutrophils. Biochim Biophys Acta. 1996; 1273(2):87–107.

9. Pithon-Curi T, Leveda AC, Lopes R, Doi SQ, Curi R. Glutamine plays a role in superoxide production and the expression of p47phox, p22phox and gp91phox in rat neutrorphils. Clin Sci. 2002; 103:403–8.
10. Malle E, Furtmüller PG, Sattler W, Obinger C. Myeloperoxidase: a target for new drug development? Br J Pharmacol. 2007; 152(6):838–54.
11. Aruoma OI, Murcia A, Butler J, Halliwell B. Evaluation of the antioxidant actions of gallic acid and its derivatives. J Agr Food Chem. 1998; 41:1880–5.
12. Andrew PJ, Mayer B. Enzymatic function of nitric oxide synthases. Cardiovasc Res. 1999; 43(3):521–31.
13. Beckman JS, Crow JP. Pathological implications of nitric oxide, superoxide, and peroxynitrite formation. Biochem Soc Trans. 1993; 21:330–4.
14. Bredt DS. Endogenous nitric oxide synthesis: biological functions and pathophysiology. Free Radical Res. 1999: 31(6):577–96.
15. Halliwell B. Antioxidant defence mechanisms: from the beginning to the end (of the beginning). Free Radical Res. 1999; 31(4):261–72.
16. Grace SC, Salgo MG, Pryor WA. Scavenging of peroxynitrite by a phenolic/peroxidase system prevents oxidative damage to DNA. FEBS Lett. 1998; 426:24–8.
17. Di Mascio P, Dechara EJH, Mederiros MHG, Briviba K, Sies H. Singlet molecular oxygen production in the reaction of peroxynitrite with hydrogen peroxide. FEBS Lett. 1994; 355(3):287–9.
18. Steinbeck MJ, Khan AU, Karnovsky MJ. Extracellular production of singlet oxygen by stimulated macrophages quantified using 9, 10-diphenylanthracene and perylene in a polystyrene film. J Biol Chem. 1993; 268(21):15649–54.
19. Estevam ML, Nascimento OR, Baptista MS, Di Mascio P, Prado FM, Faljoni-Alario A, et al. Changes in the spin state and reactivity of cytochrome C induced by photochemically generated singlet oxygen and free radicals. J Biol Chem. 2004; 279(38):39214–22.
20. World Wide Web. http://cardiodrops.co.nz/oxidative-stress.html. Accessed on October 28, 2013.
21. John A, Fisher H. Reactive oxygen species generation and human spermatozoa: the balance of benefit and risk. Bioassays. 1994; 16(4):259–67.
22. Schafer FQ, Qian SY, Buettner GR. Iron and free radical oxidations in cell membranes. Cell Mol Biol. 2000; 46(3):657–62.
23. Cheeseman KH, Slater TF. An introduction to free radical biochemistry. Br Med Bull. 1993; 49(3):481–493.
24. Waris G, Ahsan H. Reactive Oxygen Species: Role in the development of cancer and various chronic conditions. J Carcinog. 2006; 5–14.
25. Leeuwenburgh C, Hansen P, Shaish A, Holloszy JO, Heinecke JW. Markers of protein oxidation by hydroxyl radical and reactive nitrogen species in tissues of aging rats. Am J Physiol Regul Integr Comp Physiol. 1998; 274:453–61.
26. Stadtman ER, Oliver CN. Metal-catalyzed oxidation of proteins: physiological consequences. J Biol Chem. 1991; 266(4):2005–8.
27. Aust AE, Eveleigh JF. Mechanisms of DNA oxidation. Exp Biol Med. 1999; 222(3):246–52.
28. Van Rensburg CEJ, Staden MV, Anderson R, Van Rensburg EJ. Hypochlorous acid potentiates hydrogen peroxide-mediated DNA-strand breaks in human mononuclear leucocytes. Mutat Res. 1992; 265(2):255–61.
29. World Wide Web. http://en.wikipedia.org/wiki/Lipid peroxidation. Accessed on October 30, 2013.
30. Cadenas E. Mitochondrial free radical production and cell signaling. Mol Aspects Med 2004; 25(1-2):117–26.
31. Vaya J, Aviram M. Nutritional antioxidants mechanisms of action, analyses of activities and medical applications. Curr Med Chem Immunol Endocr Metab Agents. 2001; 1(1):99–117.
32. Rana N, Mittal S, Iqbal J. Superoxide dismutase-applications and relevance to human diseases. Med Sci Monit. 2002; 8(9):210–5.
33. Ivanova E, Ivanov B. Mechanisms of the extracellular antioxidant defend. Bulg Acad Sci. 2000; 4:22–30.
34. Krinsky NI. Mechanism of action of biological antioxidants. Proc Soc Exp Biol Med. 1992; 200(2):248–54.
35. Takebe G, Yarimizu J, Saito Y, Hayashi T, Nakamura H, Yodoi J, et al. A comparative study on the hydroperoxide and thiol specificity of the glutathione peroxidase family and selenoprotein-P. J Biol Chem. 2002; 277(43):41254–8.
36. Halliwell B, Gutteridge JM. The antioxidants of human extracellular fluids. Arch Biochem Biophys. 1990; 280(1):1–8.
37. Shahidi F. (ed.) Natural antioxidants: chemistry, health effects, and applications. American Oil Chemists Society, 1997.
38. Maguire JJ, Wilson DS, Packer L. Mitochondrial electron transport-linked tocopheroxyl radical reduction. J Biol Chem. 1989; 264(36):21462–5.
39. Borges F, Fernandes E, Roleira F. Progress Towards the discovery of xanthine oxidase inhibitors. Curr Med Chem. 2002; 9(2):195–217.
40. Dailey LA, Imming P. 12-Lipoxygenase: classification, possible therapeutic benefits from inhibition, and inhibitors. Curr Med Chem. 1999; 6(5):389–98.
41. Burton GW, Ingold KU. Autoxidation of biological molecules. 1. Antioxidant activity of vitamin E and related chain-breaking phenolic antioxidants *in vitro*. J Am Chem Soc. 1981; 103(21):6472–7.
42. Upston JM, Terentis AC, Stocker R. Tocopherol-mediated peroxidation of lipoproteins: implications for vitamin E as a potential antiatherogenic supplement, FASEB J. 1999; 13:977–94.
43. Aruoma OI. Methodological considerations for characterizing potential antioxidant actions of bioactive components in plant foods. Mutat Res. 2003; 523:9–20.
44. Lee SK, Mbwambo ZH, Chung ZH, Luyengi L, Gamez EJ, Mehta RG, et al. Evaluation of the antioxidant potential of natural products. Comb Chem High Throughput Screen. 1998; (1):35.
45. Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radical Bio Med. 1999; 26(9):1231–7.
46. Nathalie S-CG, Glories Y, Vivas G. Free radical scavenging effect of anthocyanins in red wines. Food Res Int. 1999; 32(5):327–33.
47. Yoshiki Y, Okubo K, Onuma M, Igarashi K. Chemiluminescence of benzoic and cinnamic acids, and flavonoids in the presence of aldehyde and hydrogen peroxide or hydroxyl radical by fenton reaction. Phytochemistry. 1995; 39(1):225–9.
48. Baghiani A, Ameni D, Boumerfeg S, Adjadj M, Djarmouni M, Charef N, et al. Studies of antioxidants and xanthine oxidase inhibitory potentials of root and aerial parts of medicinal plant *Capparis pinosa*. Am J Med Sci. 2012; 2(1):25–32.

49. Esterbauer H, Striegl G, Puhl H, Rotheneder M. Continuous monitoring of *in vitro* oxidation of human low density lipoprotein. Free Radical Res. 1989; 6(1):67–75.
50. Armstrong D. (ed.) Free radical and antioxidant protocols; Humana Press, Totowa, N. J: 1998; Vol. 108.
51. Richardson T, Finley JW. (ed.) Mechanisms of oxidoreductases important in food component modification in chemical changes in food during processing. Chapman and Hall: New York. 1997; pp 121–76.
52. van Acker SA, van Den Berg DJ, Tromp MN, Griffioen DH, van Bennekom WP, van der Vijgh WJ, et al. Structural aspects of antioxidant activity of flavonoids. Free Radical Biol Med 1996; 20(3):331–42.
53. Lunec J, Griffiths HR. (ed.) Measuring *in vivo* oxidative damage: a practical Approach. Wiley Publication, 2000; pp 53–61.
54. Parthasarathy S, Santanam N, Ramachandran S, Meilhac O. Oxidants and antioxidants in atherogenesis: an appraisal. J Lipid Res. 1999; 40(12):2143–57.
55. Diaz MN, Frei B, Vita JA, Keaney Jr JF. Antioxidants and atherosclerotic heart disease. N Engl J Med. 1997: 337(6):408–16.
56. Noguchi N, Niki E. Phenolic antioxidants: a rationale for design and evaluation of novel antioxidant drug for atherosclerosis. Free Radical Biol Med. 2000; 28(10):1538–43.
57. Woodford FP, Whitehead TP. Is measuring serum antioxidant capacity clinically useful? Ann Clinical Biochem. 1998; 35:48–56.
58. Lee KW, Lee HJ, Kang KS, Lee CH. Preventive effects of vitamin C on carcinogenesis. Lancet. 2002; 359:172.
59. Vatassery GT, Bauer T, Dysken M. High doses of vitamin E in the treatment of disorders of the central nervous system in the aged. Am J Clin Nutr. 1999; 70(5):793–801.
60. Clarkson PM, Thompson HS. Antioxidants: what role do they play in physical activity and health? Am J Clin Nutr. 2000; 72(2):637–46.
61. Bendich A, Machlin LJ, Scandurra A, Burton GW, Wayner DDM. The antioxidant role of vitamin C. Adv Free Radical Biol Med. 1986; 2:419–44.
62. Hamid AA, Aiyelaagbe OO, Usman LA, Ameen OM, Lawal A. Antioxidants: its medicinal and pharmacological applications. Afr J Pure Appl Chem. 2010; 4(8):142–51.
63. Stahl W, Sies H. Antioxidant activity of carotenoids. Mol Aspects Med. 2003; 24(6):345–51.
64. Singh J, Rai GK, Upadhyay AK, Kumar R, Singh KP. Antioxidant phytochemicals in tomato (Lycopersicon esculentum) Indian J Agr Sci. 2004; 74(1):3–5.
65. Harborne JB, Williams CA. Advances in flavonoid research since 1992. Phytochem. 2000; 55:481–504.
66. Bravo L. Polyphenols: Chemistry, dietary sources, metabolism, and nutritional significance. Nutr Rev. 1998; 56:317–33.
67. Cheynier V. Polyphenols in foods are more complex than often thought. Am J Clin Nutr. 2005; 81:223–9.
68. Scalbert A, Manach C, Morand C, Remesy C. Dietary polyphenols and the prevention of diseases. Crit Rev Food Sci Nutr. 2005; 45:287–306.
69. Quideau SP, Deffieux D, Douat-Casassus CL, Pouységu L. Plant polyphenols: chemical properties, biological activities, and synthesis. Angew Chem Int Ed. 2011; 50(3):586.
70. de Jesus Rivas N, Whitaker JR. Purification and some properties of two polyphenol oxidases from Bartlett pears. Plant physiol. 1973; 52: 501–7.
71. Sobolev VS, Horn BW, Potter TL, Deyrup ST, Gloer JB. Production of stilbenoids and phenolic acids by the peanut plant at early stages of growth. J Agric Food Chem. 2006; 54 (10):3505–11.
72. Milder IE, Arts IC, van de Putte B, Venema DP, Hollman PC. Lignan contents of Dutch plant foods: a database including lariciresinol, pinoresinol, secoisolariciresinol and matairesinol. Br J Nutr. 2005; 93(3):393–402.
73. Haslam E. Practical Polyphenolics: From Structure to Molecular Recognition and Physiological Action. Cambridge: Cambridge University Press; 1998. p. 422.
74. Dhingra MS, Deb PK, Chadha R, Singh T, Karan M. Synthesis, evaluation, and molecular docking studies of cycloalkyl/aryl-3,4,5-trimethylgallates as potent non-ulcerogenic and gastroprotective anti-inflammatory agents. Med Chem Res. 2014; 23:87–106.
75. Dziedzic SZ, Bertram JFH. Phenolic acids and related compounds as antioxidants for edible oils. Food Chem. 1984; 14(1):45-51.
76. Zong L, Inoue M, Nose M, Kojima K, Sakaguchi N, Isuzugawa K, et al. Metabolic fate of gallic acid orally administered to rats. Biol Pharm Bull. 1999; 22:326–39.
77. Manach C, Williamson G, Morand C, Scalbert A, Remesy C. Bioavailability and bioefficacy of polyphenols in humans. Review of 97 bioavailability studies. Am J Clin Nutr. 2005; 81:230–42.
78. Haslam E, Cai Y. Plant polyphenols (vegetable tannins): gallic acid metabolism. Nat Prod Rep. 1994; 11:41–66.
79. Rajalakshmi K, Devaraj H, Devaraj SN. Assessment of the no-observed -adverse-effect level (NOAEL) of gallic acid in mice. Food Chem Toxicol. 2001; 39:919-22.
80. Santos-Buelga C, Scalbert A. Proanthocyanidins and tannin-like compounds–nature, occurrence, dietary intake and effects on nutrition and health. J Sci Food Agr. 2000; 80(7):1094-117.
81. Masuda T, Shingai Y, Fujimoto A, Nakamura M, Oyama Y, Maekawa T, et al. Identification of cytotoxic dimers in oxidation product from sesamol, a potent antioxidant of sesame oil. J Agr Food Chem. 2010; 58(20):10880-5.