Chapter 4

Members of Antioxidant Machinery and Their Functions

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Additional information is available at the end of the chapter

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Abstract

In this modern world, due to the rapid advancement of civilization, industrialization, and overpopulation, scientific knowledge on antioxidants is important since most of the diseases are mediated through reactive oxygen species (ROS). An antioxidant is a molecule that inhibits the oxidation of another molecule. Antioxidants may work through single or combined mechanisms, and based on their activity, they have been categorized into primary, secondary, and tertiary antioxidants. Enzymatic and non-enzymatic antioxidants are the two widely accepted categories of antioxidants. In addition to natural antioxidants, synthetic antioxidants have been extensively used in medicinal and food industries. In brief, antioxidants play a significant role in ameliorating toxicity through free radical scavenging reactions and therefore have potential therapeutic value.

Keywords: Antioxidants, Free Radicals, Oxidative Stress, Drugs, Therapy

1. Introduction

Halliwell and Gutteridge [1] defined antioxidants as "any substance that delays, prevents or removes oxidative damage to a target molecule" [1, 2]. Khlebnikov et al. [3] defined antioxidants as "any substance that directly scavenges ROS or indirectly acts to up-regulate antioxidant defenses or inhibit ROS production". In other words, we can define antioxidants as any molecule that inhibits the oxidation of another molecule. A chemical reaction involving the loss of electrons and increase in the oxidative state is termed as "oxidation." Oxidation results in the formation of free radicals that are unstable atoms and molecules deficit in electrons. They have unpaired electrons and are extremely reactive and are capable of initiating chain reactions that destabilize other molecules and generate free radicals. These free radicals are also termed as reactive oxygen species or ROS and create a homeostatic imbalance that generates oxidative stress and causes cell death and tissue injury. ROS includes: superoxide ($O_2^{\cdot -}$), hydroxyl ($OH^{\cdot}$), peroxyl ($RO_2^{\cdot}$), hydroperoxyl ($HO_2^{\cdot}$), alkoxyl ($RO^{\cdot}$), peroxyl

(ROO·), nitric oxide (NO·), nitrogen dioxide (NO2 ·), and lipid peroxyl (LOO·) and the non-radicals hydrogen peroxide (H2O2), hypochlorous acid (HOCl), ozone (O3), singlet oxygen (1Δg), and lipid peroxide (LOOH) [4]. Free radicals are known to be formed as a result of environmental pollution, stress, cigarette smoke, UV Light, ionizing radiations, and xenobiotics. Toxic effect of the free radicals causes oxidative stress and results in the pathogenesis of diseases (Figure 1).

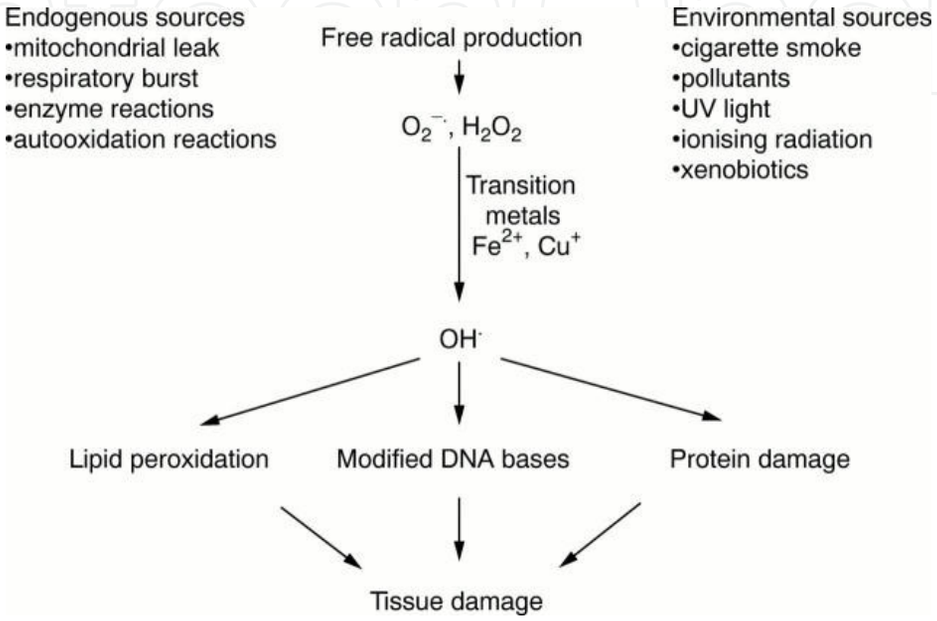

Figure 1. Free radicals: Production and damage (Adapted from [5]).

Involvement of ROS is implicated in neurodegenerative and other disorders such as Alzheimer's disease, Parkinson's disease, multiple sclerosis, Down syndrome, inflammation, viral infection, autoimmune pathology, and digestive ulcers. Recent developments in biomedical science emphasize the involvement of free radicals in many diseases, such as brain dysfunction, cancer, heart disease, and immune system [6]. Antioxidants normally terminate many reactions by removing free radical intermediates and inhibit other oxidation reactions. Thus, antioxidants often serve as reducing agents (examples: thiols, ascorbic acid, or polyphenols) [7]. Depending on the balance between ROS and the availability of antioxidants in the microenvironment of the cell, antioxidants can inhibit or delay the initiation or propagation of oxidative chain reaction and thus prevent or repair cell damage caused by reactive oxygen [8]. Antioxidants have been reported to work through single or combined mechanisms, namely, free radical scavenging, reducing activity, complexing of pro-oxidant, scavenging lipid peroxyl radicals, and quenching of singlet oxygen. Preventive oxidants are the antioxidants that act as inhibitors of free radical oxidation reactions. Chain-breaking antioxidants inhibit formation of free lipid radicals as follows: by obstructing the propagation of the autoxidation chain reactions; as singlet oxygen quenchers; as reducing agents which convert hydroperoxides into stable compounds; as metal chelators that convert metal pro-oxidants (iron and copper derivatives) into stable products; and finally as inhibitors of pro-oxidative

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enzymes (lipoxygenases) [9]. Antioxidant approach to disease management holds potential as most of the diseases are mediated through ROS, also with the rapid advancement of civilization, industrialization, and overpopulation. Epidemiological researches strongly suggest that foods containing antioxidants and scavengers have a potential protective effect against disorders caused by ROS [10].

1.1. Classification of antioxidants

Guttering and Halliwell classified the antioxidants into three categories: primary, secondary, and tertiary antioxidants [11]. Primary antioxidants are involved in the prevention of oxidant formation; secondary antioxidants are known to be scavengers of ROS, and tertiary antioxidants repair the oxidized molecules through sources like dietary or consecutive antioxidants.

Antioxidants may also be classified as enzymatic or non-enzymatic antioxidants (Figure 2).

1.1.1. Enzymatic antioxidants

The antioxidant enzymatic system directly/indirectly contributes to defense against the ROS. Catalase, superoxide dismutase (SOD), glutathione peroxidase, glutathione reductase, etc., are enzymatic antioxidants.

1.1.2. Non-enzymatic antioxidants

These antioxidants are quite a few, namely vitamins (A, C, E, and K), enzyme cofactors (Q10), minerals (Zn, Se, etc.), organosulfur compounds (allium and allium sulfur), nitrogen compounds (uric acid), peptides (glutathione), and polyphenols (flavonoids and phenolic acid).

1.1.3. Hydrophilic antioxidants

Antioxidants that react with oxidants in the cell cytoplasm and the blood plasma are termed as hydrophilic antioxidants (ascorbic acid, glutathione, and uric acid).

1.1.4. Hydrophobic antioxidants

These compounds are known to protect cell membranes from lipid peroxidation (ubiquinol, carotenes, and α-tocopherol). They are obtained either from the diet or synthesized in the body [12].

1.1.5. Endogenous antioxidants

Endogenous antioxidants can be categorized into primary antioxidants and secondary antioxidants. Primary antioxidants inactivate the ROS into their intermediates. SOD, catalase, and glutathione peroxidase are the primary antioxidant enzymes [13]. They can be water soluble or lipid soluble (ascorbate, glutathione, uric acid, etc., are water soluble, and tocopherols, ubiquinols, and carotenoids, etc., are lipid soluble). Secondary antioxidant enzymes act directly to detoxify ROS. They maintain their proper functioning by decreasing the

peroxides level and continuously supplying NADPH (nicotinamide adenine dinucleotide phosphate) and glutathione for primary antioxidant enzymes. Glutathione reductase, glucose-6-phosphate dehydrogenase, glutathione-s-transferase, and ubiquinone are the secondary antioxidants. Iron, copper, zinc, manganese, and selenium also increase the antioxidant enzyme activities [14, 15].

1.1.6. Exogenous antioxidants

Many foods and various dietary components exhibit antioxidant activities. Several herbs, spices, vitamins, foods, vegetables, etc., are reported to be sources of exogenous antioxidants. These antioxidant drugs could be used for the treatment of various pathological diseases, and therefore gained importance in clinical as well as research areas. Many polyphenolic compounds such as flavonoids, isoflavones, flavones, anthocyanins, coumarins, lignans, catechins, isocatechins, epicatechins, and phenolic acids such as hydrocinnamic acid, hydrobenzoic acid, gallic acid, ellagic acid, etc., have gained importance as antioxidant phytochemicals. These bioactive compounds are being tested in clinical and preclinical trials. Plant-derived drugs are medicinally useful as they contain phytochemicals like terpenoid, alkaloids, glycosides, polyphenolics, and steroids and are of great significance in research area [16, 17]. Dietary nutrients, protein, and amino acids are responsible for the synthesis of antioxidant enzymes and hence play an important role in the defensive mechanism. GSH, creatine, and uric acid act as the direct scavengers of reactive metabolites [18]. Antioxidants of natural origin such as polyphenols, tannins, and flavonoids act by donating electrons to the intermediate radicals formed in oxidative stress or tissue damage that help in inhibition of the lipid peroxidation.

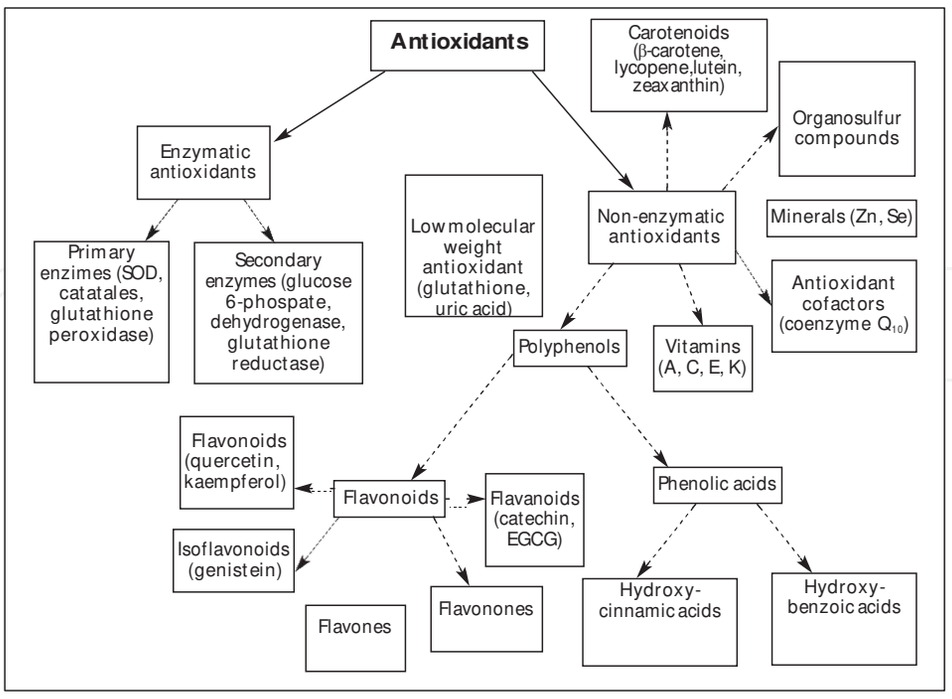

Figure 2. Antioxidants: Classification (Adapted from [19]).

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2. Enzymatic and non-enzymatic antioxidants

When cells are exposed to oxidative stress, a defense system endorses the expression and regulation of a number of antioxidant enzymes as a defense mechanism to protect them from the damage induced by free radicals. These antioxidant defenses could be non-enzymatic or enzymatic (Table 1).

2.1. Enzymatic antioxidants

Enzymatic antioxidants are categorized into primary and secondary enzymatic defenses. Primary defense is composed of three important enzymes that prevent the formation or neutralize free radicals: glutathione peroxidase, which donates two electrons to reduce peroxides by forming selenols and also eliminates peroxides; catalase, which converts hydrogen peroxide into water and molecular oxygen; and SOD, which converts superoxide anions into hydrogen peroxide as a substrate for catalase [20].

Glutathione reductase and glucose-6-phosphate dehydrogenase are involved in the secondary enzymatic defense system. Glutathione reductase reduces glutathione (antioxidant) from its oxidized form to its reduced form, thereby recycling itself to continue neutralizing more free radicals. Glucose-6-phosphate regenerates NADPH (a coenzyme used in anabolic reactions) creating a reducing environment [21, 22]. These two enzymes do not neutralize free radicals directly but have supporting roles to other endogenous antioxidants.

Glutathione peroxidase, catalase, and SOD metabolize toxic oxidative intermediates and therefore form the primary antioxidant enzymes. These form the body's endogenous defense mechanism and help protect against free radical-induced cell damage. For optimum catalytic activity, these enzymes also require co-factors such as selenium, iron, copper, zinc, and manganese. It has been indicated that an inadequate dietary intake of these trace minerals may compromise the effectiveness of these antioxidant defense mechanisms. The consumption and absorption of important trace minerals may decrease with aging.

2.1.1. Superoxide dismutase

The superoxide dismutases catalyze the dismutation of superoxide to hydrogen peroxide:

$$O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2.$$

Catalase or glutathione peroxidase removes hydrogen peroxide. Catalase converts hydrogen peroxide into water and molecular oxygen.

Mammalian tissues have three forms of superoxide dismutase, each with a specific subcellular location and different tissue distribution (Figure 3).

1. Copper zinc superoxide dismutase (CuZnSOD): CuZnSOD has a molecular mass of approximately 32,000 kDa and has two protein subunits, each containing a catalytically active copper and zinc atom and is present in the cytoplasm and organelles of all mammalian cells.

2. Manganese superoxide dismutase (MnSOD): MnSOD is found to have a molecular mass of 40,000 kDa. It consists of four protein subunits, each probably containing a single manganese atom. It is present in the mitochondria of almost all cells [23]. The amino acid sequence of MnSOD is very dissimilar to that of CuZnSOD and is not inhibited by cyanide, and thereby MnSOD activity can be distinguished from that of CuZnSOD in mixtures of the two enzymes.
3. Extracellular superoxide dismutase (ECSOD): Marklund described EC-SOD in 1982 [24]. It is a secretory copper and zinc containing SOD and is different from the CuZnSOD. Only a few cell types, including fibroblasts and endothelial cells, synthesize EC-SOD and are expressed on the cell surface where it is bound to heparin sulfates. EC-SOD is the major SOD detectable in extracellular fluids and following the injection of heparin, it is released into the circulation from the surface of vascular endothelium [25]. EC-SOD might play a role in the regulation of vascular tone because endothelial derived relaxing factor (nitric oxide or a closely related compound) is neutralized in the plasma by superoxide [26].

These superoxide enzymes are present in extracellular fluids of almost all aerobic cells. SODs contain metal ion cofactors like copper, zinc, manganese, or iron depending on the isozyme. For example, in human copper/zinc SOD is present in the cytosol while manganese SOD is present in the mitochondrion. The mitochondrial SOD is the most biologically significant of these three enzymes. SOD isozymes are present in the cytosol and mitochondria in plants, and there is also an iron SOD found in chloroplasts.

2.1.2. Catalase

Catalase was the first antioxidant enzyme to be characterized and catalyzes the two-stage conversion of hydrogen peroxide to water and oxygen. Catalases are enzymes that catalyze the conversion of hydrogen peroxide to water and oxygen, using either an iron or manganese cofactor. Here, its cofactor is oxidized by one molecule of hydrogen peroxide and then regenerated by transferring the bound oxygen to a second molecule of substrate.

$$\text{Catalase–Fe}\left(\text{III}\right) + H_2O_2 \rightarrow \text{compound I}$$
$$\text{Compound I} + H_2O_2 \rightarrow \text{catalase–Fe}\left(\text{III}\right) + 2H_2O + O_2.$$

Catalase consists of four protein subunits, each containing a heme group and a molecule of NADPH [27]. Catalase is largely located within cells in peroxisomes, which also contain most of the enzymes capable of generating hydrogen peroxide. The greatest activity is present in the liver and erythrocytes, but some catalase is found in all tissues. It is a tetrameric enzyme consisting of four identical tetrahedrally arranged subunits of 60 kDa, which contains a single ferriprotoporphyrin group per subunit and has a molecular mass of about 240 kDa

2.1.3. Glutathione enzymes

The glutathione system includes glutathione, glutathione reductase, glutathione peroxidases, and glutathione "s"-transferases. Glutathione peroxidase is an enzyme containing four

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selenium cofactors that catalyze the breakdown of hydrogen peroxide and organic hydroperoxides. Glutathione "s"-transferases show high activity with lipid peroxides. These enzymes are noticed especially in high levels in the liver. Glutathione peroxidases catalyze the oxidation of glutathione. Hydroperoxides, such as hydrogen peroxide and lipid hydroperoxides, act as substrates for these enzymes [28].

$$ROOH + 2GSH \rightarrow GSSG + H_2O + ROH.$$

Selenium is required at the active site for effective functioning of glutathione peroxidases [29]. Kidney synthesizes the plasma form of glutathione, and the highest level of glutathione peroxidases is found within liver cells, although glutathione peroxidase is widely distributed in almost all tissues. Glutathione peroxidase is the main scavenger of hydrogen peroxide in these subcellular compartments; the predominant sub-cellular distribution is in the cytosol and mitochondria. The activity of the enzyme glutathione peroxidase is dependent on the constant availability of reduced glutathione [30].

$$GSSG + NADPH^{+}H^{+} \rightarrow 2GSH + NADP^{+}.$$

The NADPH required by this enzyme to restore the supply of reduced glutathione is supplied by the pentose phosphate pathway. Glutathione reductase is a flavine nucleotide-dependent enzyme and has a similar tissue distribution to glutathione peroxidase [31].

Amino acids such as glycine, glutamate, and cysteine are utilized in the synthesis of glutathione. It is an important water-soluble antioxidant that plays a major role in xenobiotic metabolism; it can directly neutralize ROS such as lipid peroxide. When a body is exposed to xenobiotics or toxins, there is an increase in the level of detoxification enzymes (cytochrome P-450 mixed-function oxidase). Xenobiotics conjugate with glutathione, and hence a higher concentration of the enzyme is required for conjugation to make the toxin neutral and thereby making the enzyme less available as an antioxidant. Glutathione and vitamin C work interactively to neutralize the free radicals.

2.1.4. Non-enzymatic endogenous antioxidants

There are a number of non-enzymatic antioxidants: vitamins (A, C, E, and K), enzyme cofactors (Q10), minerals (Zn and Se), organosulfur compounds (allium and allium sulfur), nitrogen compounds (uric acid), peptides (glutathione), and polyphenols (flavonoids and phenolic acid).

2.1.4.1. Vitamin A

Vitamin A is produced as a result of the breakdown of β-carotene and is a carotenoid produced in the liver. It exhibits antioxidant activity due to its ability to combine with peroxyl radicals before they propagate peroxidation to lipids. Vitamin A is known to have a beneficial impact on the skin, eyes, and internal organs [32, 33].

2.1.4.2. Coenzyme Q10

Coenzyme Q10 has been reported to act by preventing the formation of lipid peroxyl radicals. It neutralizes the radicals even after their formation. An important role of this coenzyme is regeneration of vitamin E. Regeneration of vitamin E through this process is more likely than through ascorbate (vitamin C). This coenzyme is present in all cells and membranes and plays an important role in the respiratory chain and other cellular metabolism processes [34].

2.1.4.3. Uric acid

The end product of purine nucleotide metabolism in humans is uric acid. After undergoing kidney filtration, 90% of the uric acid is reabsorbed by the body, proving that it has important functions within the body. Uric acid prevents lysis of erythrocytes by peroxidation and is a potent scavenger of singlet oxygen and hydroxyl radicals. It is also known to prevent the overproduction of oxo-heme oxidants that result from the reaction of hemoglobin with peroxides [35].

2.1.4.4. Glutathione

Glutathione is an endogenous tripeptide that protects the cells against free radicals by donating either a hydrogen atom or an electron. It also plays an important role in the regeneration of other antioxidants like ascorbate [36]. However, the endogenous antioxidant system is not sufficient; humans depend on dietary antioxidants to reduce free radical concentrations [37].

2.1.4.5. Vitamin C

Ascorbic acid and tocopherols are generic names for vitamin C and vitamin E. Ascorbic acid consists of two antioxidant compounds: L-ascorbic acid and L-dehydroascorbic acid. These two compounds are absorbed through the gastrointestinal tract and can be interchanged enzymatically *in vivo*. Ascorbic acid acts by scavenging the superoxide radical anion, hydrogen peroxide, hydroxyl radical, singlet oxygen, and reactive nitrogen oxide [38].

2.1.4.6. Vitamin E

Vitamin E is the only major lipid-soluble, chain-breaking antioxidant found in plasma, red cells, and tissues, thus protecting the integrity of lipid structures, mainly membranes. It inhibits lipid peroxidation by donating its phenolic hydrogen to the peroxyl radicals forming tocopheroxyl radicals that, despite also being radicals, are unreactive and unable to continue the oxidative chain reaction. There are eight isoforms of vitamin E: four tocopherols (α-tocopherol, β-tocopherol, γ-tocopherol, and δ-tocopherol) and four tocotrienols (α-tocotrienol, β-tocotrienol, γ-tocotrienol, and δ-tocotrienol), α- tocopherol being the most potent and abundant isoform in biological systems. The antioxidant activity of tocopherols is due to the chroman head, but the phytyl tail has no effect [39]. These two vitamins also display a synergistic behavior with the regeneration of vitamin E through vitamin C from the tocopheroxyl radical to an intermediate form, therefore reinstating its antioxidant potential [40].

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2.1.4.7. Vitamin K

This vitamin has two natural isoforms: vitamins K1 and K2. Vitamin K is a group of fat-soluble compounds, essential for the post-translational conversion of protein-bound glutamates into γ-carboxyglutamates in various target proteins. The antioxidant activity is due to the 1, 4-naphthoquinone structure of these vitamins [41].

2.1.4.8. Flavonoids

Flavonoids are a group of compounds composed of diphenyl propane (C6C3C6) skeleton. It can be classified as flavonols, flavanols, anthocyanins, isoflavonoids, flavanones, and flavones. Flavanones and flavones are usually found in the same fruits and are connected by specific enzymes while flavones and flavonols do not share this phenomenon and are rarely found together. Anthocyanins are also absent in flavanone-rich plants. Flavonoids exhibit their antioxidant activity due to the phenolic hydroxyl groups attached to ring structures. They may act as reducing agents, superoxide radical scavengers, hydrogen donators, singlet oxygen quenchers, and also as metal chelators. They activate antioxidant enzymes, reduce α-tocopherol radicals (tocopheroxyls), inhibit oxidases, mitigate nitrosative stress, and increase the levels of uric acid and low-molecular-weight molecules. Some of the flavonoids of significance are quercetin, kaempferol, catechin, and catechin-gallate [42, 43].

2.1.4.9. Phenolic acids

Phenolic acids are composed of hydroxycinnamic and hydroxybenzoic acids. One of the most studied and promising compounds in the hydroxybenzoic group is gallic acid that is also the precursor of many types of tannin, while cinnamic acid is the precursor of all the hydroxycinnamic acids. They are present in plant material and sometimes present as esters and glycosides. They have antioxidant activity as chelators and free radical scavengers with special impact over hydroxyl and peroxyl radicals, superoxide anions, and peroxynitrites [44, 45].

2.1.4.10. Carotenoids

Carotenoids are a group of natural pigments and are synthesized by plants and microorganisms. They can be classified into two different groups: the carotenoid hydrocarbons known as the carotenes containing distinct end groups like lycopene and β-carotene; and the oxygenated carotenoids known as xanthophylls, like zeaxanthin and lutein. Carotenoids display their antioxidant activity due to singlet oxygen quenching which culminates in excited carotenoids that dispel the newly acquired energy through a series of rotational and vibrational interactions with the solvent, thus returning to the unexcited state and allowing them to quench more radical species. The only free radicals that completely damage these pigments are peroxyl radicals. Carotenoids are relatively unreactive, but they may also decay and form non-radical compounds and result in terminating free radical attacks by binding to these radicals [46].

2.1.4.11. Minerals

Minerals are found in trace quantities in animals and are a small part of dietary antioxidants, but play significant roles in their metabolism. The most important minerals exhibiting antioxidant activity are selenium and zinc. Selenium can be found in both organic (selenocysteine and selenomethionine) and inorganic (selenite and selenate) forms in the human body. It does not act directly on free radicals but is a vital part of most antioxidant enzymes (metalloenzymes, glutathione peroxidase, and thioredoxin reductase) that would have no effect without it [47].

Various pathways in metabolism require zinc. Zinc is essential in the prevention of free radical formation and does not directly attack free radicals. Zinc is also an inhibitor of NADPH oxidases that catalyze the production of the singlet oxygen radical from oxygen by using NADPH as an electron donor. It is present in SOD, a vital antioxidant enzyme that converts the singlet oxygen radical into hydrogen peroxide. Zinc brings about the production of metallothionein that is a scavenger of the hydroxyl radical. Finally, zinc also competes with copper for binding to the cell wall, thus decreasing the production of hydroxyl radicals [48].

2.1.4.12. Lipoic acid

Lipoic acid and its reduced form, dihydrolipoic acid (DHLA), neutralize the free radicals in both lipid and aqueous domains and are called "universal antioxidants." It is categorized as "thiol" or "biothiol."

They are sulfur-containing molecules that catalyze the oxidative decarboxylation of alpha-keto acids, such as pyruvate and alpha-ketoglutarate, in the Krebs cycle.

2.1.4.13. Peroxiredoxins

These may be of three basic types: typical 2-cysteine peroxiredoxins; atypical 2-cysteine peroxiredoxins; and 1-cysteine peroxiredoxins. Peroxiredoxins are important in antioxidant metabolism as they catalyze the reduction of hydrogen peroxide, organic hydroperoxides, as well as peroxynitrite.

2.1.4.14. Synthetic antioxidants

Synthetic antioxidants have been developed to have a standard antioxidant activity measurement system and to compare with natural antioxidants that are incorporated into food. Synthetic antioxidants are added to food so that it can withstand various treatments and conditions to prolong shelf life and prevention of food oxidation, especially fatty acids. It has been reported that synthetic antioxidants are added to almost all processed foods, which are reported to be safe, although some studies oppose this fact. The important synthetic antioxidants are BHT (butylated hydroxytoluene) and BHA (butylated hydroxyanisole). The European Food Safety Authority (EFSA) between 2011 and 2012 classified an NOAEL (No Observable Adverse Effect Level) of 0.25 mg/kg BW/day for BHT and 1.0 mg/kg BW/day for BHA in terms of daily intake and admitted that the exposure of adults and children was

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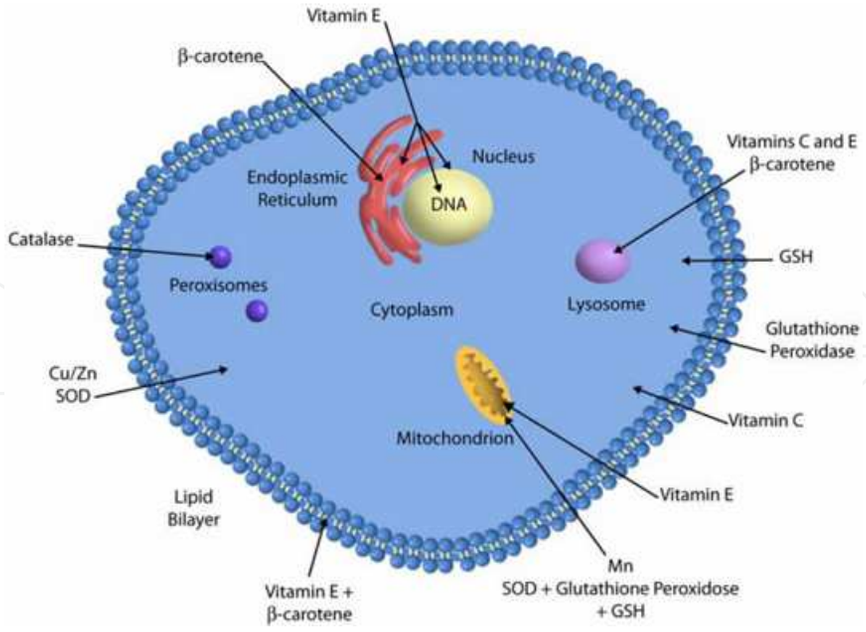

Figure 3. Diagrammatic representation of the site of enzymatic and non-enzymatic antioxidant defense system (Adapted from [49]).

unlikely to exceed these doses. TBHQ (*tert*-butylhydroquinone) stabilizes and preserves the freshness, nutritive value, flavor, and color of animal food products. Octyl gallate is considered as safe to use as a food additive because, after consumption, it is hydrolyzed into gallic acid and octanol, which are found in many plants and do not pose a threat to human health [50]. NDGA (nordihydroguaiaretic acid) despite being a food antioxidant is known to cause renal cystic disease in rodents [51].

2.1.4.15. Pro-oxidants

Pro-oxidants are defined as chemicals that induce oxidative stress, usually through the formation of reactive species or by inhibiting antioxidant systems. Free radicals are considered pro-oxidants, but sometimes, antioxidants can also have pro-oxidant behavior. Vitamin C is a potent antioxidant, but it can also become a pro-oxidant when it combines with iron and copper reducing Fe3+ to Fe2+ (or $Cu3^{+}$ to $Cu2^{+}$), which in turn reduces hydrogen peroxide to hydroxyl radicals [52].

α-Tocopherol is a powerful antioxidant, but in high concentrations, it can become a pro-oxidant. When vitamin E reacts with a free radical, it becomes a radical itself, and if there is not enough ascorbic acid for its regeneration, it will remain in this highly reactive state and support the autoxidation of linoleic acid [53].

Although not much evidence is found, it is proposed that carotenoids can also display pro-oxidant effects especially through autoxidation in the presence of high concentrations of oxygen-forming hydroxyl radicals [54]. Flavonoids may also serve as pro-oxidants. The occurrence of O_2, iron, and copper damages biological molecules [55].

Enzymatic antioxidants	Location	Properties
Superoxide dismutase (SOD)	Mitochondria and cytosol	Dismutation of superoxide radicals
Catalase (CAT)	Mitochondria and cytosol	Removes hydrogen peroxide
Glutathione peroxidase (GSH))	Mitochondria and cytosol	Removes hydrogen peroxide and organic hydroperoxide
Non-enzymatic antioxidants	**Location**	**Properties**
Vitamin C	Aqueous phase of cell	Acts as a free radical scavenger and recycles vitamin E
Vitamin E	Cell membrane	Major chain-breaking antioxidant in cell membrane
Uric acid	Product of purine metabolism	Scavenger of OH radicals
Carotenoids	Membrane tissue	Scavengers of ROS and singlet oxygen quencher
Glutathione	Non-protein thiol in cell	Serves multiple roles in the cellular antioxidant defense
Lipoic acid	Endogenous thiol	Effectual in recycling vitamin C, and also a functional glutathione substitute
Metals ions sequestration: transferrin, ferritin, lactoferrin	Mitochondria and cytosol	Scavenger of free radical and inhibitor of lipid peroxidation
Nitric oxide	Mitochondria and cytosol	Chelating of metal ions, and responsible for Fenton reactions
Ubiquinones	Mitochondria	Reduced form serve as functional antioxidants
Bilirubin	Product of heme metabolism in blood	Extracellular antioxidant

Table 1. Major enzymatic and non-enzymatic antioxidants (Adapted and modified from [56]).

3. Antioxidant defense mechanism

Free radicals are constantly being generated in the body through various mechanisms and are also being removed by endogenous antioxidant defensive mechanisms that act either by scavenging free radicals, by decomposing peroxides, or by binding with pro-oxidant metal ions (Tables 2 and 3; Figure 4).

Antioxidants are classified into three categories [56–58] as follows:

http://dx.doi.org/10.5772/61884

1. Primary antioxidants: It is involved in the prevention of oxidant formation. They act by suppressing the formation of free radicals (examples: glutathione peroxidase, catalase, selenoprotein, transferrin, ferritin, lactoferrin, carotenoids, etc.).
2. Secondary antioxidants: These exhibit scavengers of ROS. They act by suppressing chain initiation and breaking chain propagation reactions (radical scavenging antioxidants).
3. Tertiary antioxidants: They act by repairing the oxidized molecules (some proteolytic enzymes, enzymes of DNA, etc.) through sources like dietary or consecutive antioxidants.

The human body employs three general categories of antioxidants to safeguard against free radicals. They are endogenous antioxidants, dietary antioxidants, and metal-binding proteins [16].

3.1. Endogenous antioxidants

These are categorized into primary antioxidants and secondary antioxidants. SOD, catalase, and glutathione peroxidase are the primary antioxidant enzymes that inactivate the ROS into intermediates [13]. Secondary antioxidant enzymes (glutathione reductase, glucose-6-phosphate dehydrogenase, glutathione-s-transferase, and ubiquinone) detoxify ROS and supply the NADPH and glutathione for primary antioxidant enzymes for proper functioning. Metals such as copper, iron, manganese, zinc, and selenium up-regulate the antioxidant enzyme activities [14, 15].

3.2. Exogenous antioxidants

Many polyphenolic compounds such as flavonoids, isoflavones, flavones, anthocyanins, coumarins, lignans, catechins, isocatechins, epicatechins, and phenolic acids have gained importance as antioxidant drugs [16]. Dietary antioxidants act through scavenging free radicals to break the chain reaction responsible for lipid peroxidation. Vitamins C and E, carotenoids, and flavonoids are the dietary antioxidants. These vitamins are also known as chain-breaking antioxidants [16]. The metal-binding proteins (albumin, ferritin, and myoglobin) inactivate the transition metal ions that catalyze the production of free radicals [17, 18].

Antioxidant enzymes – catalase, SOD, glutathione peroxidase, glutathione reductase, and thioredoxin – act against the ROS. The non-enzymatic antioxidants are the scavengers of ROS and RNS [59].

3.3. Cellular antioxidant system

Lipid peroxidation is slowed down by the activity of chemical compounds that contain monohydroxy/polyhydroxy phenol acting as antioxidants. These compounds have low activation energy to donate the hydrogen atom and, therefore, cannot initiate the secondary free radicals. The free radical electrons are stable and, thus, slow down the oxidation. Prevention of excessive ROS and repair of cellular damage are essential for the life of cells, and cells, in turn, contain many antioxidant systems to prevent the oxidative injury [60, 61].

3.4. Mechanism of action of antioxidants

$$R + AH \rightarrow RH + A$$
$$RO + AH \rightarrow ROH + A$$
$$ROO + AH \rightarrow ROOH + A$$
$$R + A \rightarrow RA$$
$$RO + A \rightarrow ROA$$
$$ROO + A \rightarrow ROOH$$
$$\text{Antioxidant} + O_2 \rightarrow \text{Oxidized antioxidant.}$$

3.5. Mode of action of antioxidants

1. Primary or chain-breaking antioxidants: break chain reaction and the resulting radical are less reactive

$$ROO + AH \rightarrow ROOH + A$$
$$ROOH + A \rightarrow ROOA.$$

2. Secondary or preventive antioxidants:

They may act either by

- Chelating/deactivating metals,
- Scavenging singlet oxygen (highly toxic), or
- Removing ROS.

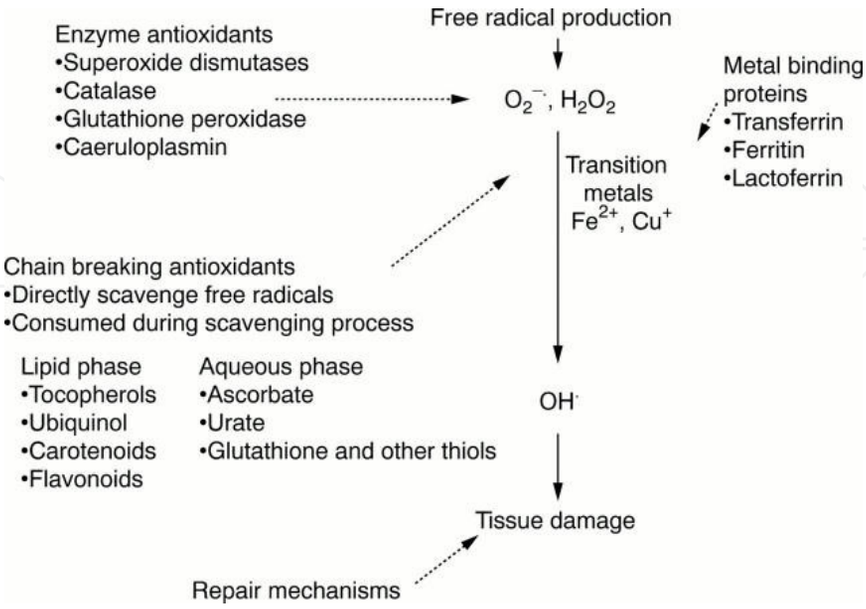

Figure 4. A schematic diagram of the antioxidant defense mechanism (Adapted from [5]).

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ROS scavengers	ROS protective enzymes	Sequestration of transition metal ions which form ROS
Glutathione	Superoxide dismutase	Transferrin
Uric acid	Catalase	Ferritin
Ascorbic acid	Glutathione peroxidase	Metallothionein
Albumin	Glutathione reductase	Ceruloplasmin

Table 2. Antioxidant defensive agents (Adapted from [66]).

Enzymatic antioxidants	Reactions catalyzed
Superoxide dismutase (SOD)	$O_2^- + O_2 + 2H \rightarrow 2H_2O_2 + O_2$
Catalase (CAT)	$H_2O_2 \rightarrow H_2O + 1/2O_2$
Glutathione peroxidase (GPX)	$H_2O_2 + GSH \rightarrow H_2O + GSSG$
Glutathione reductase (GR)	$GSSG + NAD(P)H \rightarrow 2GSH + NAD(P)^+$

Table 3. Major ROS scavenging antioxidant enzymes (Adapted from [67]).

Antioxidants are present with protective efficiency. If there is an electron-donating group, especially a hydroxyl group loaded on *o*- or *p*-positions of the phenolic compounds, it makes the compound polar, and, therefore, antioxidant activities and metal chelating ability are increased. These groups make the phenols more easily donate hydrogen atoms to activate free radicals to interrupt the chain reaction of autoxidation. Antioxidants of natural origin such as polyphenols (tannins, flavonoids, and chalcones) act by donating an electron to the intermediate radicals formed in oxidative stress or tissue damage, which helps in the inhibition of lipid peroxidation. A computational study also supports that the compounds having more electron donating potentials are better inhibitors of hydroperoxides that suggest many of the antioxidant agents [62–67].

4. Antioxidants: Health and diseases

Several human pathologies such as neurodegenerative diseases, cancer, stroke, and many other ailments are believed to be caused by ROS. Antioxidants are assumed to prevent the harmful effects of ROS and therefore treat oxidative stress-related diseases (Figure 5).

Antioxidant approach to disease management holds potential as most of the diseases are mediated through ROS; also with the rapid advancement of civilization, industrialization, and overpopulation, there has been a significant rise in oxidative stressors. Epidemiological researches strongly suggested that foods containing antioxidants and scavengers have a potential protective effect against disorders caused by ROS [66]. Many chronic diseases can be prevented, and disease progression can be slowed by increasing the body natural antioxidant

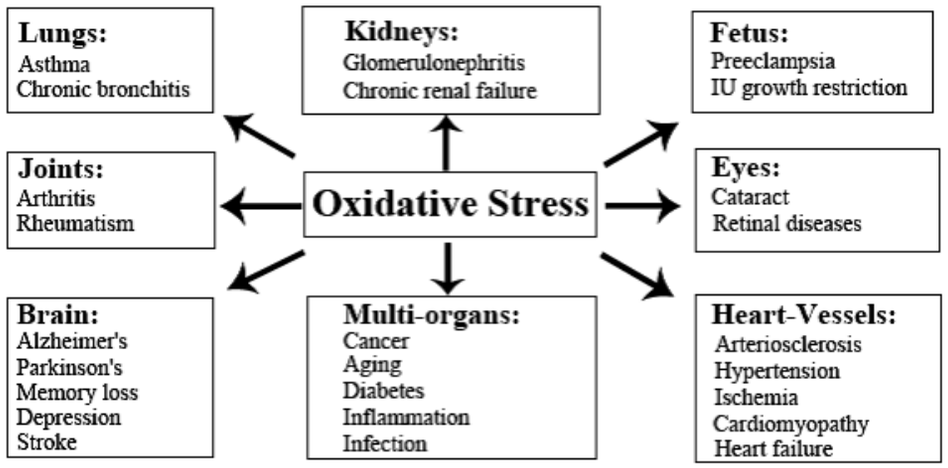

Figure 5. Oxidative stress-induced diseases in humans (Adapted from [65]).

defenses or by supplementing with dietary antioxidants. Natural antioxidants such as flavonoids, tannins, and polyphenols act by donating electrons to intermediate radicals and help in inhibition of lipid peroxidation. Antioxidants are essential to prevent the formation and oppose the actions of reactive oxygen and nitrogen species, which are generated in vivo and cause damage to DNA, lipids, proteins, and other biomolecules. The antioxidant system contains exogenous antioxidants (dietary sources) and endogenous antioxidants.

4.1. Exogenous antioxidants as drugs

Many polyphenolic compounds such as flavonoids, isoflavones, flavones, anthocyanins, coumarins, lignans, catechins, isocatechins, epicatechins, and phenolic acids have gained importance as antioxidant drugs.

4.2. Role of dietary nutrients in defensive mechanism

Protein and amino acids play an important role in the synthesis of antioxidant enzymes. Small peptides like GSH and carnosine and nitrogenous metabolites like creatine and uric acid directly scavenge the reactive metabolites [67]. iNOS expression and synthesis in various cells are controlled by taurine and taurine chloramines. Deficiency of dietary protein can have a harmful effect on the antioxidant system of the cell. Arginine and tetrahydrobiopterin deficiency directly affect the superoxide enzyme production. Decreased protein intake affects the availability of zinc, which is a cofactor of SOD. Similarly, a high-protein diet exhibits oxidative stress. Homocysteine increases inducible and constitutive NOS synthesis and stimulates ROS generation in polymorphonuclear leukocytes and monocytic cells [68–70].

4.2.1. Lipids

There is a generation of ROS due to the intake of polyunsaturated fatty acids which are neutralized by vitamins C and E and carotenoids. There is an increase in the risk of cardio-

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vascular diseases due to high intake of polyunsaturated fatty acids. On the other hand, a high-saturated-fat diet increases the risk of iNOS activity in the liver and colon. Fish oil decreases the cardiovascular risk by reducing triacylglycerol production in plasma as it contains ω-3 PUFA that is the inhibitor of ROS, iNOS expression, and NOS synthesis [71].

4.2.2. Vitamins

Vitamins exhibit anti-atherogenic and anti-inflammatory properties. Vitamin A inhibits iNOS in vascular muscle cells, endothelial cells, cardiac myocytes, and mesangial cells. Vitamins D3, K2, and niacin inhibit iNOS activity in the neuronal cells (macrophage, microglia, and astrocytes) Lipid peroxidation of the membrane is prevented by vitamin E as it inhibits the ROS generation. Irradiation decreases the concentration of vitamin C and folate, thus leading to ROS generation. It has been reported that vitamin B12 and folic acid reduce radical-induced radiation damage and improve leukocyte counts. DNA damage and hepatocellular carcinoma are prevented by vitamin C and choline. Vitamins B12, B6, and folate are essential for the synthesis of cystathionine synthase and cystathionase (B6) and methionine synthase (B12). These vitamins prevent cardiovascular diseases in humans and rodents. NADP, NADH, FAD, nicotinamide, and riboflavin protect the cells from ROS generation. NADPH and FAD are essential for glutathione reductase. NADPH is required for catalase activity [70–75].

4.2.3. Micronutrients and minerals

Copper, zinc, and manganese, the important trace elements in our body, serve as cofactors of SOD enzyme. Deficiency of either copper or zinc increases the cytochrome P_{450} activity in microsomes of the liver and lungs, and thus increases the generation of ROS and iNOS expression [76]. Selenium possesses potential antioxidant activity as it is a cofactor of glutathione transferase enzyme and other selenoproteins.

4.3. Phytochemicals

Many medicinal plants contain phytochemicals like phenolic and polyphenolic compounds such as flavonoids, isoflavones, flavones, anthocyanins, coumarins, lignans, catechin, isocatechin, gallic acid, and esculatin that possess antioxidant activities [77]. These phytochemicals are present in many plants and herbs like grapes, berry crops, tea, herbs, nutmeg, and tea. Many medicinal plants contain phenolics like gallic acids and other active constituents. *Terminalia chebula, T. bellerica, T. muelleri, Phyllanthus emblica, Hemidesmus indicus, Cichorium Intybus, Withania somnifera, Ocimum sanctum, Mangifera indica,* and *Punica granatum* are known to have potential antioxidant activities [78].

5. Antioxidant therapy

Recent human studies exploring the efficiency of antioxidants in prevention and treatment of various diseases are reviewed (Table 4).

Disease studied	Antioxidant used	Reference	Reference no.
Mortality: Primary/Secondary Prevention	Beta-carotene, vitamin A, vitamin C, vitamin E, and selenium	Bjelakovic et al.	[79]
Fatty liver disease	Vitamin A, carotenoids, vitamin C, vitamin E and selenium	Lirussi et al.	[80]
Amyotrophic Lateral Sclerosis (SLA)	Vitamin E 500 mg twice daily	Orrell et al.	[81]
Multiple Sclerosis	Omega-6 fatty acids (11-23 g/day linoleic acid)	Farinotti et al.	[82]
Alcoholic Liver Disease	S-adenosyl-L-methionine	Rambaldi et al.	[83]
Oncology Treatments	Selenium	Dennert et al.	[84]
Eye Related Macular Disease	Beta-carotene and alpha-tocopherol.	Evans et al.	[85]
Pregnancy and Pre-eclampsia	Vitamin C and vitamin E supplements	Poston et al.	[86]
Cardiovascular Risk Profile	Dietary antioxidants	EJ Brunner et al.	[87]
Neonatal Growth Under Parenteral Nutrition (PN)	Cysteine, cystine or its precursor N-acetylcysteine	Soghier et al.	[88]
Melatonin and Cognitive Impairment or dementia	Melatonin	Jansen et al.	[89]
Alzheimer Disease	Vitamins C or E	Gray et al.	[90]
Parkinson Disease	Tocopherol, CoQ10, and glutathione.	Weber et al.	[91]
. Cancer	Lipid-soluble antioxidant vitamins,	Kirsh et al.	[92]
Asthma	Vitamin C, manganese etc.	Patel et al.	[93]
Cardiovascular Diseases	Vitamins C and E	Berhendt et al.	[94]
Ischemia-Reperfusion Injury	Vitamin C.	Pleiner et al	[95]
Chronic Obstructive Pulmonary Disease (COPD)	Polyphenol-rich pomegranate juice (PJ)	Cerda et al.	[96]
Pancreatitis	Selenium, L-methionine, and vitamins C and E,	Kirk et al.	[97]
Rheumatoid Arthritis	Vitamins A, C, E or selenium or their combination	Canter et al.	[98]
Kidney Diseases	Vitamin E	Ong-ajyooth et al.	[99]
Liver Diseases	Antioxidant therapy	Gabbay et al.	[100]
Diabetes Type I and II	Probucol and statins	Endo et al.	[101]

Table 4. The efficiency of antioxidants in prevention and treatment of various diseases.

Many of these studies, either due to the small patient sample size, with uncontrolled admissions and treatment criteria, or due to relevant bias of the clinical studies failed to give precise information on effectiveness and practical advantage in taking antioxidants.

Antioxidants therapies have been in progress these days. Edaravone (for ischemic stroke), N-acetylcysteine (for acetaminophen toxicity), alfa-lipoic acid (for diabetic neuropathy), and

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some flavonoids (for chronic venous insufficiency) as well as baicalein and catechins (for osteoarthritis) have clinical importance. The evidence from human epidemiological studies about the beneficial effects of dietary antioxidants and preclinical in vitro and animal data are compelling. Attention needs to be drawn on focusing more on disease-specific, target-directed, highly bioavailable antioxidants [102]. In the recent years, due to the increase in the consumption of food and medicinal products, we are exposed to the adverse effects of various compounds noticed in the above products. For example, in our animal experimental studies, we have determined induction of oxidative stress induced by the compound cinnamaldehyde, a food flavor and also an anticancer drug [103–105]. As a therapeutic measure, addition of vegetables and fruits, the great sources of vitamins or antioxidants, in our routine diet might protect our health from toxic effects of food chemicals or drugs to a certain extent [106].

5.1. Oxidative stress test

In this advanced materialistic life, monitoring the levels of free radicals and oxidative stress is important in case of clinical practice. FORD (Free Oxygen Radicals Defense) is an easy, cheap, and reliable diagnostic device to monitor oxidative stress [19, 107]. It discriminates the high risk of oxidative damage on sick or healthy individuals, monitoring with precise laboratory parameters in the clinical situation at the baseline and in the follow-up of a medical prescription.

FORD (Free Oxygen Radicals Defense) is a colorimetric test based on the influence of antioxidants present in plasma to reduce the activity of free radicals. The principle of the assay is that at an acidic pH (5.2) and in the presence of a suitable oxidant solution (FeCl3), 4-amino-n, n- diethylaniline, the FORD chromogen, can form a stable and colored radical cation. Antioxidant molecules (AOH) present in the sample which are able to transfer a hydrogen atom to the FORD chromogen radical cation, reduce it, quenching the color and producing a discoloration of the solution which is proportional to their concentration in the sample. This instrument will be helpful in understanding the problem of the individual bioavailability of each antioxidant molecule which can be monitored during the administration, with a pre-post measure of the oxidative balance. In order to achieve the evidence of the oxidative background related to the outcome of specific symptoms and diseases, epidemiological studies can be encouraged, and the role of nutrition and targeted antioxidant therapy can be better defined.

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