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Nonenzymatic Exogenous and Endogenous Antioxidants

Ziad Moussa, Zaher M.A. Judeh and Saleh A. Ahmed

Abstract

Nonenzymatic exogenous and endogenous antioxidants play an important role in human health and act as preservatives for cosmetics, pharmaceuticals, and food products. This chapter will discuss the chemical structure and mechanism of action of the most important nonenzymatic small exogenous and endogenous organic molecules that act as antioxidants. The chapter will focus on the structural features, functional groups, properties, biosynthetic origin, and mechanism of action of such antioxidants. It also covers damages that free radicals create and the mechanisms by which they are neutralized by the various antioxidants. The scope of this chapter will be limited to nonenzymatic exogenous and endogenous antioxidants since enzymatic antioxidants have been discussed extensively in several reviews.

Keywords: antioxidants, nonenzymatic, endogenous, exogenous, low-molecular weight antioxidants, mechanism

1. Introduction

Antioxidants are structurally diverse group of small organic molecules and large enzymes that comprise complex systems of overlapping activities working synergistically to enhance cellular defense and to combat oxidative stress resulting from various reactive oxygen species (ROS) and reactive nitrogen species (RNS) [1]. The former substances are byproducts of metabolism and are ironically produced from oxygen, an indispensable element for life. Many of these reactive species are free radicals possessing one or more unpaired electrons and as such rendered highly reactive. The reactive species generated in cells include hydrogen peroxide (H_2O_2), hypochlorous acid (HClO), the hydroxyl radical ($\cdot OH$), the superoxide anion radical (O_2^-), the nitric oxide radical ($NO\cdot$), and the lipid peroxyl radical ($LOO\cdot$) [2, 3]. The term antioxidants may refer to either industrial chemicals that may be added to products to combat oxidation or to natural products that are found in foods and tissue. While the former act as preservatives for cosmetics, pharmaceuticals, and food products, the latter play an important role in human health as well. There are many reactive oxygen species conducting unwanted oxidation reactions in a variety of cell and tissue sites [4]. Likewise, each antioxidant targets specific types of ROS and provides protection in distinct environments. Antioxidants reduce reactive oxygen species which otherwise participate in oxidation reactions that can generate free radicals and cause damage to cellular components such as DNA, proteins, carbohydrates, and lipids [4]. It is noted, however, that reactive oxygen species mediate certain cellular functions like redox signaling and gene expression as well as defend against pathogens [5, 6]. Thus, the role of antioxidant systems is not to

eliminate oxidants completely, but instead maintain them at an optimum level. Despite the presence of the antioxidant defense mechanism to counteract oxidative stress, damage due to oxidation has a cumulative effect and has been implicated in several chronic conditions and disease states such as cancer [7], cardiovascular disease [8], and neurodegenerative disorders [9]. Antioxidant compounds and antioxidant enzyme systems display synergistic and interdependent effects on one another. Antioxidants found in nature can be classified in a number of ways. Based on their activity, they can be classified as enzymatic and nonenzymatic antioxidants (phytochemicals and vitamins). While antioxidant enzymes like superoxide dismutase (SOD) [10], glutathione peroxidase (GPx) [11], glutathione reductase (GSR) [12], peroxiredoxin I-IV and catalases (CAT) [13] are macromolecules, the vast majority of the remaining natural antioxidants classified as phytochemicals and vitamins are relatively smaller organic molecules with low molecular weights [14, 15]. Antioxidants have also been categorized as water-soluble or fat-soluble molecules.

This chapter will highlight the chemical structures and mechanism of action of important nonenzymatic small exogenous (natural) and endogenous (synthetic/physiological) organic molecules that act as antioxidants in plants and animals. The antioxidants described in this chapter are among the most important, although certainly they are not the only ones known. Special focus on the structural features, functional groups, properties, biosynthetic origin, and mechanism of action will be undertaken with special coverage of damages that free radicals create and the mechanisms by which they are neutralized by the various antioxidant molecules.

2. Enzymatic versus nonenzymatic antioxidants

Based on their activity, antioxidants are classified as enzymatic and nonenzymatic antioxidants. While enzymatic antioxidants [10–13] function by converting oxidized metabolic products in a multi-step process to hydrogen peroxide (H_2O_2) and then to water using cofactors such as iron, zinc, copper, and manganese, nonenzymatic antioxidants intercept and terminate free radical chain reactions. Examples of natural nonenzymatic antioxidants are vitamin E, A, C, flavonoids, carotenoids, glutathione, plant polyphenols, uric acid, theaflavin, allyl sulfides, curcumin, melatonin, bilirubin, and polyamines [14, 15]. Some of these antioxidants are water-soluble and predominantly found in the cytosol or cytoplasmic matrix, while others are liposoluble and are present in cell membranes. The enzymatic antioxidants and their mechanism of action have been discussed extensively in several review articles [16–18]. The scope of this chapter will be limited to nonenzymatic exogenous and endogenous antioxidants.

3. Generation of free radicals in living organisms

The production of ROS in biological systems occurs during oxygen metabolism and plays an important role in homeostasis and cell signaling [5]. However, under conditions of environmental stress, the concentration of ROS can increase significantly and inflict damage on cell structures. The generation of ROS begins with the reduction of molecular oxygen with NADPH to produce the superoxide anion radical ($O_2.-$), a precursor to most remaining reactive oxygen and nitrogen species (**Figure 1**). Subsequent dismutation of two molecules of the superoxide anion catalyzed by the enzyme superoxide dismutase (SOD) generates oxygen and

hydrogen peroxide. The latter in turn may undergo partial reduction to hydroxyl radical through the Fenton reaction or alternatively *via* the Haber-Weiss process [19]. While hydrogen peroxide is more damaging to DNA, the hydroxyl radical is highly reactive and turns biomolecules into free radicals, thus perpetuating a free radical chain reaction. Hydrogen peroxide may also be converted to the potent oxidant hypochlorous acid in the presence of the chloride ion, an omnipresent species. This transformation is catalyzed by the enzyme myeloperoxidase (MPO). Reaction of HOCl with H_2O_2 regenerates chloride ion and produces singlet oxygen as yet another ROS. On the other hand, RNS such as nitric oxide (NO·) are produced by the enzyme nitric oxide synthase (NOS) starting from the precursor L-arginine [20]. Nitric oxide functions as a superoxide quencher forming peroxynitrite (ONOO–), a strong oxidant that reacts indiscriminately with biological targets. Further, it may disintegrate into a pair of hydroxyl and nitric dioxide radicals and cause damage through such species (**Figure 1**).
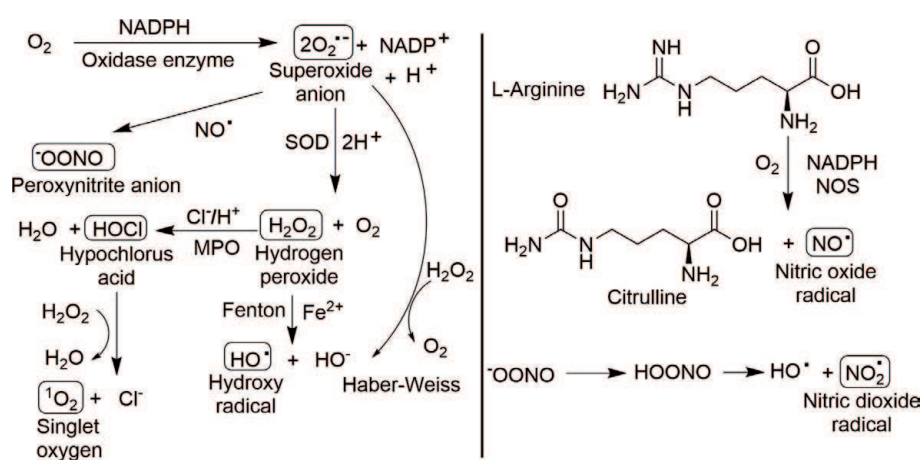
Figure 1.
*Generation of ROS and RNS in living species.*

4. Damaging chemical reactions of free radicals in living organisms

4.1 Free radical damage to the deoxyribose moiety of DNA

The highly reactive hydroxyl radical (·OH) reacts with the sugar moiety of DNA causing structural modification and strand breaks by a variety of mechanisms [21]. The OH radical reacts with the 2′-deoxyribose sugar residue in DNA by abstracting H· from all its carbon atoms forming five carbon-centered radicals. The H4′ and H5′ atoms are more accessible to H· abstraction by the OH radical than the H1′, H2′, and H3′. The C4′ C-centered radical appears to be the major radical generated by H· abstraction from 2′-deoxyribose in DNA [22]. These radicals undergo further reactions, producing a variety of 2′-deoxyribose oxidative adducts. While some products detach from DNA, others remain tethered as end groups of fragmented DNA strands [22]. In the absence of oxygen and as depicted in **Figure 2**, one of the byproducts formed from C4′-radical of 2′-deoxyribose as an end group of a severed DNA strand is 2,5-dideoxypentose-4-ulose. The product is formed by heterolytic cleavage of the phosphate group at C5′ to give a C4′/C5′-radical cation which in turn undergoes hydration and subsequent one-electron reduction and base elimination (**Figure 2**). Other products formed from the C4′ radical include 2-deoxypentose-4-ulose and 2,3-dideoxypentose-4-ulose. However, in the presence of oxygen, rapid addition of O_2 to the C4′-radical forms a peroxyl radical which undergoes a series of fragmentation reactions yielding 3′-phosphoglycolate as an end group [23].

Oxidation of the C1′, C2′, and C5′ radicals yields products such as 2-deoxypentonic acid lactone, erythrose, 2-deoxytetradialdose, and 5′ aldehyde [22].
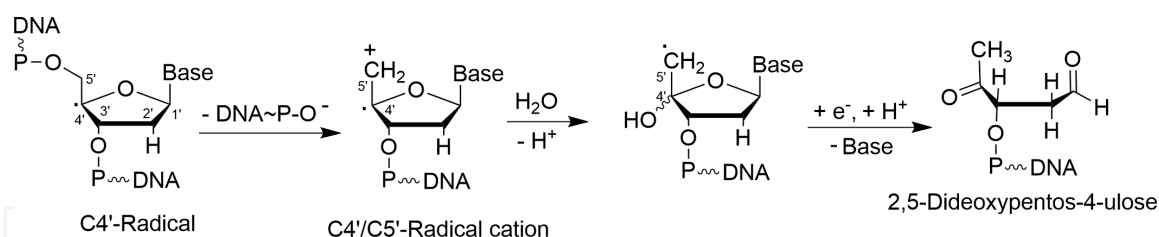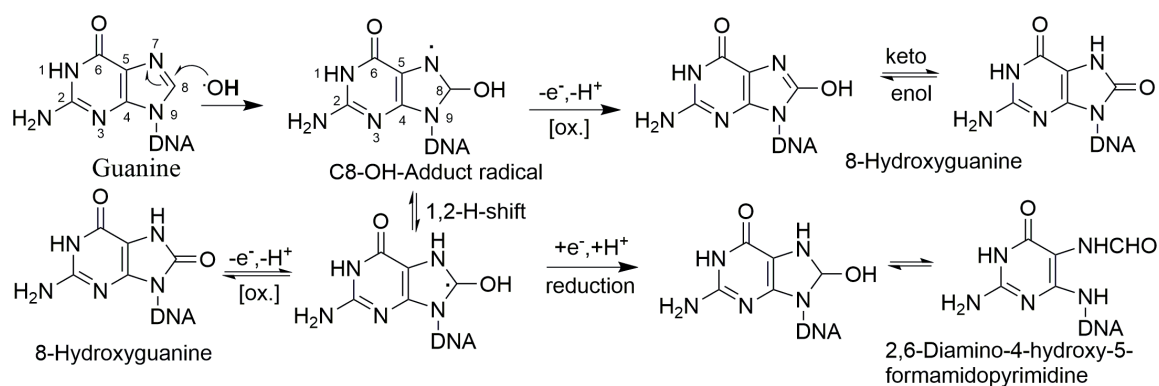

Figure 2.
Mechanism of product formation from reactions of the C4′-radical of 2′-deoxyribose, leading to 2,5-dideoxypentose-4-ulose as an end group of a broken DNA strand.

4.2 Free radical damage to DNA bases

Besides reacting with the sugar moiety of DNA, the highly reactive hydroxyl radical (·OH) reacts with the heterocyclic bases guanine (**Figure 3**), thymine, and cytosine, causing free radical-induced DNA damage by several different pathways. Guanine, however, possesses the lowest reduction potential (1.29 V) among the four DNA bases, rendering the motif the best electron donor and prone to preferential oxidization [24]. The hydroxyl radical reacts with the C4-, C5-, and C8-positions of guanine and to a lesser extent with the C2-position, generating a plethora of products. Interestingly, the HO-adduct radicals generated from the addition reactions of HO· may exhibit reducing or oxidizing properties (redox ambivalence), yielding the relevant products accordingly. Hence, while the C5-OH– and the C8-OH–adduct radicals are reducing, the C4-OH–adduct radical is predominantly oxidizing. The last two adduct radicals form in yields of 17% and 65–70%, respectively, whereas the yield of the C5-OH– adduct radical is lower than 10% [25]. Although formed in relatively lower yields, the C8-OH–adduct radical produces the major byproducts of guanine reactions (**Figure 3**). Thus, as shown in **Figure 3** and following reaction of the hydroxyl radical with the C-8 position of guanine, one-electron oxidation of the resulting C8-OH–adduct radical yields the enol form of 8-hydroxyguanine which undergoes tautomerization to generate the predominant keto form [21]. The latter may also form via a pathway involving 1,2-hydride-shift and subsequent oxidation of the C8-OH–adduct radical. The 1,2-hydride-shift radical product may also undergo single electron reduction, followed by ring opening reaction to form 2,6-diamino-4-hydroxy-5- formamidopyrimidine. The preceding radical damage to DNA has been directly correlated to several disease states such as genetic mutation, atherosclerosis, Alzheimer's disease, and the aging process [26, 27].
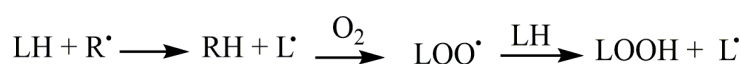

Figure 3.
Oxidative and reductive product formation from reactions of the C8-OH–adduct radical of guanine.

4.3 Free radical damage to polyunsaturated fatty acid groups of cell membranes

While free radicals react with all major classes of biomolecules, peroxidation of the polyunsaturated fatty acid groups (PUFA) of cell membranes comprises the main target of oxidative damage, resulting in a destructive self-propagating chain reaction. The general mechanism of PUFA peroxidation involves abstraction of hydrogen from a lipid molecule (LH) by an initiator (R·) to generate a carbon-based free radical (L·) which reacts rapidly with molecular oxygen to form the peroxyl radical (LOO·) known to propagate the chain reaction (**Figure 4**). As such, the peroxyl radical reacts with PUFA moieties, producing lipid hydroperoxides (LOOH) and perpetuating the chain reaction. The hydroperoxides can further dissociate to dangerous radical species like bioactive aldehydes which inflict damage on other cellular components. Lipid hydroperoxidation has been linked to a number of physiological conditions and tissue injuries [28].

$$LH + R^{\bullet} \longrightarrow RH + L^{\bullet} \xrightarrow{O_2} LOO^{\bullet} \xrightarrow{LH} LOOH + L^{\bullet}$$

Figure 4.
*General process of lipid peroxidation.*

5. Regulation of free radicals with nonenzymatic small natural exogenous antioxidants

5.1 Vitamins

5.1.1 Vitamin E

Vitamin E is a collection of optically active methylated phenolic compounds comprising four tocopherols and four tocotrienols [29] where α-tocopherol is the most common and biologically active species (**Figure 5**) [30]. The structures feature two primary parts: a densely substituted polar chromanol aromatic ring and a lipophilic long polyprenyl side chain. The main chemical structural difference between different forms of Vitamin E is that tocotrienols feature unsaturated isoprenoid hydrocarbon side chains with three carbon-carbon double bonds versus saturated isoprenoid side chains for tocopherols. Within each group, the vitamers are differentiated by the number and positions of the methyls in the chromate ring. The polyprenyl precursor for the biosynthesis of tocopherols and tocotrienols is phytyl pyrophosphate (PPP) and geranylgeranyl pyrophosphate (GGPP), respectively [31]. Vitamin E is biosynthesized though the shikimate pathway, and while α-tocopherol and α-tocotrienol are considered structurally unique, the remaining compounds in each class are constitutional isomers. The presence of three stereogenic centers (position C_2 of the chromate ring, position C_4 and C_8 of the phytyl side chain) produces 8 different stereoisomers (four pairs of enantiomers) depending on the position and orientation of the groups in each of the chiral centers. Since the discovery of vitamin E in 1920, it has been shown to be the most powerful membrane-bound antioxidant utilized by cells to scavenge reactive nitrogen and oxygen species with consequent disruption of oxidative damage to cell membrane phospholipids during cellular lipid peroxidation of the polyunsaturated fatty acids (PFA) and low-density lipoprotein (LDL) [32]. The antioxidant is liposoluble and localized to cell membranes. Vitamin E functions by reducing lipid peroxyl radicals (LOO·) by transferring the phenolic hydrogen atom of the chroman ring (**Figure 5**), resulting in a relatively stable and unreactive resonance-stabilized tocopheroxyl radical which is unable to trigger

further lipid peroxidation itself. The α-tocopherol radical can be reduced back to the original active α-tocopherol form by ascorbic acid or coenzyme Q10 [33, 34]. Alternatively, it may quench a second peroxyl radical where the resulting tocopheryl peroxide eliminates a peroxide leaving group, forms a hemiketal after reacting with water, and lastly hydrolyses to the tocopherolquinone. This is an essential foundation and benchmark of a good antioxidant. The synergistic antioxidation interactions between vitamin E and the ascorbate ion of vitamin C position the former at the forefront of the anti-radical defense system. Vitamin E is exogenous and hence is essential and must be obtained through diet in small amounts since the organism cannot synthesize it. Its biosynthesis is restricted to plants, photosynthetic algae, and certain cyanobacteria. Although vitamin A deficiency is rare, the most frequent manifestations of its lack comprise a number of disorders and disease states which include encephalomalacia, exudative diathesis, muscular dystrophy, and ceroid pigmentation. α-Tocopherol exhibits the highest bioactivity (100%), with the relative activities of β-, γ-, and δ-tocopherols being 50, 10, and 3%, respectively [35].
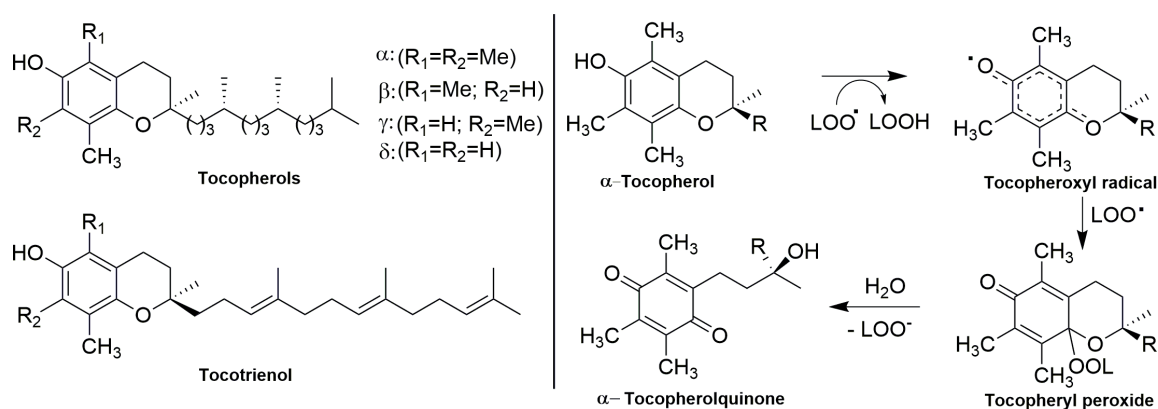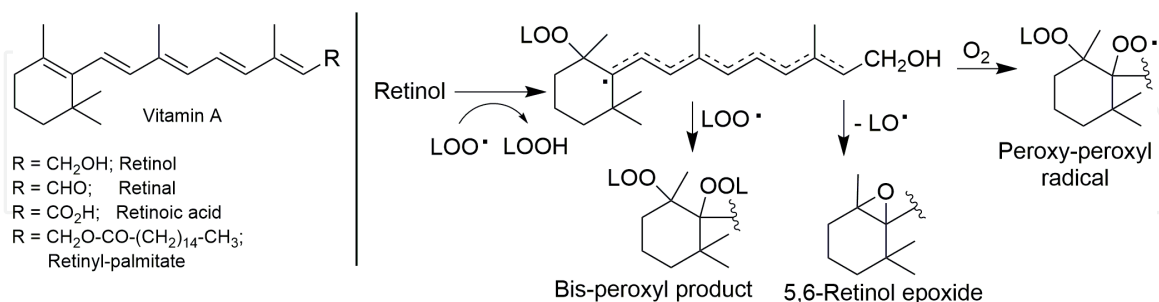

Figure 5.
Chemical structures of the tocopherols and tocotrienols that comprise vitamin E and termination of lipid peroxidation with α-tocopherol.

5.1.2 Vitamin A

Vitamin A, just like vitamin E, is a term that designates a family of unsaturated liposoluble organic compounds that include retinol, retinal, retinoic acid, and retinyl palmitate, and many provitamin A carotenoids such as beta-carotene (**Figure 6**). All forms share a beta-ionone ring to which an isoprenoid tether known as retinyl group is attached. It is noteworthy that both features are essential for vitamin A activity. The common chemical structure is a diterpene ($C_{20}H_{32}$) where the various molecular forms differ by the terminal side chain functional group. Thus, retinol contains a hydroxyl group, retinal contains an aldehyde function, retinoic acid has a terminal carboxylic acid group, and retinyl palmitate bears an ester moiety. The discovery of the antioxidant activity of vitamin A dates back to 1932 when Schmitt and Monaghan reported that vitamin A prevents lipid rancidity [36]. Several reviews outlining the antioxidant role and metabolic functions of vitamin A have appeared in the literature [37, 38]. Besides eliminating free radicals, it plays a major role in maintaining good vision. The aldehyde form of vitamin E is required by the retina to form the light-absorbing molecule rhodopsin necessary for both color and scotopic vision [39]. On the other hand, the fully irreversibly oxidized form of retinol functions in a very different way as a growth factor for epithelial and other types of cells [38]. As an antioxidant, vitamin A scavenges lipid peroxyl radicals (LOO·) according to the mechanism shown in **Figure 6**. Thus, by trapping the peroxyl radical through an addition reaction to the

beta-ionone ring of retinol, the resultant tertiary and highly conjugated trans-retinol carbon radical intermediate is relatively stable and under normal conditions is not reactive enough to induce further lipid peroxidation itself. However, the intermediate may continue reacting with lipid peroxyl radicals or molecular oxygen to produce a bis-peroxyl adduct or retinol-derived peroxyl radical, respectively. Alternatively, it may eliminate LO radical and oxidizes to 5,6-retinol epoxide [15].
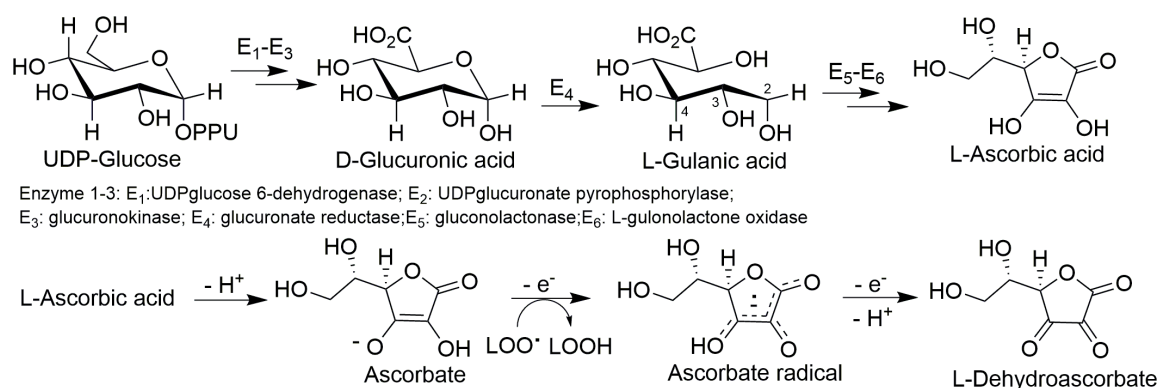

Figure 6.
Chemical structure of vitamin A and termination of lipid peroxidation with retinol.

5.1.3 Vitamin C

Vitamin C (L-ascorbic acid) is an optically-active hydrosoluble free radical scavenger that bears a highly acidic hydroxyl group (pKa = 4.2) known to be completely ionized at neutral pH [35, 40]. Thus, the acidic vitamin readily loses a proton from the 3-hydroxyl group affording a resonance-stabilized ascorbate anion (AscH⁻) (**Figure 7**). The unusual acidity of the alcohol is related to the presence of two conjugated double bonds which stabilize the deprotonated monoanionic conjugate base. Furthermore, these same electronic factors impart stability to the radical form of vitamin C when it undergoes one electron oxidation by lipid radicals to generate the ascorbate radical (**Figure 7**), a much less reactive species than most other free radicals. As such, vitamin C is able to assume the role of a free-radical scavenger. The low standard 1-electron reduction potential (282 mV) renders vitamin C an excellent electron donor. As well, at low ascorbate concentrations, it may function as a pro-oxidant reducing agent and is able to reduce redox-active copper and iron metals. Vitamin C is therefore required as a cofactor for a number of metabolic processes that mediate essential biological functions in all animals and plants [41]. The structure features a chiral 3,4-dihydroxyfuran-2(5*H*)-one ring and a 1,2-dihydroxyethyl tether containing another stereogenic center. The 6-carbon ketolactone is structurally related to glucose. Although four stereoisomers are expected depending on the position of the substituents around the stereogenic centers, only the L-enantiomer exhibits antioxidant capacity in biological systems, both in vitro and in vivo. While vitamin C is biosynthesized by nearly all animals, humans comprise a notable exception. Consequently, it is an essential nutrient and must be obtained through dietary means. In biological species, the vitamin exists in the protonated form at low pH, but in media with pH above 5, it is found in the dissociated ascorbate form [42]. This species is a 2-electron donor and gets oxidized to a molecule of dehydroascorbate (DHA) which does not have any antioxidant capacity. However, regeneration of the ascorbate from DHA is possible by the addition of two electrons and has been proposed to be carried out by oxidoreductase [43]. In animals, the biosynthesis of ascorbic acid is carried out by several enzymes in the liver from glucose [42], by a synthetic route which initially involves oxidation to D-glucuronic acid via uridine diphosphate (UDP) derivatives. Subsequent reduction of the open-chain aldehyde form of D-glucuronic acid to the primary

alcohol (L-gulonic acid), lactone formation between the carboxyl and 4-hydroxyl group, oxidation of the secondary hydroxyl function to a carbonyl, and subsequent enolization result in L-ascorbic acid. The latter, specifically in the ascorbate form, acts as a reducing agent, donating electrons to lipid radicals in order to terminate the lipid peroxidation chain reaction (**Figure 7**). Another main function of Vitamin C as an antioxidant is to regenerate vitamin E (HO-tocopherol) from its oxidized form (.O-tocopherol) back to its active state by reducing vitamin E radicals formed when vitamin E scavenges oxygen radicals. The recycling of vitamin E is carried out in cell membranes in conjunction with glutathione (GSH) or other sacrificial reductants [33, 34]. Likewise, vitamin C acts as an antioxidant and reducing agent by donating electrons to various enzymatic and nonenzymatic reactions. It reduces the transition metal ions of several biosynthetic enzymes, thus preventing biological oxidation of macromolecules. In plants, vitamin C is a substrate for the enzyme ascorbate peroxidase which catalyzes the reduction of toxic hydrogen peroxide (H_2O_2) to water (H_2O) [44]. Currently, this vitamin is the most widely employed vitamin in drugs, premedication, and dietary supplements worldwide.

Figure 7.
Biosynthesis, chemical structure, and reduction mechanism of ascorbic acid.

5.2 Flavonoids

Flavonoids are exogenous antioxidants displaying rich structural diversity and are ubiquitous in plants and certain photosynthetic organisms. More than 8000 of these benzo-γ-pyran derivatives have been identified and characterized [45, 46]. The general structure features a C6-C3-C6 15-carbon flavone skeleton, which comprises two phenyl rings (A and B) linked by a heterocyclic ring (C) (**Figure 8**). Flavonoids have been classified into flavones, flavanones, flavanols, flavonols, and anthocyanins. While flavones have a double bond between C2 and C3, flavanones have a saturated C2–C3 bond. Compared to flavones, the corresponding flavonols have an additional hydroxyl group at the C3 position while flavonols are C2-C3 saturated analogs of flavonols. Flavonoid groups are differentiated based on the number of hydroxyl and other substituents on the phenyl rings [47].
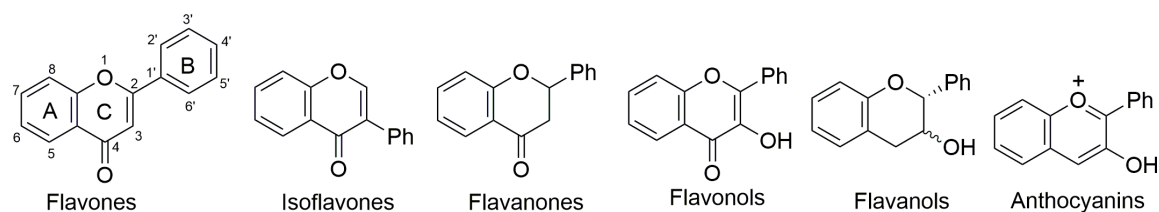
Figure 8.
*General skeletal structure of the various flavonoid classes.*

Quercetin (3,5,7,3′,4′–pentahydroxyflavone) (**Figure 9**) is the most ubiquitous polyphenolic flavonoid known to prevent oxidative damage to DNA oligonucleotides brought about by H_2O_2, $HO·$, and $O_2^{·-}$. On the other hand, anthocyanidin is a strong inhibitor of lipid oxidations. Thus, as shown in **Figure 9**, the antioxidant mechanism of lipid peroxyl radicals scavenging capability of anthocyanidin is based on its hydrogen radical donation ability from the *p*-hydroxyl group of ring B to generate a resonance-stabilized anthocyanidin radical incapable of participating in other radical reactions. In addition, the effectiveness of anthocyanidin in inhibiting lipid peroxidation has been correlated to their metal-ion chelating power [48, 49]. In particular, the ortho-dihydroxy groups in the B-ring confer upon this class of compounds antiperoxidative properties [50]. However, phenolic compounds can also act as prooxidants if present in high concentrations with metal ions and high pH [47].
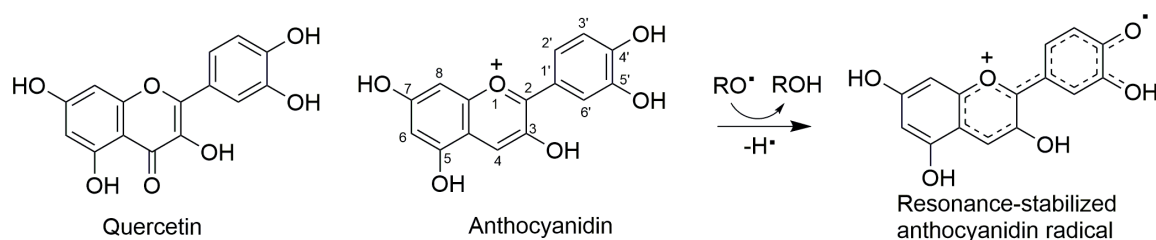
Figure 9.
Structure of quercetin and mechanism of radical scavenging activity of anthocyanidin.

5.3 Carotenoids

Carotenoids, also known as tetraterpenoids, are a group of phytonutrients produced by plants and algae, as well as some bacteria and fungi [51]. The long unsaturated hydrocarbon alkyl chain renders carotenoids highly liposoluble. Hence, they play a key role in the protection of lipoproteins and cellular membranes from lipid peroxidation and exhibit particularly efficient scavenging capacity against peroxyl radicals as compared to any other ROS and they are known to be the most common lipid-soluble antioxidants [52, 53]. Over 1100 carotenoids have been identified and classified primarily into two groups: the oxygen-containing xanthophylls and those that are purely hydrocarbons, carotenes (**Figure 10**). Biosynthetically, all carotenoids are tetraterpenes comprising 40 carbon atoms which are produced from eight isoprene units. The structural backbone consists of isoprenoid units biosynthesized either by head-to-tail or by tail to-tail process. The basic building blocks of carotenoids are isopentyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) which produce the major carotenoid precursor geranylgeranyl pyrophosphate (GGPP) [54]. GGPP undergoes several different reactions within the carotenoid biosynthetic pathway to afford carotenes or xanthophylls. Carotenoids reduce the peroxyl radicals to form a resonance-stabilized carbon-centered radical product. Lycopene and carotene are the most prominent and potent carotenoid antioxidants. The former is notably a strong singlet oxygen quencher due to the high number of conjugated *trans*-configuration double bonds present in the structure. In general, the extended conjugated system in carotenoids is strongly-reducing, facilitating abstraction of hydrogen atoms from the allylic positions to this conjugation, as well allowing free-radical addition reactions to proceed with ease. Lycopene for instance reduces peroxyl radicals through electron transfer to afford an unreactive resonance stabilized carbon-centered radical (**Figure 10**).
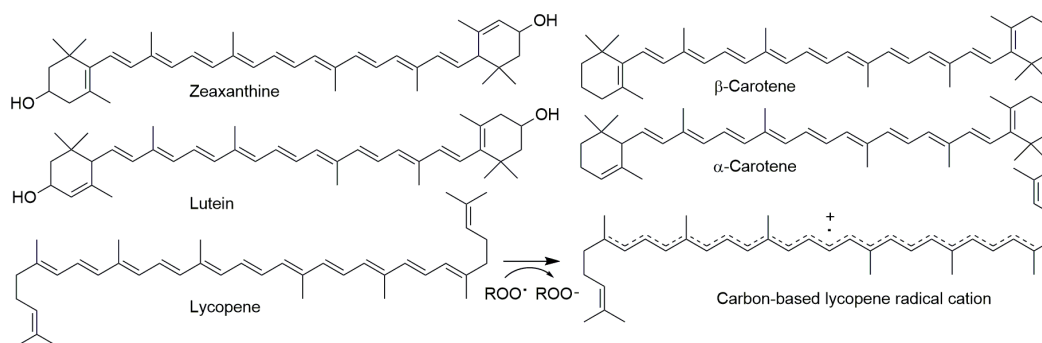
Figure 10.
Structure and radical scavenging mechanism of some prominent xanthophyll and carotene carotenoids.

5.4 Hydroxycinnamic acids

Hydroxycinnamic acids (hydroxycinnamates) are a class of phenylpropanoids possessing a C_6–C_3 skeleton. These compounds are hydroxy derivatives of cinnamic acid which is their common biosynthetic precursor. Mechanistically, bimolecular elimination of ammonia from the side chain of L-Phenylalanine generates *trans*-*(E)*-cinnamic acid (**Figure 11**). A subsequent cytochrome P-450-dependent direct hydroxylation reaction of cinnamic acid mediated by cinnamate 4-hydroxylase enzyme (E2) produces the first member of this class, *p*-coumaric acid. The substitution patterns of the remaining cinnamic acids are constructed sequentially by further hydroxylation and methylation reactions, which is typical of shikimate pathway metabolites. Hence, direct hydroxylation of *p*-coumaric acid mediated by *p*-coumarate 3-hydroxylase enzyme (E3) generates caffeic acid. Subsequent methylation of the latter by caffeic acid *O*-methyltransferase (E4) produces ferulic acid. Hydroxylation of ferulic acid by ferulate-5-hydroxylase (E5), a cytochrome P450-dependent monooxygenase enzyme, followed by methylation with SAM produces the last member, sinapic acid. As chain-breaking antioxidants, hydroxycinnamic acids prevent oxidation of LDL, although in varying efficiencies, depending on their standard one-electron reduction potential, hydrogen or electron donating ability, and the capacity to delocalize and stabilize the resulting phenoxyl radical within their structural framework [55, 56]. The antioxidant activity of the derivatives is correlated with the methylation and hydoxylation substitution pattern of the benzene ring. Thus, the antioxidant efficiency of the hydroxycinnamate conjugates on human LDL oxidation has been found to increase in the order of *p*-coumaric acid, ferulic acid, sinapic acid, and caffeic acid [57]. The general mechanism of free radical scavenging by which these antioxidants act involves donation of a *p*-hydroxyl hydrogen atom to ROS and generation of resonance stabilized carbon-based radical. Additionally, the presence of *ortho*-dihydroxyl groups allows metal-ion chelation much like flavonoids and enhances their antioxidant capacity against lipid peroxidation.
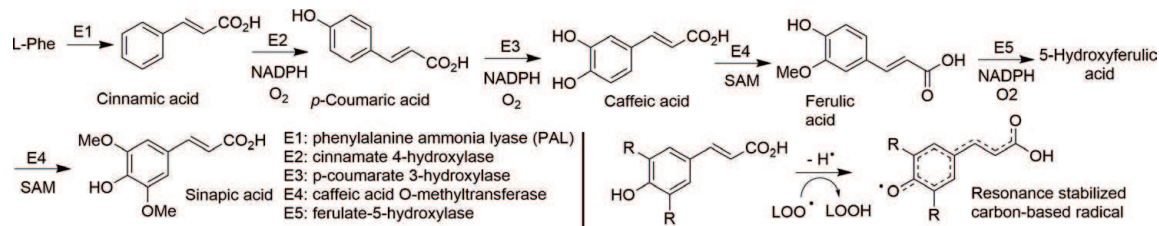
Figure 11.
Structures, biosynthesis, and free radical scavenging mechanism of hydroxycinnamic acids.

5.5 Other natural exogenous antioxidants (allyl sulfides & curcumin)

Allicin (diallyl thiosulfinate), a compound mainly found in garlic, and curcumin are biologically active compounds possessing antioxidative properties. The active form responsible for the antioxidant activity of allicin is 2-propene-sulfenic acid [58], formed via a cope elimination reaction of the former precursor (**Figure 12**) [59, 60]. The radical-scavenging mechanism of allicin involves H-atom abstraction by a peroxyl radical from the sulfenic acid residue [61, 62]. The bis-α,β-unsaturated β-diketone, curcumin, is a liposoluble free radical scavenger that displays remarkable chain breaking ability similar to that of vitamin E [63]. As shown in **Figure 11**, the methylene group of the β-diketone residue and the phenolic hydroxyl (OH) function are sites that can transfer electrons or H-atoms to quench free radicals and generate extended resonance-stabilized carbon- or oxygen-centered radicals. The phenoxyl radical, which has been credited for the antioxidative properties of curcumin [64], generates a quinone methide as it moves through the carbon framework and reacts with molecular oxygen to produce a peroxyl radical. Subsequent reduction of the peroxyl radical and dehydration of the resulting hydroperoxide, followed by rearrangement into a spiro-epoxide and hydrolysis, give the final bis-cyclopentadione product.
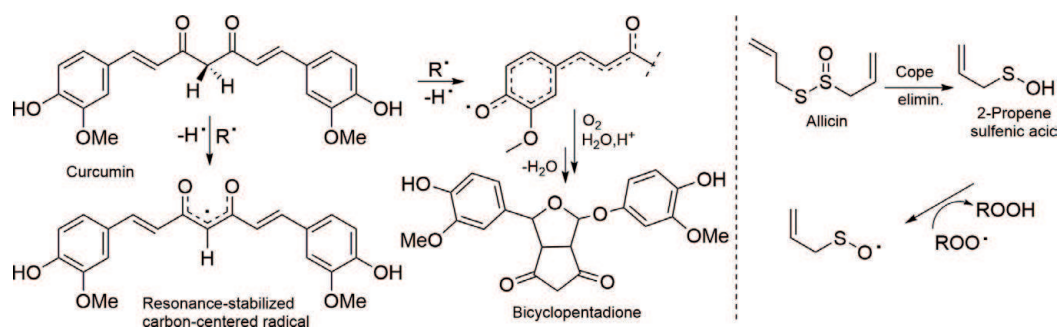
Figure 12.
Structures and radical-scavenging activities of curcumin and allicin.

6. Regulation of free radicals with nonenzymatic small endogenous (synthetic/physiological) antioxidants

6.1 Uric acid

Uric acid (UA) is a hydrophilic antioxidant generated during the metabolism of purine nucleotides and accounts nearly for 66% of the total oxygen scavenging activity in the blood serum. Mammals and humans are capable of producing UA, making it the most predominant aqueous antioxidant present in humans [65, 66] with an approximate blood level of 3.5–7.5 mg/dL. UA is a strong electron donor and a selective scavenger of peroxynitrite ($ONOO^-$), requiring the participation of ascorbic acid and thiols in its cycle for complete scavenging of such species [67, 68]. Peroxynitrite is formed by the reaction between nitric oxide ($\cdot NO$) and superoxide radical ($O_2^{\cdot-}$) (**Figure 1**) and has been implicated in many pathologies. Besides scavenging peroxynitrite, UA reacts with hydroxyl radicals, singlet oxygen, lipid peroxides, and hypochlorous acid, itself getting converted to innocuous chemical species like urea and allantoin. Furthermore, it has been implicated in scavenging carbonate ions ($CO_3^{\cdot-}$) and nitrogen dioxide ($NO_2^{\cdot}$) [69], and in complexation with copper and iron ions, resulting in the inhibition of deleterious free radical reactions like the Fenton and the Haber-Weiss reactions [65]. Some have suggested that UA

does not directly scavenge peroxynitrite since UA cannot compete for the reaction of peroxynitrite with CO_2. The antioxidant effect of uric acid may thus be related to the scavenging of the radicals $CO_3\cdot^-$ and $NO_2\cdot$ which are formed from the reaction of peroxynitrite with CO_2 [67]. As shown in **Figure 13**, UA displays a keto-enol tautomerism where the enol form predominantly exists as the monobasic urate anion at physiological pH [70]. The complete scavenging of peroxynitrite requires the presence of ascorbic acid and thiols whereby the urate anion is regenerated following reduction of the urate free radical with ascorbate ($AscH^-$). ESR studies on UA radical production by hydrogen atom abstraction provided evidence that the unpaired electron resides primarily on the five-membered ring of the purine structure. The radical was described as a delocalized π radical as the odd electron showed spin density on all four nitrogen atoms [71].
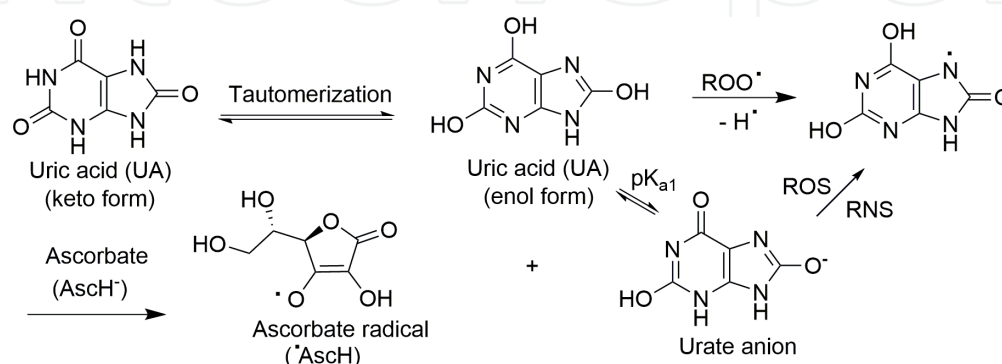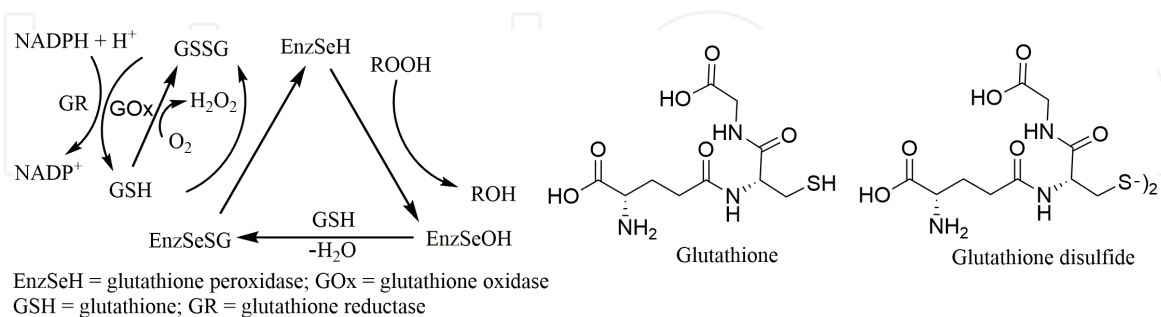

Figure 13.
Chemical structure and radical scavenging mechanism of uric acid.

6.2 Glutathione

Glutathione (GSH) is present in all plant and animal cells and comprises three amino acids: glycine, cysteine, and glutamic acid. It is mainly synthesized in the liver [72] and exists in several redox forms, among which the most predominant is the reduced glutathione. GSH is a hydrosoluble antioxidant present in high cellular concentrations (1–10 mM) in the nucleus, mitochondria, and cytoplasm. GSH is involved in several lines of defense against ROS. First, the thiol group confers GSH with the ability to protect other thiol functions in proteins against oxidative damage [73]. Thiol groups (-SH) are widespread and highly reactive chemical entities in cells. They complex with metal ions, participate in oxidation reactions by getting oxidized themselves to sulfonic acids, and form thiol radicals and disulfides [74]. As an antioxidant, GSH reduces ROS during the enzymatic and nonenzymatic reactions. It regenerates other oxidized antioxidants like vitamin C and vitamin E [75] and is involved in the repair of lipids damaged in peroxidation processes and in the maintenance of sulfhydryl moieties of proteins in the reduced form [76, 77]. GSH functions in conjunction with three groups of enzymes to maintain an intracellular reducing environment and combat excessive formation of harmful ROS. These enzymes are glutathione peroxidase (GSHPx), glutathione reductase (GR), and glutathione oxidase (GOx). Glutathione peroxidase (GSHPx) is a selenium-containing enzyme that mediates catalytic reduction of peroxides using GSH as a sacrificial reductant [78]. The enzyme is a tetramer featuring a selenocysteine residue in each subunit [11]. The oxidation-reduction chemistry of the selenol functional group found in each selenocysteine is responsible for the activity of GSHPx, and the catalytic cycle is displayed in **Figure 14** [79]. In the first step, the selenol functional group (EnzSeH) gets oxidized by the peroxide to the corresponding selenenic acid (EnzSeOH). The thiophilic acid reacts with GSH to generate a selenenyl sulfide
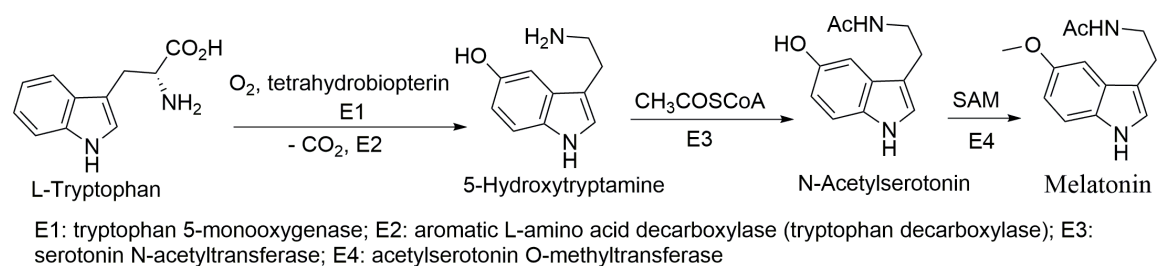
intermediate (EnzSeSG) which is highly reactive and is susceptible to nucleophilic displacement at the sulfur atom. Thus, attack by a second molecule of GSH at the sulfur atom regenerates the original selenol and eliminates oxidized glutathione (GSSG) as a byproduct. The latter is recycled back to GSH in an NADPH-dependent reduction process mediated by glutathione reductase (GR). GSH is also a substrate for glutathione oxidase (GOx) which catalyzes the reduction of oxygen to hydrogen peroxide and GSSG.

EnzSeH = glutathione peroxidase; GOx = glutathione oxidase
GSH = glutathione; GR = glutathione reductase

Figure 14.
Structure and role of glutathione (GSH) in the catalytic cycle of glutathione peroxidase (GSHPx), glutathione reductase (GR), and glutathione oxidase (GOx).

6.3 Melatonin

Since its discovery in 1993, melatonin's ability to reduce oxidative stress induced in all cells and organs by both oxygen- and nitrogen-based radicals has been reported in over one thousand publications. The structure of this endogenous antioxidant features an indoleamine and is biosynthesized in animals from L-tryptophan, an intermediate product of the shikimate pathway [80]. The biosynthetic process includes hydroxylation, decarboxylation, acetylation, and a methylation (**Figure 15**). Melatonin, which is produced mainly by the pineal gland in the brain [81], indirectly reduces free radical formation primarily through a process known as radical avoidance by stimulating the expression of endogenous antioxidant enzymes that metabolize reactive species and maintain redox homeostasis within cells [82]. These include superoxide dismutase (SOD), glutathione peroxidase (GSHPx), glutathione reductase, and catalase. In addition, it induces the synthesis of the antioxidant glutathione and inhibits certain enzymes that normally produce free radicals like nitric oxide synthase (generates $NO^{\bullet}$). Melatonin can also directly scavenge free radicals along with several of its metabolites that are formed during radical neutralization [83, 84]. For example, it is a very effective scavenger of the hydroxyl radical, singlet oxygen, peroxynitrite anion, and nitric oxide. Interestingly, melatonin has been shown to exhibit double the activity of vitamin E and ranks among as the most effective lipophilic antioxidant.

E1: tryptophan 5-monooxygenase; E2: aromatic L-amino acid decarboxylase (tryptophan decarboxylase); E3: serotonin N-acetyltransferase; E4: acetylserotonin O-methyltransferase

Figure 15.
Structure and biosynthesis of melatonin.

6.4 Bilirubin

Bilirubin (BIL) is an endogenous antioxidant produced from the enzymatic degradation of hemoglobin and other heme proteins (**Figure 16**). The process involves oxidative cleavage, catalyzed by the enzyme heme oxygenase, of one porphyrin exocyclic double bond of a heme residue of hemoglobin to generate biliverdin. Subsequent enzymatic reduction of biliverdin by biliverdin reductase yields bilirubin. This process is reversible and the oxidation of bilirubin by lipophilic ROS results in the formation of biliverdin. Notable structural features of bilirubin include an open chain of four connected pyrrole rings and a *Z,Z*-double bond geometry. In biological systems, bilirubin shows potent antioxidant properties [85, 86] especially against peroxyl radicals [87].
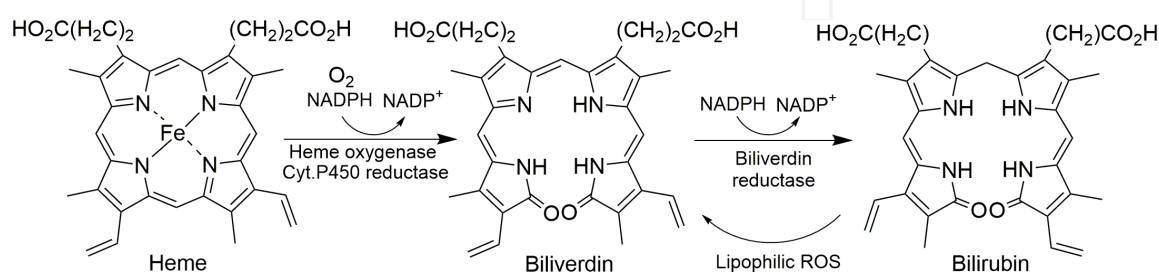
Figure 16.
Enzymatic degradation of hemoglobin heme to bilirubin.

6.5 Polyamines

Putrescine (H_2N-$(CH_2)_4$-NH_2), spermidine ($[H_2N$-$(CH_2)_3]_2$-NH), and spermine (H_2N-$(CH_2)_3$-NH-$(CH_2)_4$-NH-$(CH_2)_3$-NH_2) are biogenic unbranched polyamines (PAs) that exhibit antioxidant activities [88–90]. These amines are present in minute quantities in virtually all living species. While putrescine (1,4-diaminobutane) bears two primary amine groups at both terminal carbons, spermidine (triamine) and spermine (tetraamine) contain one and two additional secondary amine moieties, respectively. As antioxidants, PAs mediate protection of DNA against oxidative damage induced by hydrogen peroxide [90], scavenge free radicals [88], and reduce oxidative haemolysis of erythrocytes [90]. The amines also function as positive modulators of antioxidant genes under conditions of strong oxidative stress [88]. The protective effect of PAs is related to the stabilization of polyunsaturated phospholipids in cell membranes from peroxyl radicals, superoxides, and hydrogen peroxide [89]. In regard to their role in DNA protection against ROS, PAs are positively charged at physiological pH, enabling them to remain in proximity to negatively charged macromolecules, thus protecting them against oxidative damage [90]. Biosynthetically, the three polyamines are biosynthesized from L-ornithine, known to supply C_4N building block, and L-methionine [91]. In animals, L-ornithine undergoes a pyridoxal phosphate (PLP)-dependent decarboxylation to generate putrescine. Thereafter, aminopropylation of putrescine by the enzyme spermidine synthase and decarboxy-S-adenosyl methionine produces spermidine. Repetition of the same sequence of reactions in the presence of the enzyme spermine synthase generates spermine.

7. Conclusions

In addition to the oxidative damage that reactive oxygen and nitrogen species inflict on macromolecules, they also participate in damage caused by microbial

infections, tumor progression, and neurodegenerative diseases. In response to such oxidative injuries, tissues protect themselves by expressing genes encoding antioxidant enzymes and endogenous antioxidants to maintain oxidants at harmless levels. Oxidants themselves mediate certain cellular functions and cannot be eliminated completely. This fact emphasizes the significance of the antioxidant defense system in maintaining homeostasis and normal physiological processes, and in combating diseases and promoting immunity. The regulation of gene expression by employing oxidants and antioxidants represents a novel approach with promising therapeutic implications. Exogenous antioxidants are also critical for maintaining healthy living and longevity and must be obtained through dietary means. However, excessive dietary supplementation may disrupt the activation of the endogenous antioxidant defense system. Consequently, further research is required to fully elucidate the importance of antioxidants in the therapy of several human disease states and promotion of health span.

Acknowledgements

Abbreviations

AscH⁻	ascorbate
BIL	bilirubin
CAT	catalase
DHA	dehydroascorbate
DNA	deoxyribonucleic acid
DMAPP	dimethylallyl diphosphate
ESR	electron spin resonance
EnzSeSG	glutathione peroxidase selenenyl sulfide
EnzSeOH	glutathione peroxidase selenenic acid
EnzSeH	glutathione peroxidase selenol
GSH	glutathione
GSSG	glutathione disulfide
GPx or GSHPx	glutathione peroxidase
GR	glutathione reductase
GOx	glutathione oxidase
GSR	glutathione reductase
H_2O_2	hydrogen peroxide
HO·	hydroxyl radical
HClO	hypochlorous acid
IPP	isopentyl diphosphate
LDL	low-density lipoprotein
LOOH	lipid hydroperoxides
LOO·	lipid peroxyl radical
MPO	myeloperoxidase
NOS	nitric oxide synthase
NADPH	nicotinamide adenine dinucleotide phosphate
NO·	nitric oxide radical
ONOO–	peroxynitrite

$O_2\cdot^-$	superoxide anion radical
PLP	pyridoxal phosphate
PAs	polyamines
PUFA	polyunsaturated fatty acids
PPP	phytyl pyrophosphate
ROS	reactive oxygen species
RNS	reactive nitrogen species
SOD	superoxide dismutase
SAM	S-adenosyl methionine
UA	uric acid
UDP	uridine diphosphate

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