Chapter 12
In-vitro Models to Assess Antioxidant Potential

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12.1 Introduction

The events of World War II directly led to the genesis of free radical biochemistry and the deadly effects of ionizing radiations were attributed to the formation of reactive oxygen species (ROS). Since then ROS and reactive nitrogen species (RNS) have earned bad reputation (Gilbert 1981). ROS also include free radicals and non-radical derivatives of oxygen (Namiki 1990). During respiration in biological systems, a substantial fraction of oxygen is partly reduced. Such partially reduced oxygen molecules and their derivatives are known as ROS, which are extremely reactive pro-oxidants and lethal. The reactivity of ROS can cause functional impairment in biological systems, eliciting a number of degenerative complications like mutagenesis, circulatory disturbances, carcinogenesis and aging (Halliwell and Gutteridge 1985). Inside the cell, free radicals are routinely produced in the metabolism within the peroxisomes, mitochondria through xanthine oxidase, phagocytosis, inflammation processes, physical exercise, arachidonate pathways and ischemia. Many external factors stimulate the production of free radicals viz., smoking, pollutants, drugs, radiation, pesticides, industrial solvents and ozone. It is an irony that essential elements for survival such as oxygen have harmful effects on the human body through ROS generation by prooxidative enzyme systems, lipid peroxidation, glycoxidation etc. which creates an imbalance leading to oxidative stress (Lobo et al. 2010; Kumar and Pandey 2015). However, living systems have natural enzymatic and non-enzymatic antioxidant defenses which counteract the harmful effects of free radicals and other oxidants (El-Bahr 2013; Kumar and Pandey 2014).

An antioxidant in biological systems has been defined as any compound that when present at small level compared to that of an oxidizable substrate, considerably retards or averts oxidation of the substrate (Halliwell 1990). But in food systems, antioxidants have been categorized as compounds, which are able to prevent or delay

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the oxidation of easily oxidizable substances viz., lipids in small amounts (Chipault 1962). Antioxidants may interfere at any of the three main stages of the free radical mediated oxidative processes i.e., initiation, propagation, and termination (Cui et al. 2004; Kumar et al. 2013a; Sharma et al. 2018). In foods, it is necessary to measure the effectiveness of natural antioxidants for preservation and defense against oxidative destruction to evade harmful changes and loss of nutritional and commercial values. Thus, it is desirable to have a rapid method for qualitative and quantitative assessment of the potential antioxidant capacity in food products. Such a method would be a useful tool to make a choice among diverse species, varieties, degree of maturation, and culture conditions, so as to obtain high content of natural antioxidants in food products (Leonardi et al. 2000). Techniques that measure total antioxidant activities of foods can be used to assess the relationship between important dietary factors and disease prevention, thus reducing human suffering by helping to pick vital dietary sources of antioxidants. Various in-vitro methods used to study the antioxidant property of food products, plant extracts, commercial antioxidants and pure compounds are described below.

12.2 Peroxyl Radical Scavenging Capacity Assay

One of the most important radicals generated due to oxidative stress is the peroxyl radical ($ROO^{\cdot}$). It is associated with the oxygenation of unsaturated fatty acids which is a reactive target in the biological system. Peroxyl radicals are naturally produced by the electrophilic addition of molecular oxygen and the activated singlet oxygen by abstraction of an allylic hydrogen atom from the 1,4-pentadien system of the major part of unsaturated fatty acid carbon chains and is further stabilized by a double-bond conjugation. $ROO^{\cdot}$is involved in further decomposition and free radical stabilization till production of fatty acid rancidity marker compounds. Hence, these events explain the loss of membrane function triggered by the damage to the lipid bilayer and eventually undesirably affecting the biological function. Generation of free radicals and reaction monitoring are the most vital factors for the evaluation of antioxidant activity against peroxyl radicals. Free radicals can be generated either by the action of enzymes like lipoxygenase, or by the thermal decomposition of an azo-bis compound (Fig. 12.1a) (Scalzo et al. 2012). A sensitive assay has been developed to monitor peroxyl radical scavenging capacity of water- and lipid-soluble antioxidant compounds, food stuffs and plant extracts. The assay is based on the degree of inhibition of oxidation of the dye dichlorofluorescin diacetate (DCFH-DA) by antioxidants that have the competence to scavenge peroxyl radicals, produced by thermal degradation of 2,2′-azobis(amidinopropane) (ABAP). Thermal degradation of ABAP produces peroxyl radicals ($ROO^{\bullet}$) which oxidize non-fluorescent dichlorofluorescein (DCFH) to fluorescent dichlorofluorescein (DCF) (Fig. 12.1b). The degree of retardation of DCFH oxidation, by antioxidants that scavenge peroxyl radicals is used as the basis for calculation of antioxidant activity (Adom and Liu 2005).

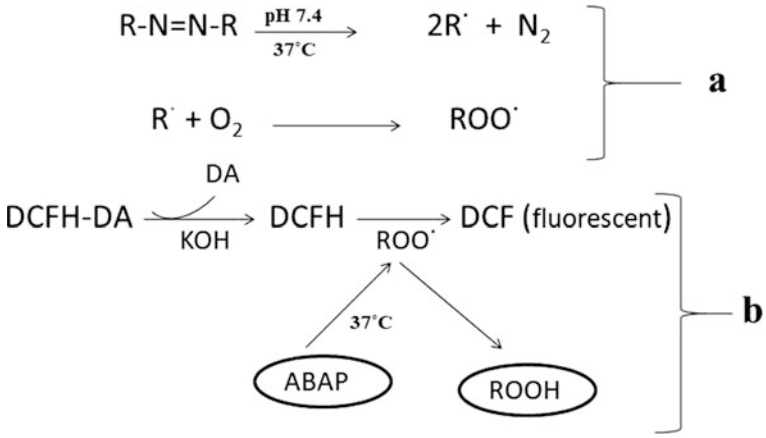


Fig. 12.1 Mechanism of peroxyl radical production and scavenging action. (**a**) Thermal decomposition of an azo-bis compound (R–N=N–R) by oxygen at physiological pH to yield peroxyl radical (ROO˙). (**b**) Reaction mechanism of peroxyl radical scavenging capacity assay. *Abbreviations*: *DCFH-DA* dichlorofluorescindiacetate, *ABAP* 2,2′-azobis(amidinopropane), *DCFH* nonfluorescentdichlorofluorescein, *DCF* fluorescent dichlorofluorescein

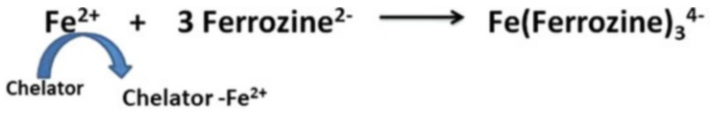


Fig. 12.2 Reaction mechanism of metal ion chelating assay

12.3 Metal Ion Chelating Assay

The metal ion chelating assay is based on the binding affinity of Ferrozine with Fe^{2+} ions. Ferrozine is a sulfonated derivative of the parent compound 3-(2-pyridyl)-5,6-bis-(4-phenylsulfonic acid)-1,2,4-triazine and forms a pink colored water soluble iron (II) complex (Carter 1971) (Fig. 12.2). Thus, the chelating activity of the plant extracts/samples and standard antioxidant compounds (BHA, BHT and EDTA) for ferrous ions is measured based on this mechanism. Redox chemistry of iron has been implicated in both the occurrence and the rate of lipid peroxidation (LPO). Reaction of Fe_3^+ with lipid hydroperoxides produces radicals that begin a chain reaction by reacting with other molecules producing MDA, a typical peroxidation marker. LPO also inflicts damage to unsaturated fatty acids results in decreased membrane fluidity and consequently leading to several pathological alterations. The transition metal ion, Fe_2^+ has the capacity to move single electrons by which it can permit the production and propagation of many radical reactions, even beginning with comparatively non-reactive radicals. To circumvent ROS generation related with redox active metal catalysis, chelation of the metal ions is the major approach. Iron can trigger LPO by the Fenton reaction and also speeds up peroxidation by breaking down lipid

hydroperoxides into peroxyl and alkoxyl radicals that can abstract hydrogen and further propagate the peroxidation chain reaction. Ferrozine can quantitatively form complexes with Fe_2^+. However, formation of pink-red colored complexes decreases in the presence of compounds possessing chelating ability which can be measured spectrophotometrically at 562 nm. Therefore, monitoring the rate of reduction of color intensity helps to measure the chelating activity of the co-existing chelator present in the reaction mixture (Dinis et al. 1994; Kumar and Pandey 2012).

12.4 Superoxide Anion Radical Scavenging Capacity Assay

Superoxide anion radical ($O_2^{\bullet -}$) isproduced by the reduction of molecular oxygen. It is also formed in aerobic cells due to leakage of electrons from the electron transport chain. Superoxide radicals ($O_2^{\bullet -}$) are also produced by activated phagocytes such as monocytes, eosinophils, neutrophils and macrophages and play an important role in the phagocytosis of bacteria. In living organisms, $O_2^{\bullet -}$ is removed by the enzymes called superoxide dismutases (SOD). Thus, SOD keeps in check the excess production of superoxide radicals which may lead to oxidative damage (Halliwell and Gutteridge 1985). The in-vitro superoxide radical scavenging activity is based on the chemical anion radical generating system which includes phenazine methosulfate (PMS), nicotinamide adenine dinucleotide (NADH) and nitroblue tetrazolium (NBT). Superoxide radicals are produced within PMS-NADH systems by the oxidation of NADH and are examined by the reduction of NBT. Briefly, the generation of superoxide anion is measured in a reaction mixture containing NADH, NBT and PMS in phosphate buffered saline (PBS) at pH 7.4. The reduction of NBT is detected by measuring the change in absorbance at 560 nm (Liu et al. 1997; Shareef et al. 2014).

12.5 Hydroxyl Radical ($OH^{\bullet}$) Scavenging Assay

In cell metabolism the hydroxyl radicals ($^{\bullet}OH$) are the extremely reactive creations of ROS generated by successive 1-electron reduction of molecular oxygen (O_2). Hydroxyl radicals are mainly accountable for the cytotoxic effects in aerobic organisms. Several studies have reported protection from the harmful effects of hydroxyl radical by several medicinal plants, thus endorsing their antioxidant properties. The hydroxyl radical is one of the strongest known oxidants. It is capable of nonspecifically oxidizing all classes of biological macromolecules including lipids, carbohydrates, proteins, and nucleic acids at almost diffusion-limited rates. Therefore, disturbance in $^{\bullet}OH$ homeostasis may inflict oxidative injury that leads to many diseases like atherosclerosis, diabetes, cancer, arthritis etc. (Ozyurek et al. 2008). $OH^{\bullet}$ radicals are mainly generated in the body via the Haber-Weiss reaction where the superoxide anion radical ($O_2^{\bullet -}$) reduces cellular Fe(III) to Fe(II). Thus, it initiates the Fenton reaction between Fe^{2+} and $H_2O_2^-$ (Fig. 12.3).

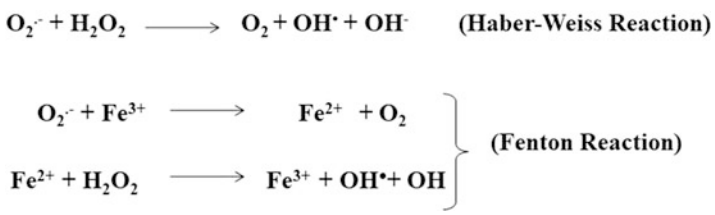


Fig. 12.3 Hydroxyl radical generation by Haber-Weiss reaction and Fenton reaction

The hydroxyl radical scavenging assay is based on the inhibition of p-nitrosodimethylaniline (pNDA) bleaching. Hydroxyl radicals are generated through Fenton reaction in which iron (II) is oxidized by hydrogen peroxide to iron (III) and are capable of bleaching pNDA specifically. The scavenging of these radicals is measured by the extent of inhibition of bleaching in the presence of antioxidants (Kunchandy and Rao 1990).

12.6 DPPH Radical Scavenging Assay

DPPH (2,2-diphenyl-1-picrylhydrazyl) is an organic compound composed of stable free radical molecules. DPPH assay has been mostly used as a reliable, quick, and reproducible method for screening in-vitro antioxidant activity of pure compounds, food products as well as natural plant extracts. The violet coloured DPPH is converted to a yellow product in the presence of antioxidants. Antioxidants react with DPPH, a nitrogen-centered free radical, which is a stable, and convert it to α, α-diphenyl-β-picryl hydrazine. The degree of discoloration indicates the scavenging potentials of the antioxidant extracts. The decrease in absorbance by the DPPH radical with increasing concentration of the extracts in dose dependent manner results in the rapid discolouration of the purple DPPH, suggesting that compounds present in food material or plant extracts have radical scavenging antioxidant activity owing to its proton donating ability. For assessment of the antioxidant prowess of compounds or extracts, they are permitted to react with DPPH in methanolic medium. A deep violet color of the DPPH radical in solution due to a strong absorption band centered at about 520 nm and it turn into colorless or pale yellow after neutralization (Fig. 12.4). The percentage inhibition (% scavenging activities) at diverse concentrations of the test compounds or extracts are calculated using the formula (Eq. 12.1).

$$(\%\text{Inhibition}) = \left[(A_c - A_s)\right] \times 100 \tag{12.1}$$

where A_c and As are the absorbance values of the control and the sample, respectively. Three replicates are made for each sample and results are expressed as mean ± SD.

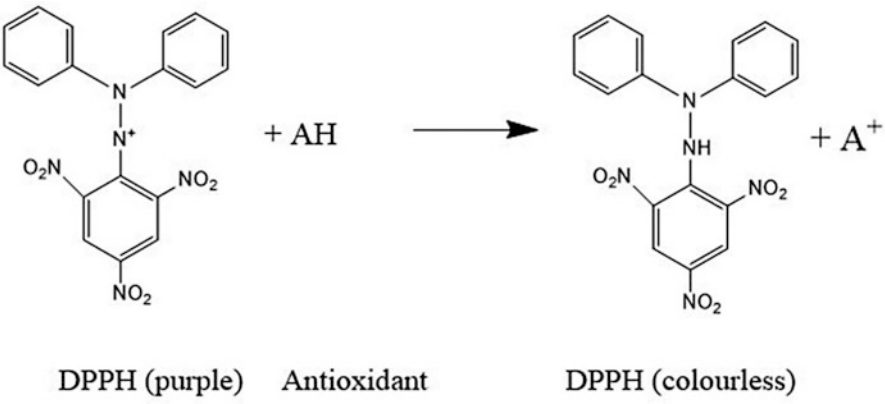


Fig. 12.4 Reaction mechanism of DPPH radical scavenging assay

The reduction of DPPH molecules could be correlated with the number of available hydroxyl groups. Chemical constituents present in food products or plant extract fractions exhibit significantly higher inhibition percentage (stronger hydrogen –donating ability) which can be positively correlated with total phenolic content (Sharma et al. 2016).

12.7 ABTS Radical Scavenging Assay

ABTS [2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)] is a chemical compound commonly used as a free radical in in-vitro antioxidant assays. Generation of the ABTS radical cation forms the basis of ABTS radical scavenging assay, which measures the total antioxidant activity of sample compounds. Initially, this method was based on the stimulation of metmyoglobin with H_2O_2 in the presence of ABTS to generate the radical cation, in the presence/absence of antioxidants, in which case the faster reacting antioxidants could also add to the reduction of the ferryl myoglobin radical. Therefore, the results in this method could be ambiguous. The decolorization technique is a relatively more targeted and absolute form of the assay as the radical is directly generated in a stable form prior to reaction with the antioxidants. In this system, the reaction between ABTS and potassium persulfate results in the direct formation of the blue/green ABTS chromophore which has absorption maxima at 734 nm (Fig. 12.5). Addition of antioxidants to the pre-formed radical cations reduces it to ABTS, which varies with the concentration and activity of the antioxidant as well as the reaction duration. Therefore, the magnitude of decolorization as % inhibition of the ABTS radical cation is measured with respect to concentration and time and is computed in comparison with the reactivity of a standard under the same conditions. The method is applicable for the study of both water-soluble and lipid-soluble antioxidants, pure compounds, food and plant extracts (Re et al. 1999).

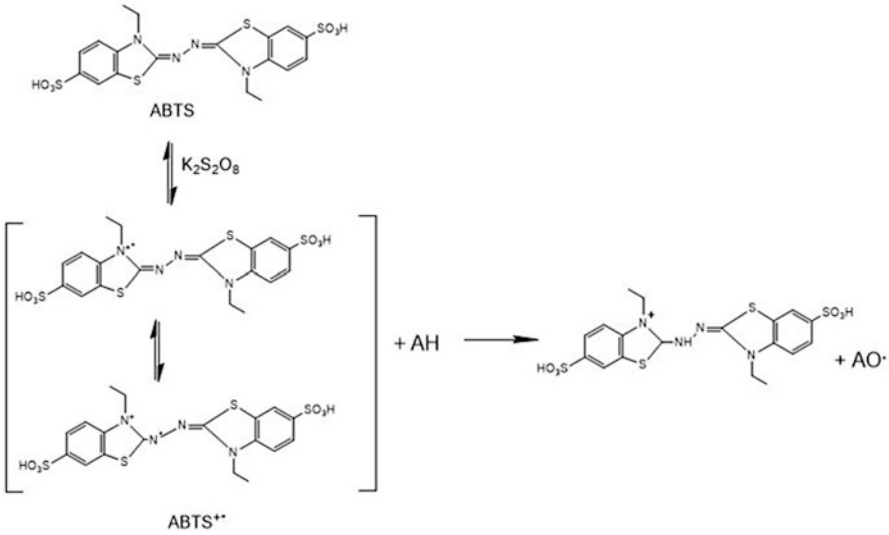
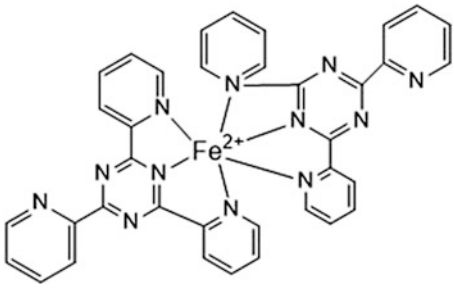


Fig. 12.5 Oxidation of ABTS molecule by potassium persulfate ($K_2S_2O_8$) to generate radical cation ABTS$^{+\bullet}$ (absorption maxima 734 nm) and its reaction with an antioxidant compound (AH)

Fig. 12.6 Fe^{2+}-TPTZ complex



12.8 FRAP Assay

The ferric reducing antioxidant power (FRAP) assay is another in-vitro antioxidant capacity assay. At low pH, when a ferric-tripyridyltriazine (FeIII-TPTZ) complex is reduced to the ferrous (FeII) form, a dark blue color develops which absorbs spectrophotometrically at 593 nm (Fig. 12.6). This reaction is nonspecific, and any half-reaction with a less-positive redox potential than the Fe-III/Fe-II-TPTZ half-reaction, under similar reaction conditions, will impel the FeIII-TPTZ reduction. The complex is reduced in the presence of an antioxidant which acts as a reductant and blue color is developed under favorable conditions. Ferrozine is commonly used with ascorbic acid, which acts as an antioxidant and a reductant to measure iron (Benzie and Strain 1996; Sharma et al. 2014, 2017a). FRAP values can be calculated

by relating the change in absorbance of the reaction mixture with those obtained from increasing concentrations of Fe^{3+} using calibration curve and expressed as mM of Fe^{2+} equivalents per kg (solid food) or per litre (liquid) of sample.

12.9 Nitric Oxide (NO) Radical Scavenging Assay

Nitric oxide (NO) and RNS are free radicals that originate from the interplay of NO with oxygen or ROS. NO exhibits reactivity with certain proteins and other free radicals such as superoxide due to its unpaired electron. The production of NO at lower levels plays crucial role in physiological processes. However, the sustained NO production can cause damaging effect in tissues and plays role in the pathological manifestation of disease states like inflammation and endotoxin shock (Gomes et al. 2006). Moreover, NO and $O_2^{\bullet -}$, produced by cells of the immune system during the oxidative burst, may react with each other, generating significant amounts of the highly reactive peroxynitrite anion ($ONOO^-$), which is a strong oxidative molecule. Therefore, toxicity of NO greatly increases when it reacts with the superoxide radical, and hence scavenging of NO by antioxidants is crucial for biological system homeostasis (Carr et al. 2000; Boora et al. 2014). The NO radical scavenging assay is based on the reaction of NO produced from aqueous sodium nitroprusside solution with oxygen to generate nitrite ions, at physiological pH. The nitrite ions are further quantified by Griess Illosvoy reaction (Hazra et al. 2008). Nitrite is detected and measured by formation of a red pink colour upon reaction with Griess reagent, which typically comprises of naphthylethylene diamine dihydrochloride, sulphanilamide and phosphoric acid.

12.10 Hydrogen Peroxide (H_2O_2) Scavenging Capacity Assay

Among ROS, H_2O_2 is a relatively stable, non-radical oxidant, having the ability to diffuse across biological membranes. It is produced by reduction of molecular oxygen via two electrons or by dismutation with or without the enzyme SOD (Fig. 12.7).

The biological system generates the superoxide anion radical $O_2^{\bullet -}$ from molecular oxygen and unquestionably produces H_2O_2 by the dismutation reaction, the rate of which depends on pH and O_2 concentration. H_2O_2 induces the oxidative

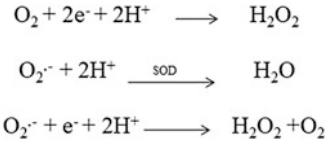


Fig. 12.7 Hydrogen peroxide production by reduction of molecular oxygen

degradation of most biological macromolecules such as lipids, proteins or enzymes, carbohydrates and nucleic acids, through generation of the hydroxyl radical ($OH^{\cdot}$), the most potent and reactive ROS. H_2O_2 in presence of Cu(I) or Fe(II) salts in-vivo produces OH radicals by using transition metal ion-catalyzed Fenton and Haber–Weiss reactions (Ozyurek et al. 2010). Besides that, the most frequently used in-vitro H_2O_2 scavenging assay is carried out on the principle that there is a decrease in absorbance of H_2O_2 upon oxidation in the presence of antioxidants. A solution of H_2O_2 prepared in phosphate buffer (pH 7.4) is used and the solution of sample extract or pure compound in phosphate buffer is added which acts as an antioxidant. The absorbance of the reaction mixture is documented spectrophotometrically at 230 nm (Ak and Gulcin 2008). The percentage H_2O_2 scavenging by standard compounds is calculated as per equation (Eq. 12.2).

$$H_2O_2 \text{ scavenging effect } (\%) = (1 - A_s/A_c) \times 100 \tag{12.2}$$

where A_c is the absorbance of the control solution and A_s is the absorbance in the presence of standard compound which acts as a scavenger.

12.11 Hypochlorous Acid (HOCl) Radical Scavenging Capacity Assay

Hypochlorous Acid (HOCl) radicalis produced in the living system by the heme-peroxidase enzyme myeloperoxidase from activated neutrophils, monocytes, and macrophages. The HOCl is the well-known strongest oxidant formed by neutrophils and is recognized as a powerful pro-inflammatory agent. Moreover, it can also react with O_2 to produce one more microbicidal species $OH^{\bullet}$. These HOCl radicals may be regulated by the internal antioxidant systems such as SOD, catalase, glutathione peroxidase (GPx), and glutatione S-transferase. However, when HOCl levels exceed beyond control, they may directly react with amino acids, proteins, carbohydrates, thiol-containing antioxidants, membrane lipids, and nucleic acids. These processes cause several inflammatory diseases such as arthritis, cystic fibrosis, asthma, heart diseases, and even certain forms of cancer. Thus, the use of external antioxidants from natural sources is required for treating these oxidative stress mediated anomalies. The HOCl radical scavenging capacity assay is based on the elimination of the catalase peak due to the destruction of the heme prosthetic group by HOCl and is determined spectrophotometrically at 404 nm. The HOCl scavenging capacity of the compounds is measured by the inability of HOCl to reduce the peak in a concentration dependent manner. A solution of catalase is mixed with HOCl with increasing concentrations of sample compounds acting as HOCl radical scavengers (Arguello-Garcia et al. 2010).

12.12 Reducing Power Assay

Basic principle underlying the reducing power assay is that substances with a reduction potential react with potassium ferricyanide (Fe^{3+}) to form potassium ferrocyanide (Fe^{2+}), which subsequently reacts with ferric chloride to form ferric ferrous complex exhibiting an absorption maxima at 700 nm. Antioxidants acting as electron donor reduce the Fe^{3+} complex to its Fe^{2+} (Fig. 12.8) (Kumar et al. 2014a).

Ascorbic acid (various concentrations) is routinely used as standard antioxidant compound and increased absorbance of the reaction mixture specifies increased reducing power (Jayanthi and Lalitha 2011; Kumar et al. 2013b). Conversion of the yellow coloured reaction mixture to the bluish green during assay signifies reducing action of the compound and intensity of the colour produced further depends upon the reducing ability of the test compound. Natural foods/vegetables/plant extracts show considerable reducing power at higher concentrations as shown by absorbance values signifying dose dependent response. There are several articles on dose dependent reducing power of plant extracts. Phytochemicals particularly flavonoids have been stated to function as reducing agents. So the reducing ability of plant based natural products could be ascribed to the presence of phenolic compounds which might act as reductones.

12.13 Phosphomolybdate Assay

The phosphomolybdate assay is based on the reduction of Mo(VI) at acidic pH. It is regularly used to assess the total antioxidant capacity of food supplements/plant extracts and other compounds using propyl gallate as standard (Negi and Jayaprakasha 2004). In this method molybdenum (VI) is reduced to a green coloured molybdenum (V) complex by antioxidants which shows maximum absorption at 695 nm. The total antioxidant capacity is expressed as μg propyl gallate equivalents per gram of sample. The difference in antioxidant capacity of different samples is a function of differences in their chemical composition. The antioxidant activities of the specific phenolic compounds depend on structural features namely, the number and position of phenolic hydroxyl or methoxyl groups, flavone hydroxyl, free carboxylic groups, keto groups (Kumar et al. 2014b).

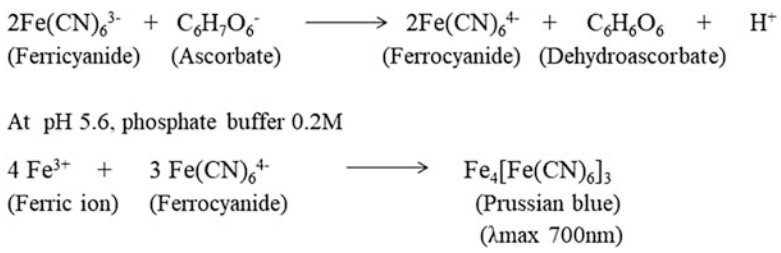


Fig. 12.8 Mechanism of reducing power assay

12.14 Singlet Oxygen ($^1O_2$) Scavenging Capacity Assay

Singlet oxygen ($^1O_2$) is a high energy form of oxygen which exists in the singlet state with a total quantum spin of zero. It was first predicted in 1931 as a meta-stable intermediate state of oxygen in dye-sensitized photo-oxygenations. However, in 1964, it was finally recognized that $^1O_2$ was same entity whether generated in sensitized photo-oxygenations, through radio frequency or via chemical reactions with H_2O_2/NaOCl. It is an important free radical in the living system associated with cell signaling and oxidative stress (Luqman et al. 2016). The $^1O_2$-scavenging capacity is determined by a fluorescence method that consists of monitoring the oxidation of the non-fluorescent dihydrorhodamine 123 (DHR) to fluorescent rhodamine by the reaction with $^1O_2$. The singlet oxygen species is generated by thermal breakdown of an earlier synthesized water-soluble endoperoxide [disodium 3,3′-(1,4-naphthalene)bispropionate] at 37 °C. The results are expressed in terms of percentage inhibition of $^1O_2$-induced oxidation of DHR (Rebeiro et al. 2015).

12.15 Lipid Peroxidation Inhibition Assay

The magnitude of oxidative damage caused in a lipid based system is difficult to determine because of the short half-lives of free radicals as well as many of the products primarily formed by attack of free radical on polyunsaturated fatty acids, the electron rich substrates. Therefore, the revealing of oxidative stress depends typically on the quantification of compounds known as thiobarbituric acid reactive substances (TBARS) such as malondialdehyde (MDA). The TBARS are the byproducts resulting from the degradation of primary products of free radical attack on lipids (Antolovich et al. 2002; Kumar and Pandey 2013). The reaction between MDA and 2-thiobarbituric acid at high temperature and low pH produces a pink colored compound which is a measured spectrophotometrically at 532 nm. The intensity of the color produced varies with the degree of lipid peroxidation induced by free radicals. It is very frequently used method for lipid peroxidation assay (Oakes and Kraak 2003; Sharma et al. 2017b).

12.16 Conclusion

Interest in antioxidants, especially those intended to thwart the apparent harmful effects of free radicals in the human system along with the deterioration of lipids and other components of foodstuffs, is rising. This chapter describes a comprehensive account of the in-vitro methods commonly used for the evaluation of antioxidant potential of different food substances, plant extracts and other compounds. Most of the in-vitro models for determining antioxidant activity rely on three important

mechanisms namely, free radical scavenging, reducing property and metal ion chelating abilities. Mechanisms related with different in-vitro assays have been described in specific methods. These methods are very useful for the assessment of antioxidant evaluation of vegetables, fruits and other food products, food supplements, neutraceuticals and pharmaceutical agents.

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References

Adom KK, Liu RH. Rapid peroxyl radical scavenging capacity (PSC) assay for assessing both hydrophilic and lipophilic antioxidants. J Agric Food Chem. 2005;53:6572–80.

Ak T, Gulcin I. Antioxidant and radical scavenging properties of curcumin. Chem Biol Interact. 2008;174:27–37.

Antolovich M, Prenzler PD, Patsalides E, et al. Methods for testing antioxidant activity. Analyst. 2002;127:183–98.

Arguello-Garcia R, Medina-Campos ON, Perez-Hernandez N, et al. Hypochlorous acid scavenging activities of thioallyl compounds from garlic. J Agric Food Chem. 2010;58:11226–33.

Benzie IFF, Strain JJ. The ferric reducing ability of plasma as a measure of antioxidant power: the FRAP assay. Anal Biochem. 1996;239:70–6.

Boora F, Chirisa E, Mukanganyama S. Evaluation of nitrite radical scavenging properties of selected zimbabwean plant extracts and their phytoconstituents. J Food Process. 2014;2014:918018.

Carr AC, Mccall MR, Frei B. Oxidation of LDL by myeloperoxidase and reactive nitrogen species reaction pathways and antioxidant protection. Arterioscl Throm Vas. 2000;20:1716–23.

Carter P. Spectrophotometric determination of serum ion at the submicrogram level with a new reagent (Ferrozine). Anal Biochem. 1971;40:450–8.

Chipault JR. Antioxidants for use in foods. In: Lundberg WO, editor. Autoxidation and antioxidants. 2nd ed. New York: Interscience; 1962. p. 477–542.

Cui K, Luo X, Murthy MRV. Role of oxidative stress in neurodegeneration: recent developments in assay methods for oxidative stress and nutraceutical antioxidants. Prog Neuro Psycopharma Biol Psychiatry. 2004;28:771–99.

Dinis TCP, Madeira VMC, Almeida LM. Action of phenolic derivatives (acetaminophen, salicylate, and 5-aminosalicylate) as inhibitors of membrane lipid peroxidation and as peroxyl radical scavengers. Arch Biochem Biophys. 1994;315:161–9.

El-Bahr SM. Biochemistry of free radicals and oxidative stress. Forensic Sci Int. 2013;1:111–7.

Gilbert DL. Oxygen and living processes: an interdisciplinary approach. New York: Springer; 1981.

Gomes A, Fernandes E, Lima JLFC. Use of fluorescence probes for detection of reactive nitrogen species: a review. J Fluoresc. 2006;16:119–39.

Halliwell B. How to characterize a biological antioxidant. Free Radic Res Commun. 1990;9:1–32.

Halliwell B, Gutteridge JMC. Free radicals in biology and medicine. Oxford: Oxford University Press; 1985.

Hazra B, Biswas S, Mandal N. Antioxidant and free radical scavenging activity of *Spondiaspinnata*. BMC Complement Altern Med. 2008;8:63.

Jayanthi P, Lalitha P. Reducing power of the solvent extracts of *Eichhornia Crassipes* (Mart.) Solms. Int J Pharm Pharm Sci. 2011;3:126128.

Kumar S, Pandey AK. Antioxidant, lipo-protective and antibacterial activities of phytoconstituents present in *Solanum xanthocarpum* root. Intl Rev Biophys Chem. 2012;3:42–7.

Kumar S, Pandey AK. Phenolic content, reducing power and membrane protective activities of *Solanum xanthocarpum* root extracts. Vegetos. 2013;26:301–7.

Kumar S, Pandey AK. Medicinal attributes of *Solanum xanthocarpum* fruit consumed by several tribal communities as food: an in vitro antioxidant, anticancer and anti HIV perspective. BMC Complement Altern Med. 2014;14:112.

Kumar S, Pandey AK. Free radicals: health implications and their mitigation by herbals. Br J Med Med Res. 2015;7:438–57.

Kumar S, Gupta A, Pandey AK. *Calotropis procera* root extract has the capability to combat free radical mediated damage. ISRN Pharmacol. 2013a;2013:691372.

Kumar S, Mishra A, Pandey AK. Antioxidant mediated protective effect of *Parthenium hysterophorus* against oxidative damage using in vitro models. BMC Complement Altern Med. 2013b;13:20.

Kumar S, Kumar R, Dwivedi A, Pandey AK. In vitro antioxidant, antibacterial and cytotoxic activity and in vivo effect of *Syngonium podophyllum* and *Eichhornia crassipes* leaf extracts on isoniazid induced oxidative stress and hepatic markers. Biomed Res Int. 2014a;2014:459452.

Kumar S, Pandey S, Pandey AK. In vitro antibacterial, antioxidant, and cytotoxic activities of *Parthenium hysterophorus* and characterization of extracts by LC-MS analysis. Biomed Res Int. 2014b;2014:495154.

Kunchandy E, Rao MNA. Oxygen radical scavenging activity of curcumin. Int J Pharm. 1990;58:237–40.

Leonardi C, Ambrosino P, Esposito F, et al. Antioxidative activity and carotenoid and tomatine contents in different typologies of fresh consumption tomatoes. J Agric Food Chem. 2000;48:4723–7.

Liu F, Ooi VEC, Chang ST. Free radical scavenging activities of polysaccharide extracts. Life Sci. 1997;60:763–71.

Lobo V, Phatak A, Chandra N. Free radicals and functional foods: impact on human health. Pharmacogn Rev. 2010;4:118–26.

Luqman S, Fatima K, Masood N. A high through put spectrophotometric method for singlet oxygen quenching. Protocol Exch. 2016; https://doi.org/10.1038/protex.2016.054.

Namiki M. Anatioxidants/antimutagens in food. Food Sci Nutr. 1990;29:273–300.

Negi PS, Jayaprakasha GK. Control of foodborne pathogenic and spoilage bacteria by garcinol and *Garcinia indica* extracts, and their antioxidant activity. J Food Sci. 2004;69:61–5.

Oakes KD, Kraak GJ. Utility of the TBARS assay in detecting oxidative stress in white sucker (*Catostomus commersoni*) populations exposed to pulp mill effluent. Aquat Toxicol. 2003;63:447–63.

Ozyurek M, Bektasolu B, Guclu K, et al. Hydroxyl radical scavenging assay of phenolics and flavonoids with a modified cupric reducing antioxidant capacity (CUPRAC) method using catalase for hydrogen peroxide degradation. Anal Chim Acta. 2008;616:196–206.

Ozyurek M, Bektasolu B, Guclu K, et al. Novel hydrogen peroxide scavenging assay of phenolics and flavonoids using cupric reducing antioxidant capacity (CUPRAC) methodology. J Food Compos Anal. 2010;23:689–98.

Re R, Pellegrini N, Proteggente A, et al. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radic Biol Med. 1999;26:1231–7.

Rebeiro AB, Berto A, Chiste RC, et al. Bioactive compounds and scavenging capacity of extracts from different parts of *Vismia cauliflora* against reactive oxygen and nitrogen species. Pharm Biol. 2015;53:1267–76.

Scalzo RL, Todaro A, Rapisarda P. Methods used to evaluate the peroxyl (ROO) radical scavenging capacities of four common antioxidants. Eur Food Res Technol. 2012;235:1141–8.

Shareef MI, Reddy PJM, Gopinath SM, et al. Superoxide anion scavenging activity of *Carthamus tinctorius* flower. Int J Inn Res Sci Eng Technol. 2014;3:10101–4.

Sharma AK, Kumar S, Pandey AK. Ferric reducing, anti-radical and cytotoxic activities of *Tinospora cordifolia* stem extracts. Biochem Anal Biochem. 2014;3:153.

Sharma UK, Sharma AK, Pandey AK. Medicinal attributes of major phenylpropanoids present in cinnamon. BMC Complement Altern Med. 2016;16:156.

Sharma AK, Sharma UK, Pandey AK. Protective effect of *Bauhinia variegate* leaf extracts against oxidative damage, cell proliferation and bacterial growth. Proc Natl Acad Sci India Sect B Biol Sci. 2017a;87:45–51.

Sharma UK, Sharma AK, Gupta A, et al. Pharmacological activities of cinnamaldehyde and eugenol: antioxidant, cytotoxic and anti-leishmanial studies. Cell Mol Biol. 2017b;63:73–8.

Sharma UK, Kumar R, Gupta A, et al. Renoprotective effect of cinnamaldehyde in food color induced toxicity. 3 Biotech. 2018;8:212.