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Chemical and molecular mechanisms of antioxidants: Experimental approaches and model systems

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Abstract

Free radicals derived from oxygen, nitrogen and sulfur molecules in the biological system are highly active to react with other molecules due to their unpaired electrons. These radicals are important part of groups of molecules called reactive oxygen/nitrogen species (ROS/RNS), which are produced during cellular metabolism and functional activities and have important roles in cell signaling, apoptosis, gene expression and ion transportation. However, excessive ROS attack bases in nucleic acids, amino acid side chains in proteins and double bonds in unsaturated fatty acids, and cause oxidative stress, which can damage DNA, RNA, proteins and lipids resulting in an increased risk for cardiovascular disease, cancer, autism, and other diseases. Intracellular antioxidant enzymes and intake of dietary antioxidants may help to maintain an adequate antioxidant status in the body. In the past decades, new molecular techniques, cell cultures and animal models have been established to study the effects and mechanisms of antioxidants on ROS. The chemical and molecular approaches have been used to study the mechanism and kinetics of antioxidants and to identify new potent antioxidants. Antioxidants can decrease the oxidative damage directly via reacting with free radicals or indirectly by inhibiting the activity or expression of free radical generating enzymes or enhancing the activity or expression of intracellular antioxidant enzymes. The new chemical and cell-free biological system has been applied in dissecting the molecular action of antioxidants. This review focuses on the research approaches that have been used to study oxidative stress and antioxidants in lipid peroxidation, DNA damage, protein modification as well as enzyme activity, with emphasis on the chemical and cell-free biological system.

Keywords

ROS; antioxidants; cell-free systems; radical scavenging; antioxidant enzymes; lipid peroxidation; DNA damage; protein modification

Introduction

Free radicals are atoms, molecules or ions with unpaired electrons, which are highly active to chemical reactions with other molecules. In the biology system, the free radicals are often derived from oxygen, nitrogen and sulfur molecules. These free radicals are parts of groups of molecules called reactive oxygen species (ROS), reactive nitrogen species (RNS) and

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reactive sulfur species (RSS). For example, ROS includes free radicals such as superoxide anion ($O_2^{-\bullet}$), perhydroxyl radical ($HO_2^{\bullet}$), hydroxyl radical (·OH), nitric oxide (NO), and other species such as hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), hypochlorous acid (HOCl) and peroxynitrite ($ONOO^-$) [1]. RNS are derived from NO through the reaction with $O_2^{-\bullet}$ to form $ONOO^-$. RSS are easily formed from thiols by reaction with ROS [2]. ROS are produced during cellular metabolism and functional activities, and have important roles in cell signaling, apoptosis, gene expression and ion transportation (Figure 1) [1]. However, excessive amounts of ROS can have deleterious effects on many molecules including protein, lipid, RNA and DNA since they are very small and highly reactive. ROS can attack bases in nucleic acids, amino acid side chains in proteins and double bonds in unsaturated fatty acids, in which ·OH is the strongest oxidant. ROS attacking macromolecules is often termed oxidative stress. Cells are normally able to defend themselves against ROS damage through the use of intracellular enzymes to keep the homeostasis of ROS at a low level. However, during times of environmental stress and cell dysfunction, ROS levels can increase dramatically, and cause significant cellular damage in the body. Thus, oxidative stress significantly contributes to the pathogenesis of inflammatory disease, cardiovascular disease (CVD), cancer, diabetes, Alzheimer's disease, cataracts, autism and aging [2–6]. In order to prevent or reduce the ROS-induced oxidative damage, the human body and other organisms have developed an antioxidant defense system that includes enzymatic, metal-chelating, and free radical-scavenging activities to neutralize these radicals after they have formed. In addition, intake of dietary antioxidants may help to maintain an adequate antioxidant status in the body.

Antioxidants may be molecules that can neutralize free radicals by accepting or donating electron(s) to eliminate the unpaired condition of the radical. The antioxidant molecules may directly react with the reactive radicals and destroy them, while they may become new free radicals which are less active, longer-lived and less dangerous than those radicals they have neutralized. They may be neutralized by other antioxidants or other mechanisms to terminate their radical status. For example, many antioxidants have aromatic ring structures and are able to delocalize the unpaired electron (Figure 1). Vitamin C ($AscH^-$) in the aqueous phase and vitamin E (TOH) in the lipid phase will directly react with or neutralize hydroxyl, alkoxyl (LO·) and lipid peroxyl (ROO·) radicals and form H_2O, alcohol and lipid hydroperoxides, respectively. Vitamin E itself becomes a phenyl radical (TO·) and vitamin C turns to a very stable radical ($Asc^{-\bullet}$), due to its delocalized structure (Figure 2A and B). Furthermore, vitamin C can also neutralize the radical form of other antioxidants such as glutathione radical and vitamin E radical, and regenerate these antioxidants (Figure 2C). Vitamin C itself is readily regenerated from $Asc^{-\bullet}$ with NADH or NADPH-dependent reductases [7]. Many antioxidants may directly react with ROS and/or free radical intermediates induced by ROS and terminate the chain reaction, thereby stopping the ROS-induced damage [8].

Small molecules such as vitamin C, vitamin E, uric acid and glutathione play important roles as cellular antioxidants. Synthetic antioxidants such as tert-butylhydroxyl-toluene, tert-butylhydroxyanisole and *tert*-butylhydroquinone have been widely used in the food industry to retard lipid oxidation. However, such synthetic antioxidants are not preferred for pharmacologic use due to toxicological concerns. Thus, more and more interests have focused on identifying plant extracts to use as dietary antioxidant supplements [9]. Most of these natural antioxidants come from fruits, vegetables, spices, grains, and herbs such as ginseng, curcuma, ginkgo, rosemary, green tea, grape, ginger and garlic. They contain a wide variety of antioxidant compounds, such as phenolics (phenol and polyphenols), flavonoids, carotenoids, steroids and thiol compounds [10]. These antioxidants may help to protect cellular damages from oxidative stress and also lower the risk of chronic diseases. For example, ginseng contains steroid-like compounds, ginsenosides, which show

antioxidant activities against free radical damage on the vascular endothelium [11]. Ginkgo has been reported to have strong antioxidant activities due to flavone glycosides that scavenge free radicals [8]. Flavonoids such as catechin and epicatechin in green tea and grape seed extracts could be responsible for their potent antioxidant activities [10,12].

Another important function of antioxidants is to regulate ROS-related enzymes. Antioxidants may decrease the cellular level of free radicals either by inhibiting the activities or expressions of free radical generating enzymes such as NAD(P)H oxidase (NOX) and xanthine oxidase (XO) or by enhancing the activities and expressions of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) [13,14]. These antioxidant enzymes produced in the body provide an important defense against free radicals. Animal cells containing the enzyme SOD can convert two $O_2^{-\bullet}$ into a H_2O_2 and an oxygen. Also, organisms use CAT and/or GPX to eliminate H_2O_2 before the Fenton Reaction can create a ·OH. These observations of inhibition or enhancement of enzyme activities by antioxidants have opened a new door for us to further understand the molecular mechanisms of action of antioxidants.

Since 1990s, antioxidant research has expanded dramatically due to its potential benefit in disease prevention and health promotion. The antioxidant activity of pure compounds, foods, and dietary supplements has been extensively studied in biological systems such as cell cultures, animal models and clinical trials [10,12,15,16]. Many research models have been established in chemical and/or biological systems for the studies of mechanisms of action of antioxidants as well as identification of new antioxidants especially from natural substances. Generally, the antioxidant ability was measured and presented as total antioxidant activity [17,18], total antioxidant capacity [19,20], total antioxidant potentials [21], Trolox equivalent antioxidant capacity [22], total radical absorption potentials [23], ferric reducing/antioxidant power [24], and oxygen radical absorption capacity [25]. Mechanistically, these methods are based on either a single-electron transfer reaction or a hydrogen atom transfer reaction from an antioxidant or oxidant to a free radical. The change of optical absorbance of either antioxidant or oxidant is measured for the quantitation for antioxidant capability. The chemical approaches are simple and easy to study the total antioxidant activity, which includes the radical scavenging ability and reductive activity [18]. Radicals are commonly used to measure the radical scavenging ability of antioxidant. This article describes the scavenging of active radicals such as ·OH and $O_2^{-\bullet}$, stable radicals such as 1,1-diphenyl-2-picryl-hydrazyl free radical (DPPH), 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) cation radical ($ABTS^{+\cdot}$) and other species such as HOCl, H_2O_2, $ONOO^-$ The scavenging of ROS in the biology system is the most important.

Further studies in cell cultures and animal models give critical information of the bioavailability, metabolism and toxicity issues of antioxidants, implicating potential clinical applications of these substances. However, animal models and human studies are expensive and not suitable for the initial antioxidant screening of foods and dietary supplements. Thus cell culture models have been used for initial antioxidant screening and antioxidant research prior to animal studies and human clinical trials [15]. However, cell systems are very complicated and too many factors may affect the results compared with cell freee systems. Many chemical and cell-free systems offer unique advantages to delineate the chemical and molecular mechanisms of action of new antioxidants. It is possible that some antioxidants can work chemically, while they may not work cellularly and physiologically. The aim of this review is to update the current approaches to study properties and mechanisms of action of antioxidants with emphasis on the chemical and cell-free biological systems. This review may greatly benefit researchers and scientists who are interested in the study of detail chemical and molecular mechanisms of antioxidants. It is also helpful for physicians who

are interested in antioxidant therapy or prevention for certain oxidative stress-related diseases or inflammation conditions.

Free radical scavenging

Searching and identifying natural and safe antioxidants, especially of plant origin, have been notably increased in recent years [26,27]. Assays based on the use of $O_2^{-\bullet}$ and ·OH, DPPH, $ABTS^{+\cdot}$, and N,*N*-dimethyl-*p*-phenylenediamine dihydrochloride cation radical ($DMPD^{+\cdot}$) are among the most popular spectrophotometric methods for determination of the antioxidant capacity of foods, beverages and vegetable extracts [26–32]. DPPH and $ABTS^{+\cdot}$ scavenging methods have been the most commonly used to evaluate the antioxidant activity of compounds due to their simple, rapid, sensitive, and reproducible procedures. Thus, the radical scavenging assays in the cell-free systems for antioxidant studies are often considered by researchers before further studies in cellular lines and/or animal models.

Scavenging Superoxide and other ROS—Superoxide ($O_2^{-\bullet}$), a predominant cellular free radical, is involved in a large number of deleterious changes often associated with an increase in peroxidative processes and linked to a low antioxidant concentration. Although $O_2^{-\bullet}$ itself is not so reactive to biomolecules, it helps in generation of more powerful ·OH and $ONOO^-$ [33]. In phagocytes, $O_2^{-\bullet}$ is produced in large quantities by the enzyme NADPH oxidase for killing pathogens. $O_2^{-\bullet}$ is also a byproduct of mitochondrial respiration, as well as several other enzymes such as NADH oxidase, XO, monooxygenases, and cyclooxygenases [34].

Direct scavenging of $O_2^{-\bullet}$ has been a model for determining the antioxidant activities [28]. In the chemical systems, $O_2^{-\bullet}$can be generated enzymatically or nonenzymatically from quinone derivatives, such as 6-anilino-5, 8-quinolinequinone (LY83583), 1,4-benzoquinone, 1,4-naphthaquinone, 2-methyl-1,4-naphthaquinone, riboflavin, etc (Figure 3) [35]. In the presence of enzymes such as NADPH-cytochrome P450 reductase and mitochondrial NADH-ubiquinone oxidoreductase or thiol compounds such as glutathione and L-cysteine, LY83583 undergoes a one-electron reduction due to low redox potential (−0.3 V vs. SCE), followed by formation of LY83583 semiquinone anion radical. Under an aerobic condition, this species interacts with molecular oxygen to form $O_2^{-\bullet}$ and original quinones (Figure 3C) [35]. $O_2^{-\bullet}$ is also generated in riboflavin/methionine/illuminate and assayed by the reduction of Nitro blue tetrazolium (NBT) to form blue formazan [28,36,37]. Briefly, the reaction mixture is illuminated at 25°C for 40 min, and $O_2^{-\bullet}$ generated from the photochemically reduced riboflavin can reduce NBT to form blue formazan which has absorbance at 560 nm (Figure 2C). This system can be used to determine the radical scavenging activity of antioxidants. Antioxidants can be added to the reaction mixture to scavenge $O_2^{-\bullet}$, thereby inhibiting the NBT reduction. Decreased absorbance of the reaction mixture indicates increased $O_2^{-\bullet}$ scavenging activity. The percentage of $O_2^{-\bullet}$ scavenged is calculated by the absorption change. Nitro blue tetrazolium salt and other tetrazolium salts are chromogenic probes useful for $O_2^{-\bullet}$ determination. These probes are also widely used for detecting redox potential of cells for viability, proliferation and cytotoxicity assays [38]. In the cell culture system, $O_2^{-\bullet}$ can be increased by treating cells with a mitochondrial respiratory complex III inhibitor, antimycin A [39].

Assessment of low-level $O_2^{-\bullet}$ in nonphagocytic cells is crucial for assessing redox-dependent signaling pathways and the role of enzymes such as the NADPH oxidase complex. Many probes and methods, such as enzymatic (cytochrome c, aconitase), spectrophotometric (Nitro blue tetrazolium), chemiluminescent (lucigenin, coelenterazine, etc), fluorescent reagents dihydroethidium (DHE) and MitoSOX, as well as electron paramagnetic resonance (EPR) spin trapping, have been used to detect the production of

$O_2^{-\bullet}$ (see review [40]). Among these probes, lucigenin (Luc) luminescence is a more specific measure of $O_2^{-\bullet}$. It involves several steps such as single-electron reduction of Luc^{2+} to $Luc^{+\cdot}$, coupling of $Luc^{+\cdot}$ with $O_2^{-\bullet}$ yielding a dioxetane, decomposition of the dioxetane into two molecules of the *N*-methyl acridone, one of which is in the electronically excited state; and finally emission of a photon from the excited state acridone as it returns to the ground state (Figure 4) [41]. Since $Luc^{+\cdot}$ is readily autoxidized to Luc^{2+} and reduces O_2 to $O_2^{-\bullet}$, Luc^{2+} can not precisely determine the cellular levels of $O_2^{-\bullet}$ [40,41]. Nevertheless, lucigenin (Luc) luminescence is still widely used in control conditions due to the convenience and sensitivity of luminescence methods.

DHE is a useful fluorogenic probe for the detection of ROS including $O_2^{-\bullet}$. DHE has been used increasingly as a probe for $O_2^{-\bullet}$ in biological systems because DHE is a hydrophobic, uncharged compound that is able to cross extra- and intracellular membranes. It undergoes significant oxidation in resting leukocytes, possibly through the uncoupling of mitochondrial oxidative phosphorylation. Cytosolic DHE displays blue fluorescence, whereas after oxidation of by oxidants such as $O_2^{-\bullet}$ and H_2O_2, it becomes 2-hydroxyethidium (2-EOH) and ethidium, which intercalates cellular DNA, staining the nucleus with a bright red fluorescence (Figure 5). Its oxidation by different oxidizing systems has been used increasingly for fluorescent analysis of ROS output in cells and tissues. Determination of total DHE fluorescence in cells has been performed extensively in the literature for assessment of ROS and, more specifically, of $O_2^{-\bullet}$. Its main drawback is that the total fluorescence of DHE is a sum of the composite spectra of all different products and thus likely reflects preferentially a measure of total cell redox state rather than production of a specific intermediate. Both 2-EOH and ethidium are fluorescent products that are difficult to discriminate them by conventional fluorescence microscopy or fluorometry. Thus, high performance liquid chromatography (HPLC) analysis of DHE-derived fluorescent compounds (2-EOH and ethidium) has been developed in order to achieve separation and individual analysis of such products [42,43]. This technique provides a significant increase in the accuracy of ROS output determinations and is a meaningful advance toward the precise quantification of this species in cells and tissues. Recent studies of HPLC separation and analysis of those two main products indicated that 2-EOH is generated specifically by $O_2^{-\bullet}$ oxidation of DHE, whereas ethidium is associated mainly with pathways involving H_2O_2 and metal-based oxidizing systems, including heme proteins and peroxidases [42]. More information about the DHE fluorescent probe is available in a recent review by Laurindo et al. [40]. MitoSOX™ Red mitochondrial superoxide indicator [44], a modified DHE, is a novel fluorogenic dye for highly selective detection of superoxide in the mitochondria of live cells. It is readily oxidized by superoxide inside the mitochondrion but not by other ROS- or reactive nitrogen species (RNS)–generating systems, and oxidation of the probe is prevented by SOD. The oxidation product becomes highly fluorescent upon binding to nucleic acids [39].

Several other fluorescent reagents such as fluorescein and rhodamine are also used to detect ROS including $O_2^{-\bullet}$, although they are not specific to $O_2^{-\bullet}$. Nonfluorescent 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) is a cell-permeable indicator for ROS, and it may be extremely useful for assessing cellular oxidative stress. After the acetate groups of H_2DCFDA are removed by intracellular esterases, the nonfluorescent H_2DCFDA is oxidized to the highly fluorescent 2′,7′-dichlorofluorescein (DCF) (Figure 6), which can be monitored by a fluorometer using excitation sources and filters appropriate for the fluorescein. Fluorescein has an absorption maximum at 494 nm and emission maximum of 521 nm [45]. H_2DCF has been shown to be oxidized to DCF in human neutrophils by H_2O_2, NO, and $FeSO_4$; in the cell-free system by $ONOO^-$ and horseradish peroxidase (HRP) alone; HRP in combination with H_2O_2; $FeSO_4$ alone; and a mixture of $FeSO_4$ and H_2O_2. The oxidation by Fe^{2+} in the presence of H_2O_2 was reduced by the ·OH radical scavenger

formation and the iron chelator deferoxamine [46]. DCFH was insensitive to NO and H_2O_2 in the cell-free system. Thus Myhre et al. suggest that the DCF assay is only suitable for measurements of $ONOO^-$ and H_2O_2 in combination with cellular peroxidases, peroxidases alone, and ·OH, while it is not suitable for measurement of NO, HOCl, or $O_2^{-\bullet}$ in biological systems [46]. However, in neutrophils, H_2DCFDA has proven useful for flow cytometric analysis of NO, forming a product that has spectral properties identical to those produced when it reacts with H_2O_2 [47]. Other studies reported that oxidation of H_2DCFDA was not directly sensitive to singlet oxygen, but singlet oxygen can indirectly contribute to the formation of DCF through its reaction with cellular substrates that yield peroxy products and peroxyl radicals [48,49]. Importantly, DCF itself can also act as a photosensitizer for H_2DCFDA oxidation, both priming and accelerating the formation of DCF; thus care must be taken when using DCFH to measure oxidative stress in cells as a result of both visible and UV light exposure [49].

For the measurement of $O_2^{-\bullet}$ scavenging activity by H_2DCFDA in a cell-free system, briefly, H_2DCFDA mixed with esterase at pH 7.4 is incubated at 37 °C for 20 minutes and placed on ice in the dark until immediately prior to the study. H_2DCFDA is deacetylated to nonfluorescent 2,7-dichlorodihydrofluorescein (DCFH) by esterase and subsequently oxidized to highly fluorescent DCF by $O_2^{-\bullet}$. The extent of conversion of DCFH into DCF is stoichiometrically related to the amount of $O_2^{-\bullet}$. The fluorescence intensity of oxidized DCFH is measured by using the fluorescence reader at excitation and emission wavelengths of 485 and 530 nm, respectively, for 1 hour with or without the addition of 2-methyl-1,4-naphthaquinone (50 mM) as an $O_2^{-\bullet}$ source [50].

Dihydrorhodamine 123 (DHR123) is another commonly used fluorescent mitochondrial dye. DHR123 itself is nonfluorescent, but it readily enters most of the cells and is oxidized by oxidative species or by cellular redox systems to the fluorescent rhodamine 123 that accumulates in mitochondrial membranes (Figure 7) [51]. DHR123 is useful for detecting ROS including $O_2^{-\bullet}$ (in the presence of peroxidase or cytochrome c) and $ONOO^-$ [50].

Scavenginh hydroxyl radical and other ROS—Hydroxyl radical (·OH) is extremely reactive, more toxic than other radical species and can attack biologic molecules such as DNA, proteins and lipids. ·OH is widely believed to be generated from the Fe^{2+} (or Cu^+)/H_2O_2 Fenton reaction system, by simply incubating $FeSO_4$ and H_2O_2 in aqueous solution. Thus, ·OH scavenging activity of antioxidants can be accomplished through direct scavenging or preventing of ·OH formation through the chelation of free metal ions or converting H_2O_2 to other harmless compounds. The scavenging ability of antioxidants can be determined by Gutteridge method, which is monitored in the Fe^{3+}–EDTA–H_2O_2–deoxyribose system [36,37,52]. The extent of deoxyribose degradation by the ·OH formed can be measured directly in the aqueous phase by thiobarbituric acid reactive species (TBARS) assay at 532 nm (Figure 8). This method is based on the fact that the degradation of deoxyribose by ·OH forms a reactive species malondialdihyde (MAD), which forms an adduct with thiobarbituric acid. The adduct, MDA-TBA, has an absorption at 532 nm that can be assayed spectrophotometrically. By this assay, the ability of several antioxidants to scavenge ·OH has been studied and compared with that of DMTU, uric acid, trolox and mannitol [36,37,52].

Another method uses a spin-trapping agent 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) to trap the generated ·OH radical [53]. DMPO reacts with ·OH to form a DMPO-OH radical, which can be monitored by EPR spectrum. Comparison of the EPR intensities of DMPO-OH radical in the absence and presence of antioxidants can measure the radical scavenging ability of the antioxidants. For example, Kang et al. studied the ·OH scavenging activity of ginsenosides [53].

Stable radical scavenging—The interaction between free radicals (such as $O_2^{-\bullet}$ and ·OH) and antioxidants can show direct evidence for antioxidants to scavenge free radicals. It has been widely used to evaluate the radical scavenging ability of antioxidants. For models, radical scavenging research tends to use more stable radicals as probes. Radicals such as DPPH, galvinoxyl, $ABTS^{+\cdot}$ and $DMPD^{+\cdot}$ are stable and colored (Figure 9). DPPH and gavinoxyl radicals are commercially available, and $ABTS^{+\cdot}$ and $DMPD^{+\cdot}$ radicals can be generated freshly before the assay by oxidizing the neutral molecules with potassium persulfate and $FeCl_3$, respectively [28,32]. These radicals have strong absorption in the visible region, while their absorption decreases proportionally upon receiving an electron or hydrogen from the antioxidants. Thus, the radical scavenging capacity of the antioxidants can be obtained based on the absorption change. The detailed method description for this free radical scavenging test is available from a previous report [28], where the antioxidant and radical scavenging properties of curcumin are studied.

DPPH has been widely used to evaluate the free radical scavenging effectiveness of various antioxidant substances [27,28,30]. In the DPPH assay, the antioxidants are able to donate a hydrogen to reduce the stable radical DPPH to the yellow-colored non-radical diphenyl-picrylhydrazine (DPPH-H). DPPH is usually used as a reagent to evaluate free radical scavenging activity of antioxidants based on the absorption change of DPPH at 517 nm measured spectrophotometrically. Similar to DPPH, galvinoxyl is a stable phenoxy radical that exhibits characteristic UV absorption at 429 nm in ethanol solution. $ABTS^{+\cdot}$ radicals are more reactive than DPPH radicals, and the reactions with $ABTS^{+\cdot}$ radicals involve a single-electron transfer process. Bleaching of a preformed solution of the blue-green radical cation $ABTS^{+\cdot}$, which has an absorption at 734 nm, has been extensively used to evaluate the antioxidant capacity of complex mixtures and individual compounds. The reaction of the preformed radicals with free radical scavengers can be easily monitored by following the decrease of the sample absorbance at 734 nm. The principle of the $DMPD^{+\cdot}$ assay is very similar to that of $ABTS^{+\cdot}$. The UV–visible spectrum of $DMPD^{+\cdot}$ shows a maximum absorbance at 505 nm [54].

These free radical scavenging reactions need the antioxidant to donate an electron or an active hydrogen atom such as one in reactive hydroxyl group. Generally, antioxidants that are molecules bearing active hydroxyl groups, such as vitamins E and C, polyphenol and flavonol compounds are potent radical scavengers. Interestingly, Liu and colleagues found that ecdysteroids, which do not have active hydroxyl groups, are also active antioxidants and free-radical scavengers [55]. The most active hydrogen of the ecdysteroid may be H-9, which is an allylic hydrogen, and furthermore, conjugation with the 6-carbonyl group further weakens the C–H-9 bond. It is well known that allylic hydrogens are very active and easily abstracted by free radicals.

In the current review, we focused mainly on the scavenging of reactive species centered on oxygen, nitrogen and chlorine. These assays were commonly used to search and identify natural and safe antioxidants. Other species such as reactive sulfur species (RSS) have been considered as a separate class of oxidative stressors, which may provide new antioxidant drug targets [56,57]. RSS are formed in vivo under conditions of oxidative stress. RSS are likely to include disulfide-S-oxides, sulfenic acids, and thiyl radicals, and are predicted to modulate the redox status of biological thiols and disulfides.

Metal ion (Fe^{2+}, Fe^{3+}, Cu^{2+} and Cu^{+}) chelating

Studies demonstrate that antioxidants and metal chelators may be of beneficial use in the treatment of neurodegenerative diseases, such as Alzheimer's disease [30,58,59]. Transition metals such as copper and iron are known to aggravate oxidative stress. These metals (Fe^{2+} and Cu^{+}) react with H_2O_2, which is a product formed by the dismutation of the $O_2^{-\bullet}$ by

SOD, to produce the highly reactive ·OH (Equation 1). Unlike iron, copper generates more singlet oxygen than ·OH upon its reaction with H_2O_2. Fe^{2+} and Cu^{+} are oxidized to Fe^{3+} and Cu^{2+}, respectively. In the presence of reducing agents such as ascorbic acid ($AscH^{-}$), cellular reductant such as NADH and oxidized metal ions Fe^{3+} and Cu^{2+} are reduced and allow the recycling to react with another molecule of H_2O_2 to generate ·OH radical (Equation 2). Hydroxyl radical is highly reactive and can react directly with proteins and other macromolecules to produce carbonyls (aldehydes and ketones), cross-linking and lipid peroxidation. Chelating metal ions can decrease their activity thereby reducing the formation of ROS.

$$Fe^{2+}(\text{or}\,Cu^{+})+H_2O_2 \rightarrow Fe^{3+}(\text{or}\,Cu^{2+})+\cdot OH+OH^{-} \quad (1)$$

$$Fe^{3+}(\text{or}\,Cu^{2+})+AscH^{-} \rightarrow Fe^{2+}(\text{or}\,Cu^{+})+Asc^{-}\cdot+H^{+} \quad (2)$$

Diram et al. [30] and Ak et al. [28] studied the antioxidant and iron-binding properties of curcumin, capsaicin and S-allylcysteine. These compounds show significant DPPH and $O_2^{-\bullet}$ scavenging ability. Chelating of ferrous ion by curcumin is demonstrated by the absorbance measurement of the ferrous iron–ferrozine complex at 562 nm [60]. Specifically, curcumin is added to a solution of $FeCl_2$. The reaction is initiated by the addition of 5 mM ferrozine. Then, the mixture is shaken vigorously and left at room temperature for 10 minutes. Absorbance of the solution is then measured spectrophotometrically at 562 nm. The percentage of inhibition of ferrozine–Fe^{2+} complex formation is calculated [28]. For the ion-chelating ability in the tissue [30], the lipid peroxidation is initiated by incubating the tissue homogenate with $FeSO_4$ or $FeCl_2$ and H_2O_2 in either the absence or presence of antioxidants at 37 °C for 1 hour. The product of the lipid peroxidation, protein-bound malonedialdehyde (MDA), is extracted from the solution after the addition of TBA in the form of MDA-TBA adduct, whose concentration is determined by its absorption at 532 nm (Figure 8). The Fe^{2+} and Fe^{3+} binding activity of antioxidants such as curcumin, capsaicin, and *S*-allylcysteine can be measured electrochemically. Curcumin and capsaicin compounds can effectively bind to Fe^{2+} and Fe^{3+} and prevent the redox cycling of iron, suggesting that these agents may reduce Fe^{2+}-induced lipid peroxidation.

Cu^{+} generates damaging ·OH radical 60 times faster than Fe^{2+} [61]. It has found that selenium antioxidant or resveratrol can chelate Cu^{+} (generated in situ with Cu^{2+}/ascorbic acid) very efficiently and inhibit the DNA damage from ·OH radical (generated by Cu^{+}/H_2O_2) [62,63]. Battin et al. observed that inhibition of oxidative DNA damage requires selenium-metal coordination. This mechanism is validated by using $Cu(bipy)_2^{2+}$ (bipy=2,2′-bipyridine) as the copper source. $Cu(bipy)_2^{2+}$ is reduced by ascorbic acid to $Cu(bipy)_2^{+}$ which causes DNA damage in the presence of H_2O_2. Addition of the selenlium antioxidant does not inhibit the damage, indicating that the antioxidant does not coordinate to $Cu(bipy)_2^{+}$ since the bipy ligands completely coordinate the Cu^{+} ion. The direct evidence of antioxidant-metal coordination has been observed by the large absorption bands of the complex in the UV-vis range [62,63].

Importantly, the ·OH radical scavenging activity by several ginsenosides was thought to be related to the Fe^{2+} chelating activity of their aglycone instead of direct reaction with ·OH, studied by EPR spectroscopy [53,64,65]. The ion-chelating ability of ginsenosides was thought to be influenced by their type of hydrophilic sugar moieties. Furthermore, polyphenols also show iron binding activity to prevent ROS-induced DNA damage,

although direct scavenging of ROS by polyphenols is the generally accepted mechanism of their antioxidant activity [66–69]. The iron binding to polyphenol groups is related to their p*K*a values. Polyphenol compounds with lower p*K*a values should bind iron more readily under physiologically relevant conditions. This predicted iron-coordination mechanism for polyphenol antioxidant activity is very useful for the design of more-potent antioxidants to treat and prevent diseases caused by oxidative stress [66].

Inhibition of free radical generating enzymes

NADPH oxidases are a group of plasma membrane associated enzymes, which transfer one electron from the cytosolic donor NADPH to a molecule of extracellular oxygen, producing $O_2^{-\bullet}$ [70]. Xanthine oxidase (XO) is an enzyme involved in the formation of uric acid in the body, which catalyses the oxidation of hypoxanthine and xanthine to uric acid yielding $O_2^{-\bullet}$ and H_2O_2 and raises the oxidative level in an organism (Figure 10) [71].

These enzymes are the main sources of free radicals and have been reported in various physiological and pathological conditions. $O_2^{-\bullet}$ is also a byproduct of mitochondrial respiration, as well as several other enzymes such as NADH oxidase, monooxygenases, and cyclooxygenases. It is biologically quite toxic and is deployed by the immune system to kill invading microorganisms. In phagocytes, $O_2^{-\bullet}$ is produced in large quantities by the enzyme NADPH oxidase for use in oxygen-dependent killing mechanisms for invading pathogens. The controlled production of reactive oxygen derivatives during the respiratory burst is essential for the defense of an organism against invading microorganisms without causing a significant loss of tissue functions [34]. However, excessive ROS promote oxidative stress such as LDL oxidation. Recently, a direct association between increased phagocytic NADPH oxidase activity and elevated circulating oxidized LDL has been found in patients with metabolic syndrome. Consequently, both modulation of NADPH oxidase to prevent overproduction of ROS [7] and supplementation with antioxidants have been proposed as effective strategies to prevent the deleterious effects of oxidative stress in hemodialysis patients [72].

Physiopathological importance of ROS generating enzymes has led many researchers to develop new strategies to control the functions of these enzymes in a wide number of in vivo and in vitro experiments. A survey of the scientific literature shows that the use of inhibitors such as diphenylene iodonium and apocynin in the study of NADPH oxidase's role increased exponentially. A number of inhibitors of the $O_2^{-\bullet}$ generating NADPH oxidase have been described [73]. Treatment with an XO inhibitor largely prevents the development of endothelial dysfunction and atherosclerosis in mice [74,75]. Searching for new inhibitors has not stopped and many natural antioxidants have shown great potential to inhibit these enzymes [34,74,76–80]. These investigations have clinical significance in understanding the structure and functions of NADPH oxidases and in development of new therapeutic agents for oxidative stress-related diseases.

In a clinical trial, Castilla et al. [72] compared the effects of dietary supplementation with concentrated red grape juice (RGJ), a source of polyphenols, and vitamin E, on neutrophil NADPH oxidase activity and other cardiovascular risk factors in hemodialysis patients. They found that both RGJ and vitamin E reduced plasma concentrations of oxidized LDL and ex vivo neutrophil NADPH oxidase activity. These effects were intensified when the supplements were used in combination. This effect of RGJ consumption may favor a reduction in cardiovascular risk. The result indicates natural antioxidants are potential inhibitors of NADPH oxidase. However, the detail mechanism in this important aspect is largely unknown.

The NADPH oxidase activity can be determined by the absorption at 340 nm (a molar extinction coefficient of 6200 M^{-1} cm^{-1}) of oxidation of NADPH by NADPH oxidase in a Tris/EDTA buffer. The production of $O_2^{-\bullet}$ is determined by addition of cytochrome *c* to the reaction mixture, and the amount of $O_2^{-\bullet}$ production is calculated using a molar extinction of 21,000 M^{-1} cm^{-1} at 550 nm due to the reduction of cytochrome *c* [34,78]. Laurindo et al. also described procedures to assess NADPH oxidase activity in vascular smooth muscle cell membrane fractions using DHE-derived fluorescence oxidation products detected by either HPLC or fluorometry [40].

Diatchuk et al. [76] assayed the lithium dodecyl sulfate (LiDS)- and NADPH-dependent oxygen consumption by cell-free mixtures, consisting of membrane and recombinant cytosolic components. They examined the inhibition of NADPH oxidase by 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF) and related compounds and found that AEBSF prevents the activation of the $O_2^{-\bullet}$ generating NADPH oxidase in both intact stimulated macrophages and in cell-free systems consisting of solubilized membrane or purified cytochrome *b*559 combined with total cytosol. AEBSF did not scavenge oxygen radicals and did not affect assay reagents, while it did have a direct effect on the NADPH oxidase. Thus, NADPH oxidase can be activated in a cell-free system by certain anionic amphiphiles. The effect of AEBSF on $O_2^{-\bullet}$ production can be examined in several forms of cell-free activation systems. The simplest one consists of solubilized macrophage membranes and unfractionated cytosol, both derived from unstimulated cells. AEBSF is found to exhibit a concentration-related, inhibitory influence on LiDS-induced $O_2^{-\bullet}$ production. In addition, the effect of AEBSF on $O_2^{-\bullet}$ production can be studied in a semi-recombinant cell-free system, which consists of either solubilized macrophage membrane or purified cytochrome *b*559 incorporated in phospholipid liposomes, combined with recombinant p47*phox*, p67*phox*, and Rac1-GTPgS. AEBSF is able to act as an inhibitor of $O_2^{-\bullet}$ production in this system.

Inhibition of XO enzyme activity has also been studied in both cell-free and cell systems. Allopurinol, a potent inhibitor of XO, is clinically used for the treatment for gout by preventing urate from accumulating in joints. Natural polyphenols coumarin (known as 1,2-benzopyrone), consisting of fused benzene and pyrone rings, is an important group of low-molecular weight phenolics and has been widely used for the prevention and treatment of venous thromboembolism, myocardial infarction and strokes [81]. Coumarins acting to inhibit XO enzyme activity and scavenge free radical has been reported [74]. The XO enzyme activity can be measured spectrophotometrically by continuously measuring uric acid formation at 295 nm with xanthine as the substrate. The assay is initiated by adding the enzyme to the reaction mixture containing xanthine, EDTA and 3-(cyclohexylamino)-1-propanesulfonic acid, without or with inhibitors at 37 °C. The extent of inhibition is expressed as the chemical concentration required to inhibit 50% of the enzyme activity (IC_{50}). The XO enzyme activity is also assayed with XO assay kit (Cayman Chemical, Ann Arbor, MI, USA), based on multistep enzymatic reactions in which XO first produces H_2O_2 during oxidation of hypoxanthine. In the presence of horseradish peroxidase, the H_2O_2 reacts with ADHP (10-acetyl-3,7-dihydroxyphenoxazine) to produce the highly fluorescent compound resorufin which can be easily analyzed at 563 nm (excitation)/587 nm (emission) (Figure 11). ADHP, market name Amplex Red™ reagent, is regarded as the best fluorogenic substrate for peroxidase because it is highly specific and stable. The substrate itself is nearly colorless and nonfluorescent until it is oxidized by H_2O_2 in the presence of horseradish peroxidase (HRP) to become the highly red fluorescent resorufin (Biotium Inc, Hayward, CA, USA). Importantly, combined ROS-scavenging and XO-inhibition activities are measured using the DMPO spin adduct generated in the hypoxanthine (HPX) and XO reaction system by the spin trapping method. The structure–activity relationships (SARs) of coumarins interacting with this enzyme suggest that the number of hydroxyl groups on the

ring structure of coumarins is correlated with the effects of ROS suppression. This structure-based molecular modeling revealed interactions between coumarins and the active site, the molybdopterin region of XO. The carbonyl of coumarins points toward and closely interacts with the guanidinium group of the Arg880. The O atom of the pyrone ring forms hydrogen bonds with the hydroxyl side chain of Thr1010. One of coumarin derivative, Esculetin, which bears two hydroxyl moieties on its benzene rings, had the highest affinity toward the binding site of XO, and this was mainly due to the interaction of 6-hydroxyl with the E802 residue of XO [74].

Activation of internal antioxidant enzymes

During metabolism, ROS such as H_2O_2, $O_2^{-\bullet}$, and ·OH, etc, are produced irreversibly [82]. Thus, methods to reduce the damage induced by oxidative stress is extensively investigated. Intracellular antioxidant enzymes are an important protective mechanism against ROS. These enzymes are produced in the cell and provide an important defense against free radicals. SOD, CAT, GPX, glutathione reductase (GRd), glutathione *S*-transferase (GST), thioredoxin reductase (TrxR), heme oxygenase and biliverdin reductase are the most important antioxidant enzymes. The enzyme SOD converts two $O_2^{-\bullet}$ into a H_2O_2 and an oxygen molecule (Equations 3 and 4).

$$M^{(n+1)+} - SOD + O_2^{-\cdot} \rightarrow M^{n+} - SOD + O_2 \tag{3}$$

$$M^{n+} - SOD + O_2^{-\cdot} + 2H^+ \rightarrow M^{(n+1)+} - SOD + H_2O_2 \tag{4}$$

where M = Cu (n=1), Mn (n=2), Fe (n=2), and Ni (n=2). In this reaction, the oxidation state of the metal ion oscillates between n and n+1. SOD is an important antioxidant defense in nearly all cells exposed to oxygen. Meanwhile, CAT and/or GPX can eliminate H_2O_2 before the Fenton Reaction can create ·OH (Equations 5 and 6).

$$2\,H_2O_2 \xrightarrow{CAT} 2\,H_2O + O_2 \tag{5}$$

$$2GSH + H_2O_2 \xrightarrow{GPX} GS - SG + 2H_2O \tag{6}$$

where GSH represents reduced glutathione, and GS–SG represents glutathione disulfide. GRd then reduces the oxidized glutathione (GS-SG) to complete the cycle (Equation 7).

$$GS - SG + NADPH + H^+ \xrightarrow{GRd} 2\,GSH + NADP^+ \tag{7}$$

Both GST and GPX are involved in eliminating peroxides that are formed during metabolism. GRd regulates the equivalent of reduced glutathione (GSH) and oxidized glutathione (GSSG), and the ratio of GSH/GSSG is a well known index of oxidative stress [83]. The activation of GRd plays an important role in elevating the concentration of GSH, which maintains the oxido-redox status in the organism [14]. The brain, which is very vulnerable to free radical damage, has seven times more GPX activity than CAT activity

[84]. Accordingly, increasing evidence suggests a role of oxidative stress in the development and clinical manifestation of autism. Recently, researchers have demonstrated in a comparative cellular system between individuals diagnosed with autism and controls that in a concentration and time-dependent fashion, exposure to oxidative stress via the sulfhydryl reagent thimerosal resulted in a greater decrease in the GSH/GSSG ratio and increase in free radical generation in autism compared to control cells [83]. Moreover, GPX is found throughout the cell, whereas CAT is often restricted to peroxisomes. In humans, the highest levels of CAT are found in liver, kidney, and erythrocytes, where it decomposes the majority of H_2O_2. Nonetheless, the lifespan of transgenic mice has been extended about 20% by overexpression of human CAT targeted to mitochondria [85].

As we have addressed above, many natural substances reduces oxidative stress by radical scavenging, metal ion chelating and inhibiting the activity of radical generating enzymes. It also has been well defined that chemopreventive reagents, such as phenolic antioxidants, dithiolethiones, isothiocyanates, etc., selectively induce the activation of phase II detoxifying and antioxidant enzymes through the Keap1–Nrf2 pathway [10]. Furthermore, cell line models have been used to study the effect of antioxidants such as anthocyanins, resverratrol and curcumin on the activation of these internal antioxidant enzymes. It has been found that natural anthocyanins act as chemopreventive phytochemicals and could stimulate the intracellular antioxidant system to resist oxidant-induced injury [14]. Resveratrol, however, either has no effect on, or reduces the activities of GPX, CAT and CuZnSOD, while it dramatically and progressively induces mitochondrial MnSOD expression and activity [86]. Exposure of human cells to resveratrol increased MnSOD protein level 6-fold and activity 14-fold. Thus, long-term exposure of human cells to resveratrol for two weeks results in a highly specific upregulation of MnSOD, and this may be an important mechanism by which it elicits its antioxidant effects in human cells. Selenomethionine (SeMet), one of major dietary selenium compounds, can be converted to hydrogen selenide and selenocystein, which are ready for incorporation into proteins at specific UGA codons to form selenoproteins such as active GPX. Thus, SeMet plays a critical role to maintain enzymatic activities of GPX for antioxidation [87–89]. In addition, SeMet is able to directly interact with some oxidant molecules or oxidant generating ions [63,90,91].

The GPX and GRd activity can be determined spectrophotometrically according to the method described by Shih et al. [14]. The mixture of homogenate, EDTA, NaN_3, NADPH, GSH reductase, and GSH in PBS buffer is preincubated for 5 minutes at 37 °C. Thereafter, the overall reaction is initiated by adding H_2O_2. Enzyme activity is calculated by the change of the absorbance value at 340 nm for 5 min. The GPX activity could be expressed as nanomoles of NADPH per minute per milligram of protein. The GRd assay also monitors the oxidation of NADPH consumed in the reduction of glutathione disulfide (GSSG) by the change in absorbance at 340 nm. The following solutions are pipetted into a 1 cm spectrophotometric cuvette: homogenate, $MgCl_2 \cdot 6H_2O$, GSSG, and NADPH in PBS buffer. This mixture is preincubated for 5 minutes at 37 °C. GRd activity is calculated by the change in the absorbance value at 340 nm for 5 minutes and is expressed as nanomoles of NADPH per minute per milligram of protein.

Thioredoxin reductase (TrxR) is a key enzyme for DNA synthesis by directly serving as an electron donor to ribonucleotide reductase [19]. It catalyzes NADPH-dependent reduction of the redox-active disulfide in thioredoxin (Trx), and it plays essential roles in substrate reductions, defense against oxidative stress, and redox regulation by thiol redox control. TrxR has been found to be overexpressed by a number of human tumors. Fang et al. studied the inhibitory effect of curcumin on TrxR [92]. TrxR is first reduced with NADPH in a Tris-Cl/EDTA (TE) buffer, and incubated with curcumin at room temperature. The enzyme

activity assay is based on the reduction of DTNB [5,5′-dithiobis(2-nitrobenzoic acid)] with NADPH to 5-thio-2-nitrobenzoic acid (TNB) which produces a yellow product that is measured at 405–414 nm with spectrophotometer [79,92]. Curcumin is found to inhibit TrxR irreversibly, and the curcumin-modified TrxR enzyme showed a strong induction of NADPH oxidase activity to produce $O_2^{-\bullet}$. The curcumin modified sites and peptides of TrxR are also identified. Modification of TrxR by curcumin provides a possible mechanistic explanation for its cancer preventive activity, shifting the enzyme from an antioxidant to a prooxidant.

Native SOD has been used as a therapeutic agent to attenuate ROS-induced injuries. However, applications of exogenous SOD still have many problems including short clearance, brain inaccessibility, chemical instability, low oral bio-availability, specific tissue targeting and immunogenicity [82,93]. An increasing number of low molecular-weight SOD mimics have been developed to overcome these limitations. Most SOD catalytic mimics are designed with a redox active metal center, similar to the active site metals of the native SODs, i.e. Cu, Fe or Mn. Among them, MnSOD is thought being safe and has been well developed [94,95]. MnTBAP, Manganese (III) 5,10,15,20-tetrakis(4-benzoic acid) porphyrin (MnTBAP), is one of the most intensely explored “SOD mimics” in biology and medicine [96,97]. MnTBAP is an active, stable, nontoxic, and cell permeable SOD mimetic. Most importantly, it possesses both SOD and CAT activity [98]. MnTBAP scavenges $O_2^{-\bullet}$, H_2O_2, $ONOO^-$, and lipid peroxyl radicals, but does not scavenge NO [99,100]. It is a potent inhibitor of lipid peroxidation [98,101].

However, the molecular relationship between consumption of antioxidants and elevation of antioxidant ability is not completely clear. Many studies have been focused on the structure modification of antioxidants to improve the antioxidant property and to enhance SOD activity [1,94,95,102,103]. For example, Liu et al. modified the structure of ginsenoside Rg3 to a ocotillol type saponin and found that the lipid peroxidation (LPO) activity decreased and SOD activity increased 120 min after administration of ginsenoside Rg3 to dogs. However, the underlying mechanisms for these effects are not clear [102]. Barik et al. have found that cupper complexes of curcumin analogues had SOD activity [95]. They have established chemical models which can be used to study the SOD activity of the metal-curcumin SOD mimics. Briefly, the SOD assay is carried in a mixture containing xanthine, NBT^{2+} (NBT=Nitroblue Tetrazolium), XO, and metal-curcumin complex in PBS buffer. After incubation at room temperature for 10 minutes, the NBT^+ formed can be measured spectrophotometrically at 560 nm. SOD activity is calculated based on the rate of absorbance change per minute (DA/min) and expressed in terms of the IC50 of NBT reduction obtained from a linear regression plot of percent SOD activity vs. test compound concentrations. However, the cell-free systems for a direct interaction of antioxidants with SOD enzyme have not been established. Cell-free systems for assaying the activities of these intracellular antioxidant enzymes are necessary to elucidate the mechanisms of action of certain antioxidants.

Many diseases such as Alzheimer’s disease, audism, cardiovascular risk and cancer can be correlated with increased activity of free radial generating enzymes and decreased activity of antioxidant enzymes. One study showed that NADPH oxidase is activated in the brains of patients with Alzheimer’s disease by demonstrating the marked translocation of the cytosolic factors p47-*phox* and p67-*phox* to the membrane in Alzheimer’s disease [104]. NADPH oxidase is a highly regulated membrane-bound enzyme complex that is composed of a number of cytosolic and membrane-bound proteins. p47-*phox* and p67-*phox*, two of the components of NADPH oxidase, are normally located in the cytosol in resting cells and their translocation from the cytosol to the membrane is required for $O_2^{-\bullet}$ generation *in vitro* and *in vivo*. Activation of NADPH oxidase results in production of $O_2^{-\bullet}$. This is then rapidly

converted to secondary toxic ROS or RNS such as peroxynitrite, which can efficiently kill microorganisms. Melatonin shows significant antioxidative effects in Alzheimer's disease models in vitro and in vivo. Pretreatment of microglia with melatonin dose-dependently prevents the activation of NADPH oxidase and decreases the production of ROS. Melatonin inhibits the phosphorylation of the $p47^{phox}$ subunit of NADPH oxidase via a PI3K/Akt-dependent signalling pathway, blocks the translocation of $p47^{phox}$ and $p67^{phox}$ subunit to the membrane, down-regulates the binding of $p47^{phox}$ to $gp91^{phox}$, and impairs the assembly of NADPH oxidase [105].

Autism is a severe developmental disorder with poorly understood etiology. Oxidative stress in autism has been studied in cell culture. A mechanism has been proposed that links oxidative stress with membrane lipid abnormalities, inflammation, aberrant immune response, impaired energy metabolism and excitotoxicity, leading to clinical symptoms and pathogenesis of autism [5,6,83,106]. As reviewed by Chauhan [106], several studies have observed patients with autism showed decreased activity of glutathione peroxidase in plasma and in erythrocytes, reduced levels of total glutathione and lower redox ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG) in plasma, and decreased catalase and SOD activity in erythrocytes.

Natural substances may directly influence the activities of these enzymes [107]. For example, a clinical trial reported that both red grape juice and vitamin E reduced plasma concentrations of oxidized LDL and *ex vivo* neutrophil NADPH oxidase activity, indicating that natural antioxidants are potential inhibitors of NADPH oxidase. This may favor a reduction in cardiovascular risk [72]. However, detail mechanisms in this important aspect are largely unknown.

Prevention of lipid peroxidation

Lipid peroxidation refers to the oxidative deterioration of lipids containing any number of carbon-carbon double bonds, such as unsaturated fatty acids, phospholipids, glycolipids, cholesterol esters and cholesterol itself. ROS attack the unsaturated fatty acids which contain multiple double bonds and the methylene $-CH_2-$groups with especially reactive hydrogen atoms, and initiate the radical peroxidation chain reactions (Figure 12) [108]. Radical scavengers can directly react and quench peroxide radicals to terminate the chain reaction. Lipid peroxidation and DNA damage are associated with a variety of chronic health problems, such as cancer, ageing and atherosclerosis [109–111].

Antioxidant compounds may scavenge ROS and peroxide radicals, thereby preventing or treating certain pathogenic conditions. Lipid peroxidation has been extensively used as a research model for identifying natural antioxidants as well as the studies of their mechanisms of action. Studies on antioxidants such as vitamins, polyphenols (green tea), flavones and ginsenosides against free radical-induced lipid peroxidation have been undertaken in several systems such as lipid, human red cells, human low density lipoprotein and rat liver microsomes in homogeneous solution or micelles. The antioxidant activity of these polyphenols depends significantly on the structure of the molecules, the initiation conditions and the microenvironment of the reaction medium. In vitro lipid peroxidation such as linoleic acid can be either initiated thermally by using a water soluble azo initiator 2, 2′-azobis(2-amidinopropane) hydrochloride (AAPH) [112], or initiated by metal ions Fe^{2+} or Cu^{+} with H_2O_2 (Fenton reaction) [18,62,113]. AAPH decomposes at physiological temperature (37 °C) in aqueous solutions to generate alkyl radical (R·), which in the presence of oxygen is converted to the corresponding peroxyl radicals (ROO·). Since AAPH is water soluble, the rate of free-radical generation from AAPH can be easily controlled and measured. It has been extensively used as a free-radical initiator for biological and related studies and the hemolysis induced by AAPH provides a good approach for studying

membrane damage induced by free radicals [55]. Fe^{2+} or Cu^{+} react with H_2O_2 and generate highly reactive ·OH. These radicals (·OH, R· and ROO·) abstract an active methylene hydrogen from linoleic acid to form lipid radical and lipid peroxide in the presence of O_2, and start the chain reaction. Briefly, linoleic acid substrate (or its analogues) can be incubated with initiator (either AAPH [114] or Fe^{2+}/H_2O_2 [18]) in the absence or presence of antioxidants in homogenous solution or SDS micelle. Peroxidation of linoleic acid gives different hydroperoxides depending on the reaction conditions. Hydroperoxide substitution at the C-9 or C-13 positions produces either trans, trans or cis, trans conjugated dienes (Figure 12). These are the major products in the absence of antioxidants and show characteristic ultraviolet absorption at 235 nm that can be used to monitor the formation of the total hydroperoxides during the peroxidation after separation of the reaction mixture by HPLC [114]. In some studies [18], a color indicator benzoyl leucomethylene blue can be used to reduce the linoleic acid hydroperoxide to linoleic acid hydroxide, while itself is oxidized to methylene blue which shows a absorption at 666 nm. In the presence of antioxidants, the antioxidant first inhibits the formation of linoleic acid hydroperoxides by scavenging peroxide and ·OH radicals and also inhibits the formation of the color indicator methylene blue by reduction of the linoleic acid hydroperoxide to the linoleic acid hydroxide. By comparing the formation kinetics of hydroperoxide in the presence and absence of antioxidants, the inhibitory ability of antioxidants can be evaluated and expressed as the total antioxidant activity. This test has been used to identify the key antioxidant in bread crust [115]. By using synthesized methyl esters of linoleic acid hydroperoxide as the substrate, the reductive activity of antioxidants can be measured. The difference between the total antioxidant activity and the reductive activity is the radical scavenging activity [18].

Other models of lipid peroxidation include the lipid peroxidation of low-density lipoprotein (LDL), human red cell and pospholipid microsomes. These models are very similar to those of linoleic acid peroxidation. Instead of pure linoleic acid, human red cells, LDL or microsomes are used as the lipid substrate. Erythrocyte membranes are rich in polyunsaturated fatty acids, which are very susceptible to oxidative stress mediated by free radicals. In the human red cell system, briefly, AAPH–PBS solution is added to a suspension of erythrocytes in PBS to which an antioxidant has been in advance to a certain concentration, and the suspension is incubated at 37 °C. Samples are taken from the above incubation mixture and centrifuged, and the supernatant is analyzed for hemoglobin (hemolysis) at 540 nm [116]. Peroxidation of polyunsaturated lipids in red cell membranes causes a quick damage and the membrane losses its integrity, leading to the release of hemoglobin (hemolysis) and intracellular K^{+} ions. When antioxidants such as curcumin are present the system, peroxyl radicals can be converted to non-reactive species, and thereby the radical-induced lipid peroxidation and hemolysis can be inhibited.

LDL has a highly-hydrophobic core consisting of polyunsaturated fatty acid linoleate and about 1500 esterified cholesterol molecules [117]. The peroxidation of LDL can be initiated either thermally by a water-soluble azo initiator, AAPH, or photochemically by a triplet sensitizer benzophenone (BP). The peroxidation of LDL can be measured by the formation of conjugated dienes (absorbance at 235 nm) as above or the rate of oxygen intake [118]. A new method is to quantify the decomposition product of hydroperoxide of LDL oxidation, hexanal, by headspace GC analysis [119]. Hexanal production correlates well with the oxidation of polyunsaturated fatty acid in LDL and reflects the degree of LDL oxidation in vitro. The addition of antioxidants can inhibit the formation of lipid dienes or the rate of oxygen intake. The method has been used to study the antioxidant activities of flavonols from apple peels and green tea leaves, vitamins, and resveratrol and its analogues [118–121]. Peroxyl radical-initiated LDL oxidation in these studies is similar to the LDL oxidation under physiological conditions in humans compared with the Cu^{2+}-induced LDL oxidation model [119].

Similar to erythrocyte membranes, microsomes (especially smooth endoplasmic reticulum) are particularly susceptible to oxidative stress because of their high polyunsaturated fatty-acid content [122]. Iron (Fe^{2+} combined with a reducing reagent) is usually used for generating ·OH radicals to induce microsome peroxidation, which can be measured by the thiobarbituric acid (TBA) method [123]. Antioxidants may inhibit the formation of TBA reactive species. Thus, the antioxidation activity of the antioxidant can be determined [124,125].

Lipid peroxidation processes involve radical formations including initiator radicals (R·, ·OH), and lipid peroxyl radicals. The antioxidant may directly react with initiator radicals or lipid peroxides, and it may also inhibit the formation of active radicals. These mechanisms of action of any antioxidant are critical and warrant for further investigations with radical scavenging assays and ion chelating tests.

Prevention of DNA damage

The ·OH and $ONOO^-$ radicals generated from NO and $O_2^{-\bullet}$ can react directly with plasmid DNA macromolecules in vivo to cleave (or "nick") one DNA strand, causing oxidative DNA damage. Cell death and mutation caused by this DNA damage are implicated in neurodegenerative and cardiovascular diseases, cancer, and aging. Treating these conditions with antioxidants is of growing interest. At the same time, the DNA or plasmid damage has been used as models for the study and identification of antioxidants [27,32,62,66,91,126]. A typical research model of DNA damage caused by Cu^+ induced ·OH has been developed [62,63]. Briefly, metal-free plasmid DNA is combined with Cu^{2+}, ascorbic acid and H_2O_2 at pH 7. Cu^{2+} is reduced to Cu^+ in situ with ascorbic acid. The ·OH radical generated by Cu^+/H_2O_2 cleaves one DNA strand, causing the normally supercoiled plasmid DNA to unwind. The degree of DNA damage is assessed using electrophoresis to separate the damaged and undamaged forms. Adding antioxidants such as selenium can inhibit ·OH-induced DNA damage. Thus, the antioxidant potential of certain compounds can be quantified and directly compared. DNA damage also produces carbonyls (aldehydes and ketones), which react with TBA to form TBARS which can be measured directly in the aqueous phase by TBARS assay at 532 nm [32].

Prevention of protein modification

Besides lipid peroxidation and DNA damage, ROS also cause protein modification by nitration or chloration of amino acids. Peroxynitrite, O=N-O-O^-, formed *in vivo* by the reaction of $O_2^{-\bullet}$ with free radical NO by a diffusion-controlled reaction, is an powerful oxidant and nitrating agent [127]. $ONOO^-$ is a much more powerful oxidant than $O_2^{-\bullet}$ and can damage a wide variety of molecules including DNA and proteins in cells. Peroxynitrite and its protonated form peroxynitrous acid (ONOOH) can exert direct oxidative modifications through one- or two-electron oxidation processes [128]. $ONOO^{-\bullet}$ reacts nucleophilically with CO_2 *in vivo* to form nitrosoperoxycarbonate, which is the predominant pathway for $ONOO^-$. $ONOOCO_2^-$ homolyzes to form carbonate radical ($CO_3^{-\bullet}$) and nitrogen dioxide radical ($\cdot NO_2$). ·NO2 is also a RNS which in turn can nitrate tyrosine to nitrotyrosine. These radicals are believed to cause peroxynitrite-related cellular damage (Figure 13). Peroxynitrite ($ONOO^-$) itself is also a strong oxidant and can react directly with electron-rich groups, such as sulfhydryls, iron-sulfur centers, zinc-thiolates, and the active site sulfhydryl in tyrosine phosphatases [127]. Another pathway utilizes heme protein myeloperoxidase (MPO)-generated HOCl [129], which reacts with NO_2^- to form nitryl chloride (NO_2Cl), which may spontaneously decompose to $\cdot NO_2$ and Cl·. These products may be responsible for the chlorinating and nitrating behavior of Cl-NO_2 [130]. HOCl reacts with a wide variety of biomolecules including DNA, RNA, fatty acid groups, cholesterol

and proteins. The presence of nitrotyrosine or chlorotyrosine has been as biomarker of damage by RNS in vivo [131].

These modifications often result in the alteration of protein function or structure and, usually, the inhibition of enzyme activities. Proteins containing nitrotyrosine residues have been detected in different pathologic conditions, including diabetes, hypertension, and atherosclerosis, all associated with enhanced oxidative stress, including increased production of $ONOO^-$ [132]. In order to attenuate the protein modification caused by $ONOO^-$ and HOCl, antioxidants and antioxidant enzyme are used. Antioxidants or enzyme like CAT which can eliminate H_2O_2 should also inhibit the formation of HOCl; likewise, SOD or antioxidant such as curcumin and polyphenols may scavenge $O_2^{-\bullet}$, and inhibit the formation of $ONOO^-$. However, the formation of $ONOO^-$ from NO and $O_2^{-\bullet}$ is three times faster than the scavenging of $O_2^{-\bullet}$ by SOD. Therefore, the scavenging of $ONOO^-$ directly by using natural safe ingredients from the medicinal herbs may be a rational alternative for preventive and therapeutic interventions in diseases [133]. Indeed, reactions of $ONOO^-$ with phenoic compounds are widely reported in the literature [134]. Scavenging of $ONOO^-$ by antioxidants based on assays involving tyrosine nitration is suggested as a useful research tool which may provide additional valuable information on the antioxidant profile of the biomolecules [135,136]. Generation of $ONOO^-$ *in vivo* has been implicated in a wide range of human diseases. Agents that are able to protect against $ONOO^-$ dependent damage may be therapeutically useful.

Rosmarinic acid has been well recognized especially, in regard to its antioxidant and anti-inflammatory activities [137]. Rosmarinic acid displays a strong scavenger activity for $ONOO^-$ and other free radicals. $ONOO^-$ is responsible for a wide spread biological damage in the brains of patients with Alzheimer's disease. Amyloid β protein (Aβ), the principal component of the senile plaques, is the main cause of increased $ONOO^-$ in the brain of patients with Alzheimer's disease. In cell culture studies, rosmarinic acid protects against the ROS induced by Aβ, and in *in vivo* studies, rosmarinic acid has shown a protective effect on the memory impairment in a mouse mode induced by acute intracerebroventricular injection of $A\beta_{25–35}$ into the brains of mice. It is found that memory protective effects of rosmarinic acid in the neurotoxicity of Aβ25–35 is due to its scavenging of $ONOO^-$, and that daily consumption of rosmarinic acid may protect against memory impairments observed in Alzheimer's disease [133]. Natural product ergothioneine is also a powerful scavenger of $ONOO^-$ and is able to protect α_1-antiproteinase against inactivation and tyrosine against nitration [138].

Choi et al. characterized $ONOO^-$ scavenging constituents from twenty-eight herbs with the use of a fluorometric method in the cell-free system [139]. They found the potency of scavenging activity to be as follows: witch hazel bark > rosemary > jasmine tea > sage > slippery elm > black walnut leaf > Queen Anne's lace > Linden flower. The extracts exhibited dose-dependent $ONOO^-$ scavenging activities. Thus they found that witch hazel bark (*Hamamelis virginiana* L.) showed the strongest effect for scavenging $ONOO^-$ of the 28 herbs. Hamamelitannin, the major active component of witch hazel bark, was shown to have a strong ability to scavenge $ONOO^-$. The $ONOO^-$ scavenging activity can be measured by monitoring the oxidation of DHR 123 by $ONOO^-$ with or without an antioxidant present (Figure 7) [50]. The $ONOO^-$ scavenging ability is determined at room temperature by a microplate fluorescence spectrophotometer with excitation and emission wavelengths of 485 and 530 nm, respectively. $ONOO^-$ can be purchased from Cayman Chemical Co. or derived by 3-morpholinosydnonimine (SIN-1). SIN-1 liberates NO spontaneously when in solution, causes vasodilation and inhibits platelet aggregation. It can be used both *in vivo* and *in vitro*. Using molecular oxygen, it generates both $O_2^{-\bullet}$ and NO that spontaneously form $ONOO^-$.

HOCl is a powerful oxidant which plays an important role in bactericidal function [140]. However, excess HOCl is also highly reactive toward a range of biological substrates and may cause tissue injury. One of the major targets of HOCl in vivo is α_1-antiproteinase (α_1-AP), the most important inhibitor of the proteolytic enzymes of polymorphonuclear granulocytes especially elastase, which degrades elastin [141]. It has been reported that HOCl preferentially attacks one methionine (Met) residue of α_1-AP, Met 358, resulting in a methionine sulfoxide and the immediate loss of inhibitory function. Several sulfur-containing compounds have been demonstrated to efficiently prevent HOCl-induced deleterious effects in vitro, including reduced glutathione, oxidized glutathione, and methylglutahione, as well as lipoic acid [142,143], N-acetylcysteine [144], cefalosporins [145], and histamine H_2 antagonists [146]. Many antioxidants usually can scavenge several types of ROS. For example, S-allylcysteine, a garlic-derived compound, has in vivo and in vitro antioxidant properties. It can scavenge ROS such as $O_2^{-\bullet}$, H_2O_2, ·OH and $ONOO^-$, as reviewed by Medina-Campos et al. S-allylcysteine can also scavenge both HOCl and $^1O_2$ [147]. Sulindac and sulindac sulfone can scavenge $O_2^{-\bullet}$, ·OH, NO, and $ONOO^-$, while sulindac sulfide scavenges HOCl, $O_2^{-\bullet}$, ·OH, NO, and $ONOO^-$ [148]. HOCl scavenging assay is based on the inhibition of thio-2-nitrobenzoic acid oxidation to 5.5′-dithiobis (2-nitrobenzoic acid) (DTNB) induced by HOCl. TNB has a chromophore that has maximal absorbance at 412 nm while DTNB is colorless (Figure 14). By following the decrease of absorbance at 412 nm, the HOCl scavenging activity is measured [37,148].

Conclusions

Oxidative stress caused by ROS results in an increased risk for many diseases such as inflammatory disease, cardiovascular disease, cancer, diabetes, Alzheimer's disease, cataracts, autism and aging. Antioxidants may directly react with the reactive radicals to destroy them by accepting or donating electron(s) to eliminate the unpaired condition of the radical, or they may indirectly decrease the formation of free radicals by inhibiting the activities or expressions of free radical generating enzymes or by enhancing the activities and expressions of other antioxidant enzymes. Many research models have been established in chemical and/or biological systems for studying the mechanisms of action of antioxidants and for identifying new antioxidants, especially from natural substances. We have reviewed the antioxidant mechanisms and experimental approaches such as direct radical scavenging, ion chelating and enzyme activities in cell-free chemical systems. The preventive and inhibitory effects of antioxidants on lipid peroxidation, DNA damage, and protein modification caused by ROS are also discussed. The chemical approaches are simple and facilitate the study of the total antioxidant activity of antioxidants and the precise mechanisms of action of antioxidants. However, cell free systems do not take bioavailability and metabolic factors into consideration, and thereby the data generated from these systems require the confirmation from cell-based systems or *in vivo* studies. Recently, clinical data has shown a correlation between various types of oxidative stress measurements and clinical findings in actual individuals with diseases. For example, a significant correlation was found between the degree of mercury intoxication and oxidative stress biomarkers present in autism patients [6,149]. Also, the correlation has been observed between tissue culture models of oxidative stress and the actual pathologies observed in clinical diseases. For example, researchers have identified very similar oxidative stress pathology in human tissue culture model systems as have been observed in the brains of patients with autism, particularly following exposure to low-dose mercury from Thimerosal [150].

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Abbreviations

AAPH	2, 2′-azobis(2-amidinopropane) hydrochloride
$ABTS^{+\cdot}$	2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) cation radical
ADHP	10-acetyl-3,7-dihydroxyphenoxazine
Aβ	Amyloid β protein
CAT	catalase
DCF	2′,7′-dichlorofluorescein
DHE	dihydroethidium
DHR123	Dihydrorhodamine 123
DMPO	5,5-dimethyl-1-pyrroline-N-oxide
$DMPD^{+\cdot}$	N,*N*-dimethyl-*p*-phenylenediamine dihydrochloride cation radical
DPPH	1,1-diphenyl-2-picryl-hydrazyl free radical
DMTU	N,N′-dimethyl-thiourea
DTNB	5.5′-dithiobis (2-nitrobenzoic acid)
2-EOH	2-hydroxyethidium
GPX	glutathione peroxidase
GRd	glutathione reductase
GSH	reduced glutathione
GSSG	glutathione disulfide
GST	glutathione *S*-transferase
H_2DCFDA	2′,7′-dichlorodihydrofluorescein diacetate
$HO_2^{\bullet}$	perhydroxyl radical
H_2O_2	hydrogen peroxide
HOCl	hypochlorous acid
HPLC	high performance liquid chromatography
LDL	low-density lipoprotein
Luc	lucigenin
LY83583	6-anilino-5, 8-quinolinequinone
MAD	malondialdihyde
NBT	Nitro blue tetrazolium
NO	nitric oxide
NOX	NAD(P)H oxidase
$^1O_2$	singlet oxygen
$O_2^{-\bullet}$	superoxide anion

•OH	hydroxyl radical
$ONOO^-$	peroxynitrite
ONOOH	peroxynitrous acid
RNS	reactive nitrogen species
ROS	reactive oxygen species
SeMet	selenomethionine
SCE	saturated calomel electrode
SOD	superoxide dismutase
TBARS	thiobarbituric acid reactive species
TrxR	thioredoxin reductase
XO	xanthine oxidase

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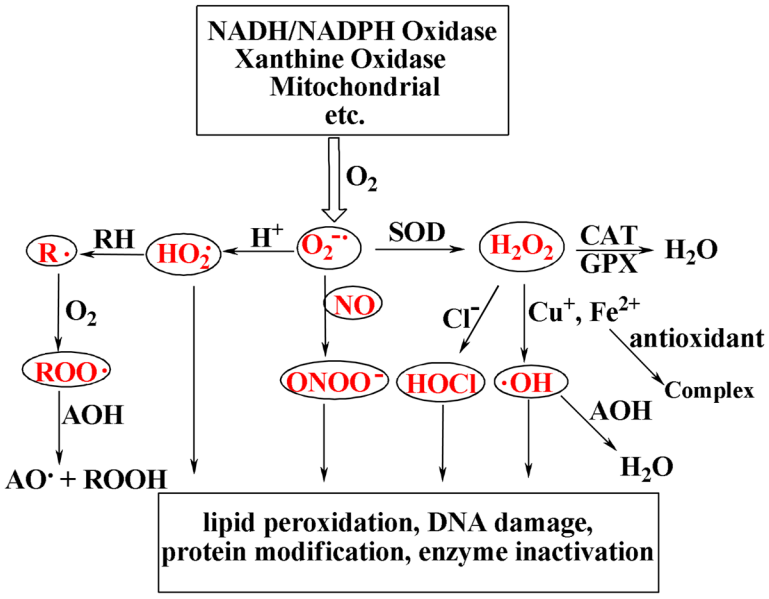


Fig. 1.

Summary of ROS types and sources, and action point of antioxidants. $O_2^{-\bullet}$, superoxide anion; $HO_2^{\cdot}$, perhydroxyl radical; ·OH, hydroxyl radical; H_2O_2, hydrogen peroxide; NO, nitric oxide; HOCl, hypochlorous acid; $ONOO^-$, peroxynitrite; R·, lipid alkyl radical; RH, lipid; ROO·, lipid peroxyl radical; ROOH, lipid hydroperoxide; SOD, superoxide dismutase; CAT, catalase; and GPX, glutathione peroxidase.

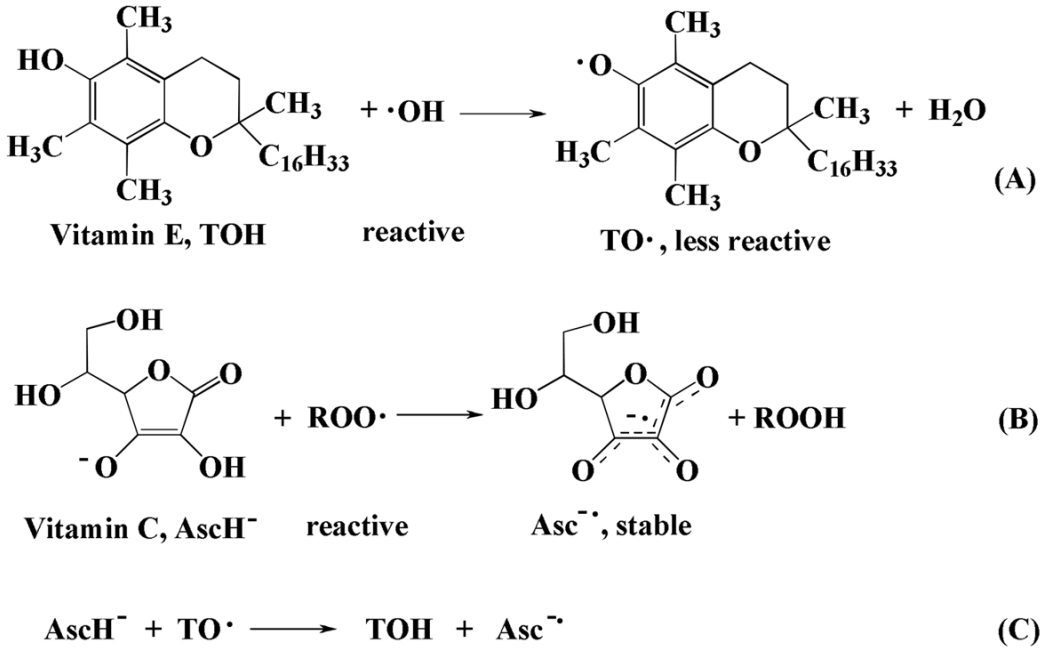


Fig. 2.

Direct reactions of vitamin E (TOH) with hydroxyl radical (A) and vitamin C ($AscH^-$) with lipid peroxyl radical (ROO·) (B), and regeneration of vitamin E from vitamin C (C).

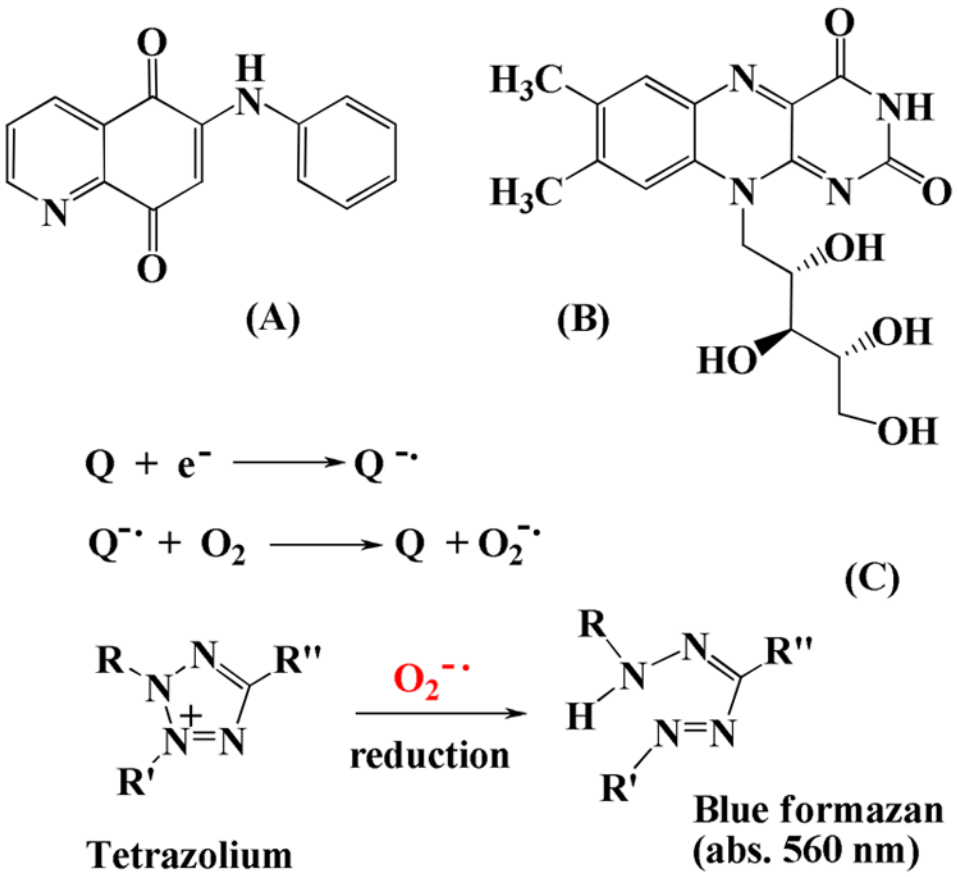


Fig. 3.

Structure of quinones (Q): LY83583 (A), riboflavin (B) and the formation of superoxide and its reaction with NBT (C), where R, R′ and R″ represent p-nitrophenyl, o-methoxyphenyl, and phenyl groups, respectively.

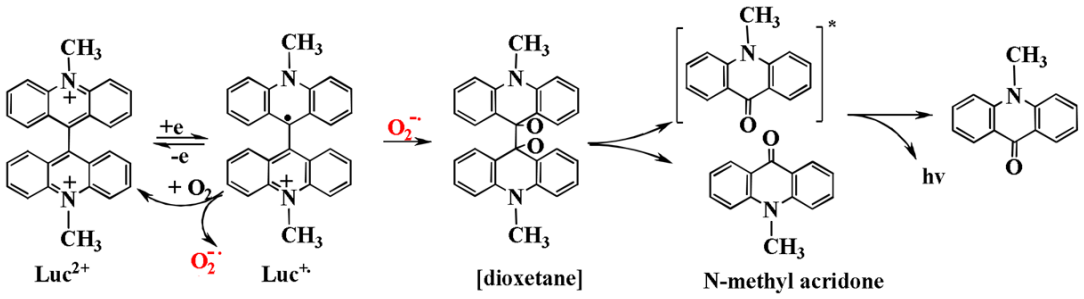


Fig. 4.
Process of lucigenin (Luc) luminescence.

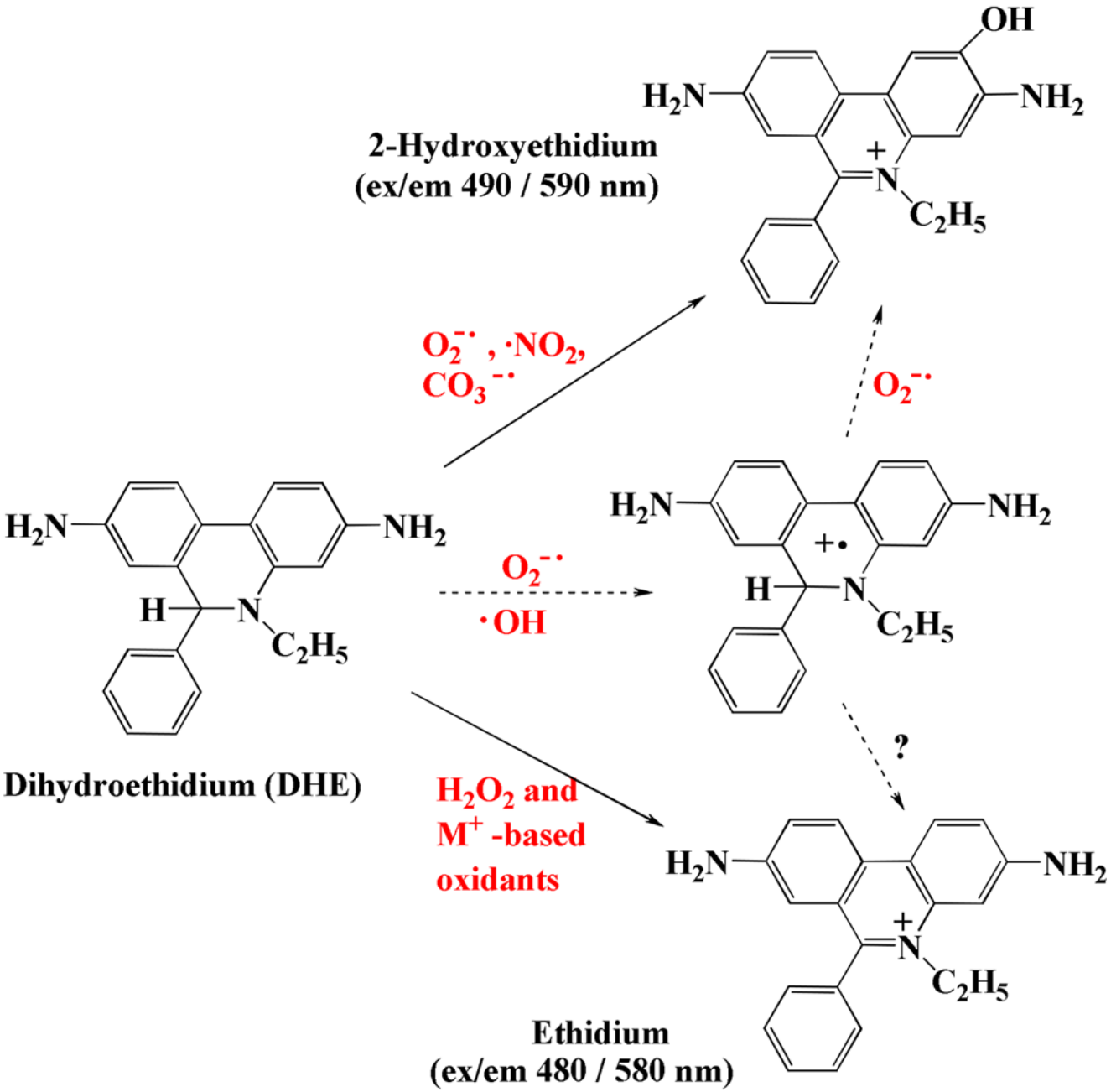


Fig. 5.

Structures of dihydroethidium (DHE), 2-hydroxyethidium (EOH) and ethidium, and hypothesized reaction pathways to account for the formation of DHE-derived red fluorescence. EOH is basically formed from superoxide and possibly involving peroxynitrite/CO_2 while ethidium is mainly formed from hydrogen peroxide pathways involving metal proteins. Dotted arrows indicate possible intermediate pathways involved in the formation of these products.

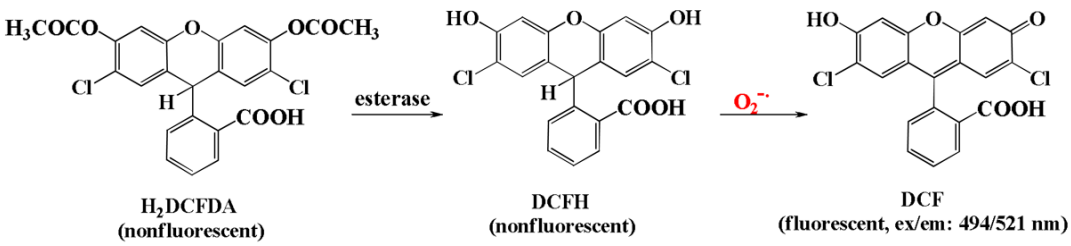
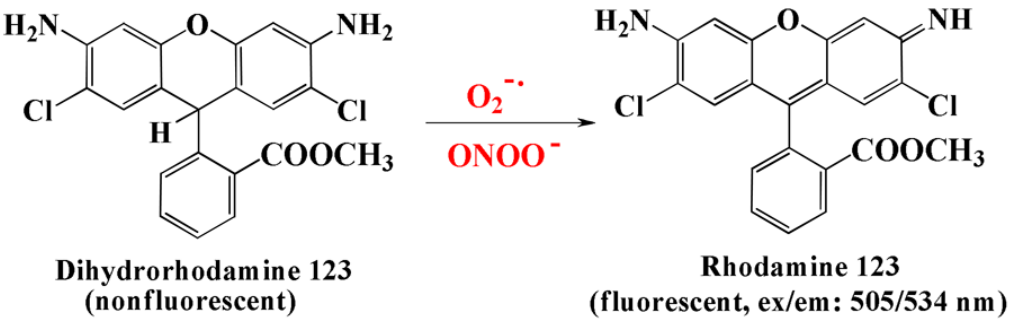


Fig. 6.

The structures of 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA), 2′,7′-dichlorodihydrofluorescein (DCFH) and 2′,7′-dichlorofluorescein (DCF); and the reaction pathway of superoxide detection. DCF has an absorption maximum at 494 nm and emission maximum of 521 nm.



Dihydrorhodamine 123
(nonfluorescent)

Rhodamine 123
(fluorescent, ex/em: 505/534 nm)

Fig. 7.
Detection of peroxynitrite and superoxide by dihydrorhodamine 123.

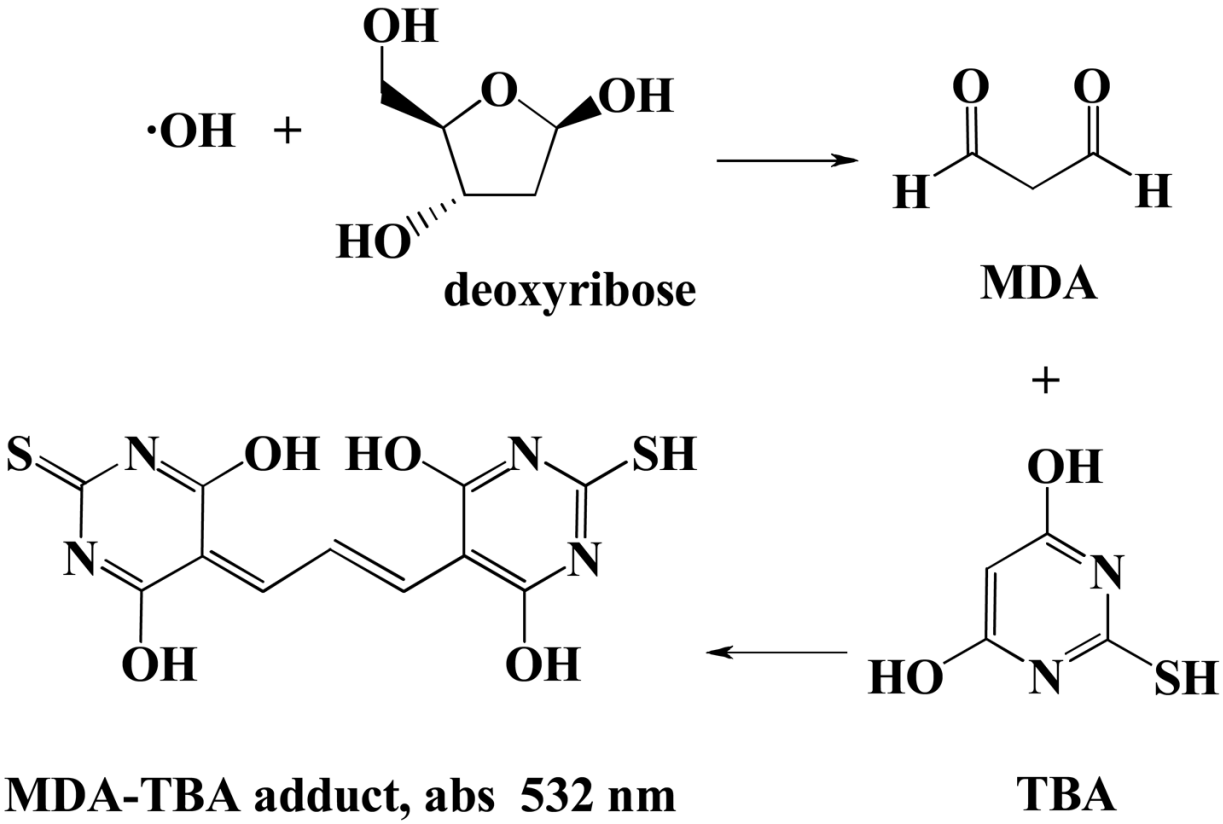
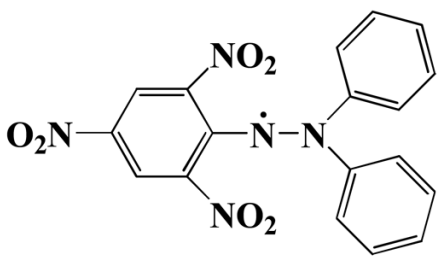
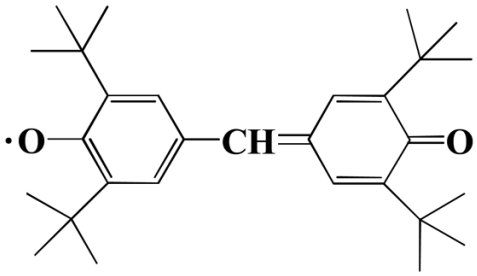


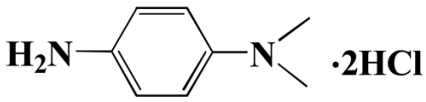
Fig. 8.
Reaction pathway of thiobarbituric acid reactive species (TBARS) assay of hydroxyl radical.



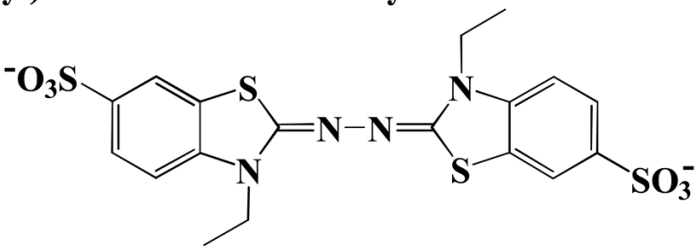
DPPH (2,2'-diphenyl-1-picrylhydrazyl)



Galvinoxyl



DMPD (N,N-dimethyl-p-phenylene-diamine dihydrochloride)



ABTS (2,2'-azinobis(3-ethylbenzo-thiazoline-6-sulfonate))

Fig. 9.
Structures of DPPH, galvinoxyl, ABTS and DMPD.

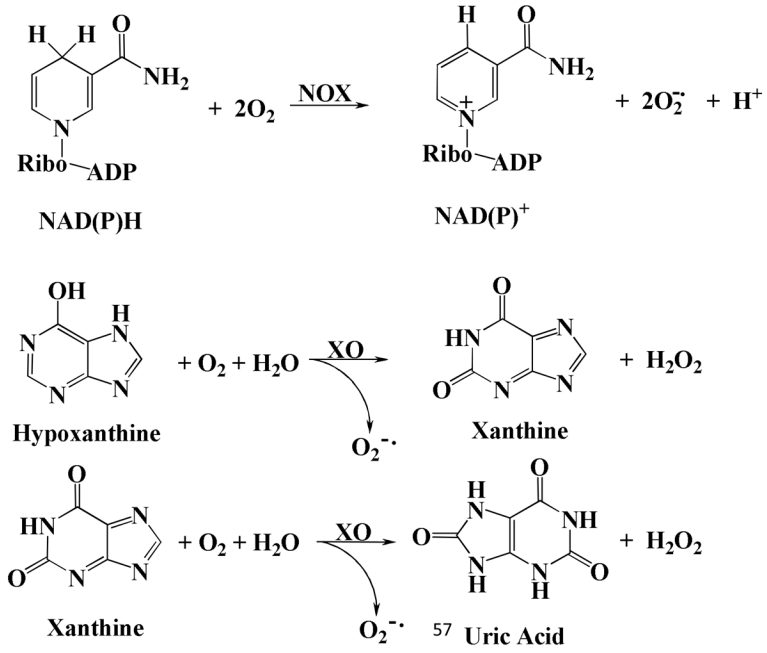


Fig. 10.
Superoxide and hydroperoxide generation from NAD(P)H oxidase (NOX) and xanthine oxidase.

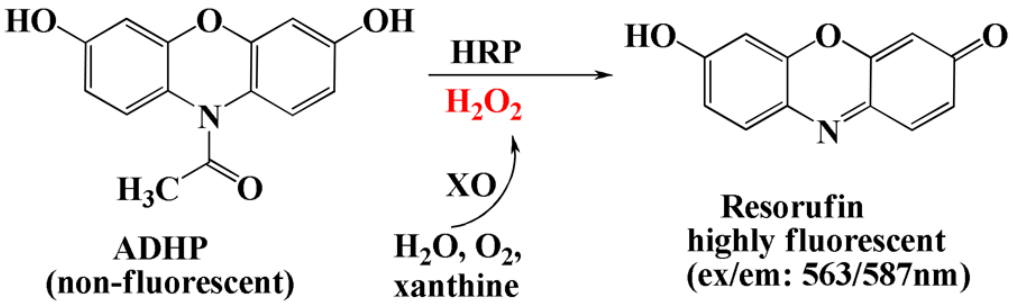


Fig. 11.
10-acetyl-3,7-dihydroxyphenoxazine (ADHP) fluorescent assay of xanthine oxidase activity.

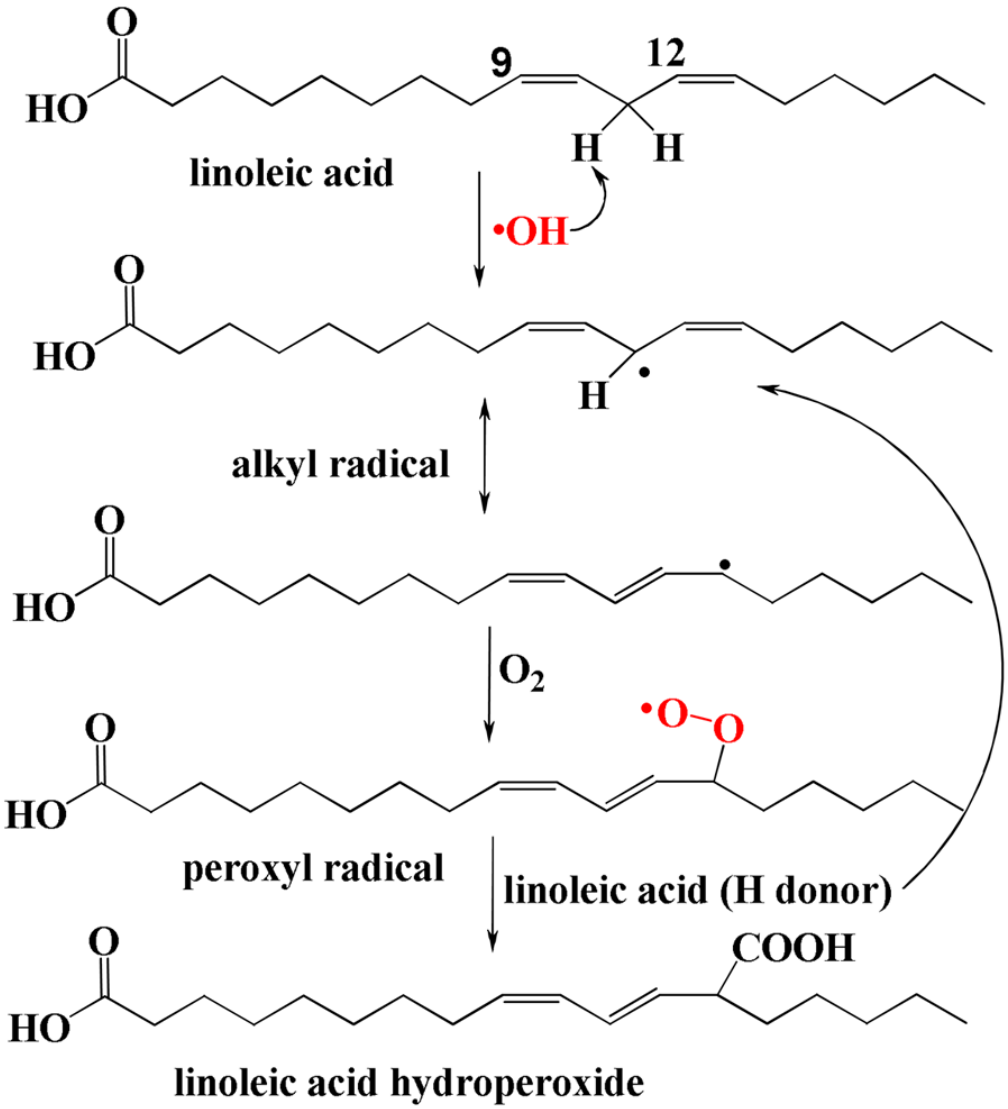


Fig. 12.
Mechanism of linoleic acid peroxidation initiated by ·OH radical.

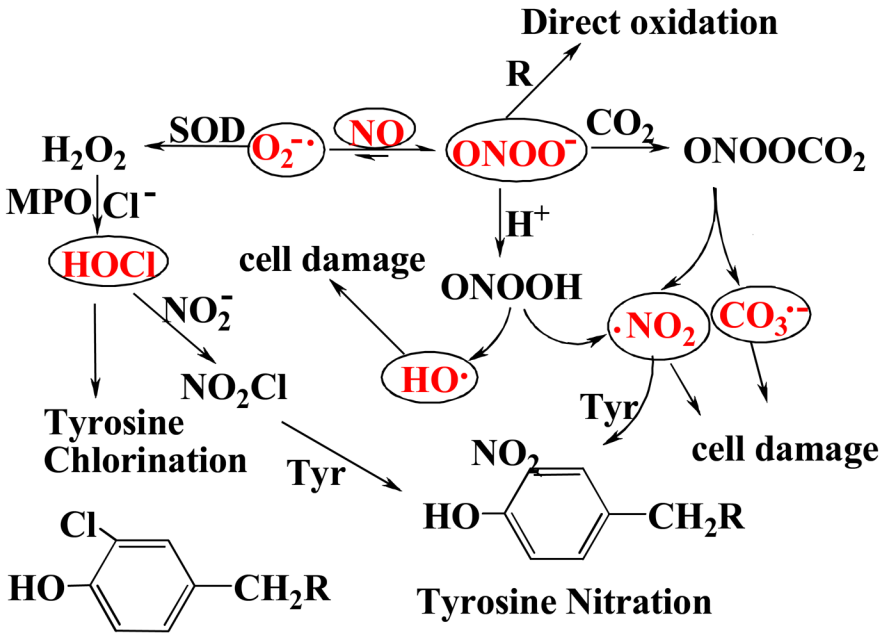
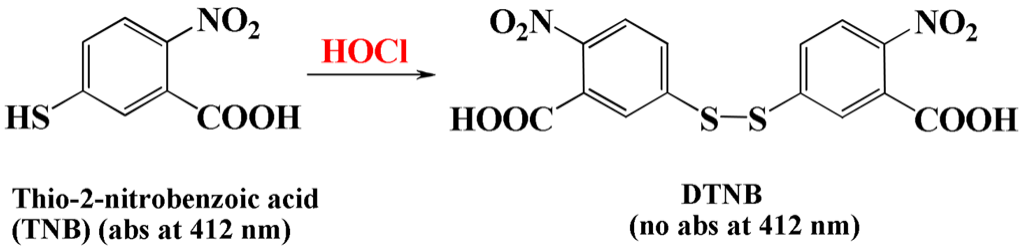


Fig. 13.

Potential pathways of nitrotyrosine and chlortyrosine formation. $ONOO^{-}$, peroxynitrite; HOCl, hypochlorous acid; NO_2Cl, nitryl chloride; Cl^{-}, chloride; H_2O_2, hydrogen peroxide; $CO_3^{-\cdot}$, carbonate radical; $\cdot NO_2$, nitrogen dioxide radical; ONOOH) NO_2^{-}, peroxynitrous acid; NO_2Cl, nitryl chloride; MPO, myeloperoxidase; Tyr, tyrosine.



Thio-2-nitrobenzoic acid (TNB) (abs at 412 nm)

DTNB (no abs at 412 nm)

Fig. 14.

Detection of hypochlorous acid by 5.5′-dithiobis (2-nitrobenzoic acid) (DTNB),