Mini-Reviews in Medicinal Chemistry, 2006, *6*, 305-312

Screening Methods for Antioxidants-A Review

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Abstract: Various environmental, physical and chemical stresses on cells may induce either an overproduction of ROS (Reactive Oxygen Species) or a deficiency of antioxidant enzymes. ROS are responsible for various cellular anomalies like protein damage, deactivation of enzymes, alteration of DNA and lipid peroxidation which in turn leads to pathological conditions like carcinogenesis, reperfusion injury, rheumatoid arthritis, diabetes etc. The regular intake of antioxidants seems to limit or prevent the dangerous effects caused by ROS. Thus, to maintain cellular health, it is important to have a specific and effective antioxidant that scavenges multiple types of free radicals so that it can be used in multiple diseases. Different *in vitro* and *in vivo* test systems are available in the literature to assess the free radical scavenging activity of various compounds. Based on the efficiency of free radical scavenging, the compounds are classified into strong, moderate and weak antioxidants. The following review explains the brief procedure and the principle behind various methods available in the literature, which can be used to determine the scavenging of different types of free radicals.

Keywords: *In vitro* tests, antioxidants, DPPH assay, superoxide anion, hydroxyl ions, nitric oxide, peroxynitrite, lipid peroxidation.

INTRODUCTION

Free radicals, the partially reduced metabolites of oxygen, are highly toxic, mutagenic and reactive. A free radical is a molecule with one or more unpaired electrons in its outer orbital. Many of these molecular species are oxygen (and sometimes nitrogen) centered. These highly unstable molecules tend to react rapidly with adjacent molecules, donating, abstracting, or even sharing their outer orbital electron(s). This reaction not only changes the adjacent, target molecule, sometimes in profound ways, but often passes the unpaired electron along to the target, generating a second free radical or other reactive oxygen species (ROS), which can then go on to react with a new target. In fact, much of the high reactivity of ROS is due to the generation of such molecular chain reactions, effectively amplifying their deleterious effects many fold (1). The later include various diseases like cancer, ischemia, atherosclerosis, diabetes, Alzheimer's disease etc.

An antioxidant is a substance that when present in low concentrations relative to the oxidizable substrate significantly delays or reduces oxidation of the substrate (2, 3). Antioxidants get their name because they combat oxidation. Some antioxidants are produced endogenously, to protect the body from damaging oxidation reactions by reacting with free radicals and other reactive oxygen species within the body, hence hindering the process of oxidation. During this reaction the antioxidant sacrifices itself by becoming oxidized. However, endogenous antioxidant supply is not unlimited as one antioxidant molecule can only react with a single free radical. Therefore, there is a constant need to replenish antioxidant resources, either endogenously or through supplementation (4).

Many antioxidant defense systems in the body such as superoxide dismutase (in mitochondria & cytosol), catalase (in peroxisomes), glutathione peroxidase, α-tocopherol (in membranes & lipoproteins) etc. limit the levels and the damage caused by free radicals [5]. When this multiple defense system fails due to increased production of reactive oxygen species or decreased level of cellular antioxidants, the net result is (i) DNA damage leading to mutations and cancer; (ii) oxidative inactivation and accumulation of metabolic enzymes and an increase in the level of oxidatively modified proteins resulting in aging; (iii) induction and initiation of lipid peroxidation and oxidative modification of low density lipoproteins leading to deleterious cardiovascular effects.

Due to the above-presented pathological implications of ROS, it is important to find an antioxidant, which may scavenge multiple ROS so that it can be used in multiple disease states and also to maintain a healthy status. The need to identify antioxidants, which can scavenge several free radicals and prevent multiple diseases, can be achieved by simple *in vitro* test systems. Further the *in vitro* results can be confirmed with *ex vivo* and *in vivo* systems.

I. *IN VITRO* METHODS

In vitro methods are qualitative and they are used to find out whether the given compound is an antioxidant or not. However, IC_{50} values (concentration which can achieve 50% scavenging) or Trolox equivalents (free radical scavenging in terms of Trolox) can be used to quantify the activity. Also the methods are simple, colorimetric and there is no use of animal tissues.

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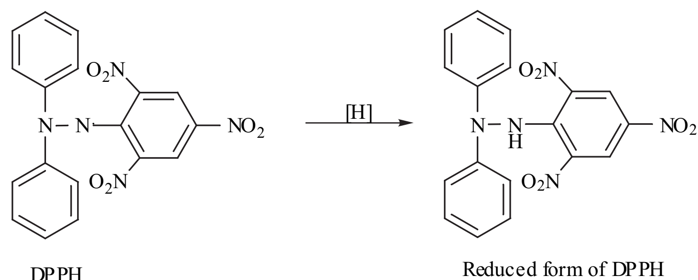


Fig. (1). Reduction of 1,1-diphenyl -2-picryl hydrazyl (DPPH) free radical.

1. General Free Radical Scavenging

a. DPPH Assay

Free radical scavenging activity is determined using 1,1-diphenyl-2-picryl hydrazyl radical (DPPH), which is a stable free radical having a purple colour. When free radical scavengers are added, DPPH is reduced and its colour is changed to yellow, based on the efficacy of antioxidants (Figure **1**). A 100 μM solution of DPPH in methanol is added to the drug solution and the absorbance is read at 515 nm after 10 min [6]. The change in absorbance with respect to the control (containing DPPH only without sample, expressed as 100% free radicals) is calculated as percentage scavenging.

b. ABTS Method

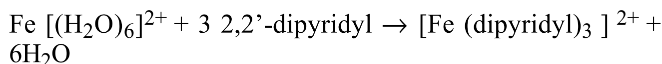
This method was developed by Miller (1996) and is based on a decolouration reaction [7]. The reaction is initiated by adding ABTS (2,2-α-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) and hydrogen peroxide to produce ABTS radical. Incubation of ABTS with peroxidase (metmyoglobin) and hydrogen peroxide results in the production of radical cation $ABTS^{\cdot+}$. This species is blue-green in colour and can be detected at 734 nm. Antioxidants or radical scavenger in the added sample cause suppression of this colour production to a degree that is proportional to their concentration. In this method the activity of tested substances is expressed as an equivalent of the millimolar concentration of a standard Trolox solution. The generation of ABTS radical is allowed to proceed until a stable color of ABTS radical occurs. Antioxidants which have scavenging activity, therefore, decolourise a mixture of ABTS radical, thus giving an index of their antioxidant capacity.

c. β-Carotene Bleaching Method

The antioxidant assay using the β-carotene discolouration is mostly used, because β-carotene is extremely susceptible to free-radical mediated oxidation. Since it contains 11 pairs of double bonds, β-carotene is decolourized easily with the oxidation of linoleic acid. In this method, the test compound (50 μl) was treated with 3 ml of an aqueous emulsion of linoleic acid and β-carotene (3 ml β-carotene solution from the stock solution of 5 mg of β-carotene in 50 ml chloroform added to 40 mg of linoleic acid and 400 mg of tween 40; chloroform is removed under nitrogen and water was added to make upto 100 ml) and kept in a water bath for 1h at 50° C. When linoleic acid undergoes oxidation, it causes bleaching of β-carotene which was measured at 470 nm [8].

d. Ferrous Sulphate-2,2'- dipyridyl Complex Formation

The concentration of free ferrous ions is estimated using chelating agent 2, 2'-dipyridyl. The reaction mixture containing 50 μM ferrous sulphate and 50 μl sodium chloride (pH 7) is incubated for 30 min with antioxidants. Then 2 ml of 2, 2'-dipyridyl (1mM) is added and the absorbance of ferrous bipyridyl complex so formed is measured at 525 nm against a blank without ferrous sulphate [9].



$Fe\ [(H_2O)_6]^{2+} + 3\ 2,2'\text{-dipyridyl} \rightarrow [Fe\ (dipyridyl)_3\ ]^{2+} + 6H_2O$

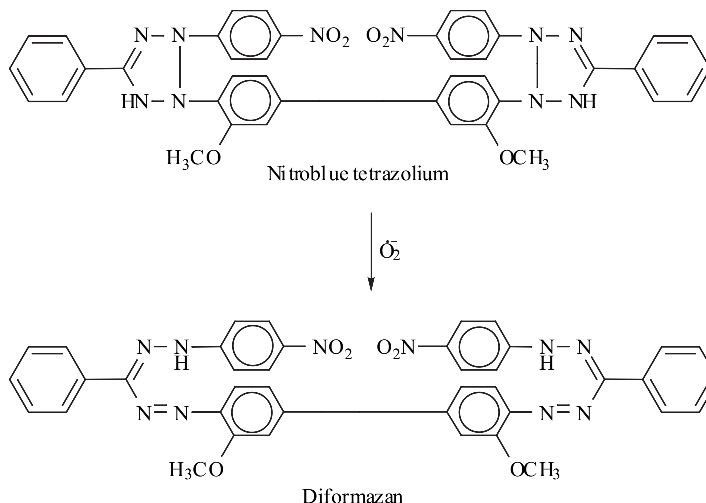


Fig. (2). Reduction of nitroblue tetrazolium to diformazan by superoxide anion.

e. Gibb's Reagent (2,6- dichloroquinonechlorimine)

Gibb's reagent couples with antioxidants containing phenolic groups in the *o*- and *p*- positions. This leads to the production of indophenols whose alkali metal and ammonium salts produce a characteristic blue colour which can be measured colorimetrically. The development of colour is pH dependent [9].

2. Superoxide Anion Scavenging

Univalent reduction of oxygen causes production of superoxide anion, which interacts with hydrogen peroxide leading to the generation of highly reactive and toxic hydroxyl radicals. Superoxide anion destroys endothelium derived relaxing factor, which affects the secretary function of vascular endothelium [10]. Superoxide anion may also react directly with lipids, catecholamines and DNA. At physiological pH (7.4), dismutation reaction yields one molecule each of hydrogen peroxide and oxygen from superoxide anion. The chemical reaction of superoxide anion with nitroblue tetrazolium dye (NBT), the basic reaction used in many *in vitro* tests is given in Figure **2**.

a. Hydroxylamine Auto Oxidation Method

When hydroxylamine undergoes auto oxidation at a pH of 10.2, it causes the production of superoxide anions which are determined by the reduction of nitroblue tetrazolium dye [11]. The reaction mixture contains 50 mM sodium carbonate buffer (pH-10.2), 24 µM NBT, 0.1 mM EDTA, 1 mM hydroxylamine and 0.03% Triton X-100 along with the compounds to be tested in a total volume of 1 ml. It is incubated for 20 min at 37°C and the absorbance is measured at 560 nm.

b. Xanthine-Xanthine Oxidase System

The reaction between xanthine and xanthine oxidase leads to the production of superoxide anions which is detected by the change in colour of NBT. The reaction mixture contains a solution of test compound, 100 µM of xanthine, 600 µM of nitroblue tetrazolium in 0.1 M phosphate buffer of pH 7.4 and 0.07 units/ml of xanthine oxidase. This mixture is incubated at 25°C for 10 min and the absorbance is read at 560 nm against a blank which does not contain the enzyme [12].

c. Phenazine Methosulphate-NADH System

The reaction between phenazine methosulphate and NADH leads to the production of superoxide anions which is detected by the change in colour of NBT. The reaction mixture consists of a solution of test compound, 10 µM of phenazine methosulphate, 78 µM of NADH and 25 µM of nitroblue tetrazolium in 0.1 M phosphate buffer of pH 7.4. This mixture was incubated at 25°C for 10 min and the absorbance was read at 560 nm against blank samples that do not contain phenazine methosulphate [12].

d. Cytochrome C reduction method

The reaction between xanthine and xanthine oxidase leads to the production of superoxide anions which reduces cytochrome C. A solution of test compound, 100 µM of xanthine, 0.07 units/ml of xanthine oxidase and 50 µM of cytochrome C when incubated at 25°C for 3 min produce a colour with an absorbance maxima at 550 nm [12].

e. Alkaline DMSO Method

In this method, superoxide anion is generated using alkaline DMSO, and the reduction of NBT by superoxide is determined in the presence and absence of a suitable test compound. The reaction mixture containing NBT (0.1 mg) and test compound at various concentrations are added to alkaline DMSO (1 ml, 1% water, 5 mM NaOH) to give a final volume of 1.4 ml and the absorbance is read at 560 nm [13].

Alkaline DMSO test is also reported by another method. Solid potassium superoxide is allowed to stand in contact with dry DMSO for at least 24 h and the solution is filtered immediately. Filtrate is added to an aqueous solution of NBT, EDTA, potassium phosphate buffer and drug solution and the absorbance was measured at 560 nm [13].

f. Riboflavin Photo-oxidation Method

In case of riboflavin photo oxidation method, the photo excitation of riboflavin leads to the generation of riboflavin radical which then auto oxidizes and generates superoxide anions. The reaction mixture contains 0.067 M phosphate buffer, 0.1 M EDTA, 1.5 mM NBT and test solutions of different concentrations. The tubes are incubated at 37°C for 5-8 min. Then riboflavin (0.12 mM) is added and the tubes are further incubated in a light box for 12 min and the absorbance is measured at 560 nm [14].

g. Pyrogallol Auto Oxidation Method

Pyrogallol (200 µmol/L) was incubated in phosphate buffered saline (PBS) with or without the addition of test compounds. Pyrogallol auto oxidation was estimated by the change in absorbance at 420 nm during a 10 min incubation [15].

3. Hydroxyl Radical Scavenging

Hydroxyl radical is generated by the reaction of hydrogen peroxide with ferrous ion, which cleaves covalent bonds in proteins and carbohydrates, causes lipid peroxidation and destroys cell membranes. This is the most reactive of the oxygen radicals and it combines with all molecules of living cells.

a. p-Nitroso Dimethylaniline (pNDA) Bleaching Method

pNDA bleaching method is a sensitive method to determine the hydroxyl radical scavenging. The reaction of hydrogen peroxide with ferric chloride leads to the production of hydroxyl radical that causes the bleaching of pNDA (Fig. **3**). The reaction mixture contains pNDA (6 µM), ferric chloride (0.1 mM), EDTA (0.1 mM) and hydrogen peroxide (2 mM) in phophate buffer of pH 7.4 (20 mM) along with drug solution in a final volume of 3 ml and the absorbance was measured at 440 nm [13].

$$H_2O_2 + Fe^{2+} \longrightarrow OH + OH^- + Fe^{3+}$$

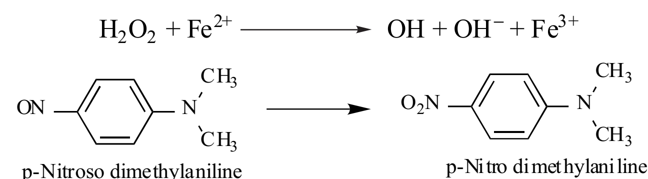


Fig. (3). Generation of hydroxyl radicals and their reaction with p-nitrosodimethyl aniline.

b. Deoxyribose Assay

The degradation of deoxyribose by hydroxyl radical generated from Fenton's reaction – ferric chloride/H_2O_2 is measured colorimetrically in the presence and absence of a suitable test compound. To the reaction mixture containing deoxyribose (3 mM), ferric chloride (0.1 mM), EDTA (0.1 mM), ascorbic acid (0.1mM), H_2O_2 (2 mM) in phosphate buffer, pH 7.4 (20 mM) are added various concentrations of antioxidant to give a final volume of 3 ml. After incubation for 30 min at ambient temperature, trichloroacetic acid (0.5 ml, 5%) and thiobarbituric acid (0.5 ml, 1%) are added. The reaction mixture is kept in a boiling water bath for 30 min, cooled and the absorbance measured at 532 nm [13].

c. Automated Radical Absorbance Capacity Assay

This assay is carried out on the COBAS FARA II spectrophotometric centrifugal analyzer. In the final assay mixture, β-phycoerythrin (β-PE; 16.7 nm) is used as a target of free radical attack with H_2O_2-Cu^{2+} (H_2O_2-0.3%; Cu^{2+}-9 μM as $CuSO_4$) as the hydroxyl radical generator. Trolox (1μM) is freshly prepared and used as a control standard. The analyzer is programmed to record the fluorescence of β-PE every 2 min after H_2O_2-Cu^{2+} is added [16]. Final results are calculated using differences of areas under β-PE decay curves between the blank and the sample. The results are expressed as μmole Trolox equivalents per μmole sample.

d. Electron Spin Trapping Method

Hydroxyl radicals were generated from auto oxidation of dihydrofuran (830 μM) in the presence of $FeCl_3$ (26 μM) chelated by ADP (250 μM). 5,5-Dimethyl-1-pyrroline *N*-oxide (DMPO; 90 mM) was dissolved in 0.9% saline and exposed to the free radical generating system in the presence and absence of test compound. The formation of radical spin adduct, DMPO-OH was monitored using a EPR spectrophotometer [17].

4. Hydrogen Peroxide Scavenging

A two electron reduction of oxygen yields hydrogen peroxide. It is generated in biological systems via the production and reaction of superoxide anions. Hydrogen peroxide is generated by a number of intracellular flavin oxidases like xanthine oxidase and also from the auto oxidation of numerous xenobiotics [18]. Hydrogen peroxide can directly affect the DNA and sulfhydryl groups apart from its participation in hydroxyl radical generation.

The hydrogen peroxide scavenging capacity of an agent can be measured by the hydrogen peroxide decomposition method. Hydrogen peroxide solution (50 μM) is added to different concentrations of drug solutions and the reduction in absorbance at 240 nm is measured after subtracting any inherent signal associated with the drug [19].

5. Nitric Oxide Scavenging

Nitric oxide is generated from the amino acid L-arginine by vascular endothelial cells, phagocytes and certain cells in the brain [20]. Nitric oxide reacts with superoxide and forms peroxynitrite radicals and is responsible for the inflammatory response by the release of prostaglandin [21]. Some scientists believe that repeated infections throughout life, cause an excessive production of NO, which, over time, causes toxic damage to the body resulting in age-related diseases such as heart disease, Alzheimer's disease and diabetes.

Nitric oxide was generated from sodium nitroprusside, which at physiological pH (7.4) liberates nitric acid. This nitric acid gets converted to nitrous acid and further forms nitrite ions on contact with air. The nitrite ions diazotize with sulphanilic acid and couple with naphthylethylenediamine (Griess reagent), producing pink colour which can be measured at 546 nm [22] (Figure **4**). Briefly, sodium nitroprusside (5 mM, 0.1 ml) in phosphate buffer (50 mM) was incubated with the test compounds at room temperature for 30 min. After 30 min, 0.5 ml of the incubated solution was added with 0.5 ml of Griess reagent and the absorbance was measured at 546 nm.

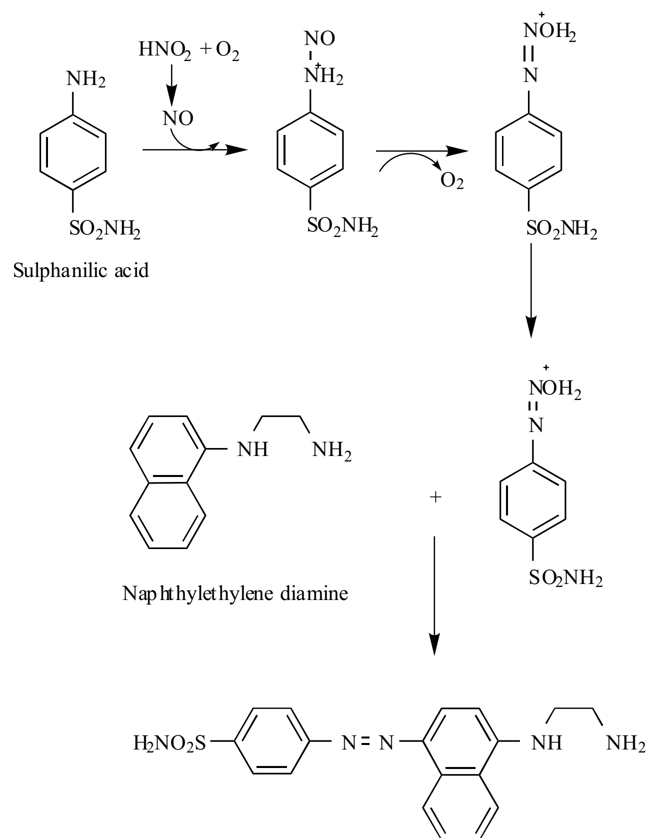


Fig. (4). Generation of nitric oxide and its reaction with Griess reagent.

6. Peroxynitrite Scavenging

Peroxynitrite, produced *in vivo* from nitric oxide and superoxide, leads to oxidation of low-density lipoprotein. Peroxynitrite is a strong nitrating species with oxidizing effect. It may oxidize all major types of biomolecules like DNA, proteins and lipids. Peroxynitrite is thought to be involved in neurodegenerative diseases like Alzheimer's disease, Parkinson's disease etc [23].

a. Peroxynitrite Decomposition

The drug solutions along with peroxynitrite are kept at room temperature for 10 min and the absorbance at 302 nm is determined. Peroxynitrite decomposition is measured based on the production of hydroxyl radical and the latter is

determined by oxidation of DMSO to formaldehyde and deoxyribose to malondialdehyde (MDA). Formaldehyde formed from DMSO can be determined by reaction with purpald [24] and MDA was determined by thiobarbituric acid method.

b. Tyrosine Nitration Assay

Tyrosine undergoes nitration by the action of peroxynitrite so that the extent of inhibition of tyrosine nitration in the presence of scavengers is used to determine the extent of peroxynitrite scavenging. 100 µl of DL-tyrosine (10 mM) was incubated with 100 µl of test compound in 795 µl of phosphate buffer of pH 7.4 in a water bath at 37°C for 15 min. After this, 5 µl of peroxynitrite (1 mM) was added and incubated for further 15 min. Then the amount of 3-nitrotyrosine formed was measured by HPLC method [25].

c. Inactivation of α1-Antiproteinase

α1-Antiproteinase is inactivated by peroxynitrite and its extent depends upon peroxynitrite concentration. α1-Antiproteinase in turn inhibits the enzyme elastase. Thus the increase in concentration of elastase is used to determine the extent of α1-Antiproteinase inactivation. α1-Antiproteinase (4 mg/ml) dissolved in phosphate buffered saline (pH-7.4) is added to 100 µl of test compound to give a final volume of 945 µl and incubated in a water bath at 37°C for 15 min. After this, 5 µl of peroxynitrite (0.5 mM) was added along with elastase in the same buffer (5 mg/ml) and the sample is incubated for further 15 min. Then 100 µl N-succinyl (Ala)-3-p-nitroanilide (SANA) is added and the residual elastase activity is measured by the rise in absorbance at 410 nm [25].

d. Oxidation of Dihydrorhodamine 123

Peroxynitrite scavenging can also be measured by the oxidation of dihydrorhodamine 123 and the fluorescence can be measured at excitation and emission wavelengths of 500 nm and 536 nm respectively using a fluorometer [26].

7. Lipid Peroxidation

ROS generated in the human body cause lipid peroxidation leading to the development of various diseases like cancer, Parkinson's disease, senile dementia and aging etc. Lipid peroxidation has received much attention recently because of its possible contributions to cancer and aging. It is the oxidative deterioration of polyunsaturated fatty acids (PUFA), by which polyunsaturated fatty acids are converted to lipid hydroperoxides (LOOH). Lipid peroxidation is a free radical-related process. Oxidants can react with polyunsaturated fatty acids in cell membranes, to form toxic metabolites [27]. Lipids that contain two or more unsaturated carbon-carbon bonds (C=C) can be attacked by reactive oxygen species [28]. Generally hydroxyl radical reacts with polyunsaturated fatty acids by abstracting a hydrogen atom from it, thus initiates lipid peroxidation

To determine the scavenging of lipid peroxide radicals by antioxidants, different test systems were used. The lipid membrane models include lecithin liposomes, linoleic acid emulsion, rat liver, kidney, brain homogenates etc. Lipid peroxidation may be induced by different agents such as hydrogen peroxide, tertiary butyl hydroperoxide or ascorbic acid along with ferrous sulphate, AAPH (2,2'-azo-bis-(2-amidinopropane) hydrochloride [29], sodium hypochlorite and ferric nitriloacetate [17].

Measurement of Lipid Peroxidation

Three types of assayable species i.e., malondialdehyde, lipid hydroperoxides and lipids containing conjugated dienes are produced during lipid peroxidation. The detection of these products are described as follows:

a. Thiobarbituric Acid Assay

Thiobarbituric acid reagent was added to the reaction mixture which interacts with malondialdehyde (MDA; the end product of lipid peroxidation) and TBARS produced (Figure **5**) was extracted with n-butanol. The absorbance of butanol layer was measured at 532 nm with a spectrophotometer [30].

b. Iodometric Method

This method is used for the measurement of lipid hydroperoxide products of peroxidation of polyunsaturated fatty acids (PUFA). This method is also used for protein peroxide measurement [9]. The principle of this method is based on the ability of I^- to reduce the hydroperoxides formed during lipid peroxidation. The reaction is as follows;

$$2H^+ + ROOH + 3I^- \; H_2O + ROH + I_3^-$$

One ml of membrane solution or aqueous suspension of lipid is mixed with 5 ml of chloroform: methanol (2:1) and centrifuged at 1000 g for 5 min to separate the phases. The chloroform layer is recovered using a syringe and it is placed in a test tube and dried at 45°C in a water bath under a stream of nitrogen. Then add 1 ml of acetic acid: chloroform and 0.05 ml of potassium iodide and the test tube is stoppered and mixed. The samples are kept at room temperature in a dark place for 5 min followed by the addition of 3 ml of cadmium acetate. The solution is mixed and centrifuged at 1000 g for 10 min and the absorbance of the upper phase is determined at 353 nm against the blank containing complete assay mixture except lipids.

c. Diene Conjugation Assay

Membrane lipids are extracted and dried by the method described under iodometric assay. Then the residue is dissolved in 1.5 ml of cyclohexane and the absorbance at

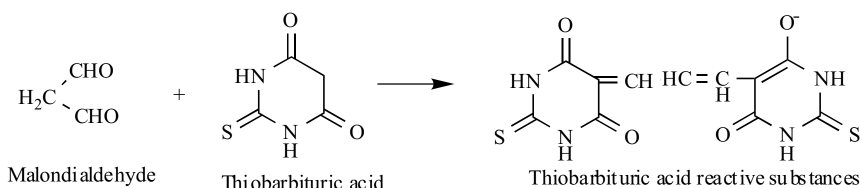


Fig. (5). Reaction of malondialdehyde and thiobarbituric acid and the formation of TBARS.

233 nm is determined by using cyclohexane as the blank. Conjugated dienes are formed during lipid peroxidation due to the rearrangement of polyunsaturated fatty acid double bonds [9].

d. FOX Method

It involves ferrous oxidation in xylenol orange and is used for measuring low levels of soluble hydroperoxides in the aqueous phase, or lipid hydroperoxides derived from membranes of lipoproteins in lipid phase. This method can also measure protein peroxides [9].

e. Ferrous Chloride and Thiocyanate System

This method is based on the determination of hydroperoxides in an ethanolic linoleic acid system containing antioxidants using the colour reaction of ferrous chloride and thiocyanate [9].

f. HPLC

Ex vivo measurement of the lipid hydroperoxide products directly is best achieved by HPLC determination following partitioning of the hydroperoxide into a polar solvent, which achieves a primary separation between less polar triacylglycerol and cholesterol hydroperoxides and the more polar free fatty acids and phospholipids hydroperoxides [9]. ***HPLC-linked TBA measurement and chemiluminescence-linked HPLC determination*** are other reliable procedures for the assay of lipid hydroperoxides [9].

g. Quantification of F_2 - Isoprostanes

This is a reliable and useful approach for assessment of lipid peroxidation and oxidative stress *in vivo.* Isoprostanes are derived from PUFA by a non-cyclooxygenase-mediated free-radical-catalyzed mechanism. The method by which F_2-isoprostanes are detected and quantified is a stable isotope dilution assay utilizing capillary gas chromatography/ negative ion chemical ionization mass spectrometry. This assay quantifies only free compounds lipids. For measurement of levels of isoprostanes esterified to tissue lipids, the isoprostanes must be hydrolyzed from tissue lipids either enzymatically, i.e. using bee venom phospholipase or chemically i.e. using alkaline saponification, prior to identification. Alkaline hydrolysis can be used to hydrolyze F_2- isoprostanes from phospholipids. Though the mass spectrometric method is very accurate, it has several disadvantages like high purchase and maintenance cost. Also, it is a time consuming and labour-intensive method. To overcome these impediments immunoassay methods for the measurement of isoprostanes are currently under development [31].

h. Bioxytech LPO-586 kit Method

In case of lipid peroxidation induced by ferric nitriloacetate in rat kidney homogenates, after incubation, the homogenates were centrifuged for 10,000 g for 10 min at 4°C and the supernatant was collected to measure MDA and 4-hydroxyalkenols. They were quantitated by using Bioxytech LPO-586 kit [32].

8. Singlet Oxygen Generation and Quenching

Singlet oxygen oxidize a number of biological molecules particularly lipid and olefinic containing molecules. Singlet oxygen is generated by using the third or second harmonic of a Q-switched Nd-YAG laser (Spectron laser system, Îem = 355 nm, 6 ns of FWHM, 60 mJ/Pulse) as the excitation source of perinaphthenone solution (500 µM in chloroform) or methylene blue (40 µM) as a sensitizer. Singlet oxygen emission at 1270 nm was measured with a nitrogen cooled Ge-photodiode with a Spectrogen BP-1270-080-s band pass filter. Drugs quench the singlet oxygen by energy transfer process [33].

II. *EX VIVO* METHODS

1. Superoxide Anion and Hydrogen Peroxide Scavenging

Chinese hamster lung fibroblast V79 cells were seeded in 60 mm petri dishes and incubated in 5 ml of minimum essential medium (MEM) with 10% heat inactivated foetal bovine serum (FBS) at 37°C. Then change the medium to MEM without FBS and add the drug solution and the cells are again incubated for 4 h. Then wash the cells with HEPES-buffered saline (HBS) of pH 7.3 and treat them with 60 µM hydrogen peroxide or 50 µM of hypoxanthine and 0.025 U of xanthine oxidase in 5 ml HBS for 30 min. Then culture the cells in MEM supplemented with 10% FBS and the number of colonies were counted after 5 days [34].

2. DPPH Assay

Cultured human gastric epithelial cells (AGS) or murine small intestinal epithelial cells (IEC-18) were exposed to DPPH (3 µM) and incubated for 24 h with antioxidants administered before 1 h of exposure. Cell number (MTT assay) and death via apoptosis or necrosis (ELISA, LDH release) was determined after 24 h [19].

3. Hydrogen Peroxide Scavenging

Cultured human gastric epithelial cells (AGS) or murine small intestinal epithelial cells (IEC-18) were exposed to H_2O_2 (50 µM) and incubated for 24 h with antioxidants administered before 1 h of exposure. Cell number (MTT assay) and death via apoptosis or necrosis (ELISA, LDH release) was determined after 24 h [19].

4. Peroxynitrite Scavenging

Cultured human gastric epithelial cells (AGS) or murine small intestinal epithelial cells (IEC-18) were exposed to peroxynitrite (300 µM) and incubated for 24 h with antioxidants administered before 1 h of exposure. Cell number (MTT assay) and death via apoptosis or necrosis (ELISA, LDH release) was determined after 24 h [19].

5. Singlet Oxygen Scavenging

Singlet oxygen was introduced into Chinese hamster ovary (CHO) and Mouse hybridoma (NS-1) cells by adding 4 µM solution of rose bengal diacetate solution under UV lighting for photosensitization. It causes cytotoxicity which was determined by cell viability and cell toxicity by using fluorescent probes designed to detect esterase activity in cellular membranes [35].

6. Hydroxyl and Superoxide Anion Scavenging

Hydroxyl and superoxide radicals were introduced into Chinese hamster ovary (CHO) and Mouse hybridoma (NS-1) cells by adding 4 μM solution of malachite green solution under UV lighting for photosensitization. It causes cytotoxicity which was determined by cell viability and cell toxicity by using fluorescent probes designed to detect esterase activity in cellular membranes [36].

III. *IN VIVO* METHODS

1. Lipid Peroxidation

Different groups of rats were used and they were given drug and ferric nitriloacetate (Fe-NTA) injection i.p. (15 mg Fe/Kg). After 60 min of Fe-NTA injection, all animals were sacrificed and their kidneys were dissected , homogenized and the MDA and 4-hydroxy alkenals were quantitated [17].

2. Nitric Oxide Scavenging-Bioassay

Nitric oxide dependent physiological response on the acetylcholine (Ach) mediated relaxation of isolated aortic rings is determined. Ach receptor stimulation results in the activation of endothelial nitric oxide synthase and the formation of nitric oxide. Nitric oxide diffuses to the muscle layer and causes the activation of guanylate cyclase leading to the accumulation of cGMP. Increase in the levels of cGMP induce protein kinase-G catalyzed phosphorylation and smooth muscle relaxation [20].

CONCLUSIONS

Knowledge of the antioxidant capacity of specific chemical scavengers and their activities with different oxidants is fundamental for understanding and predicting the susceptibility of biological tissues to oxidative stress. However, the complexity of cellular balance between oxidant challenge and antioxidant response often precludes generalization regarding the potential of ROS-mediated impact, based on the response of antioxidants to a single oxidant. The later fact is important, considering that one oxidant leads to the production of other oxidants. The *in vitro* methods reviewed in this article are useful in determining whether a particular compound or extract is an effective antioxidant. If we determine the different free radical scavenging activities of a particular compound, we explore its possible use in different disease states. However it may be noted that *in vitro* scavenging is only a preliminary test for selecting potent antioxidants. This can act as a first step of a multitier system and the compounds selected therein can be subsequently tested *in vivo* for their activity in different pathologies, so that, their potential uses can be explored.

Furthermore, depending on the type of biological activity seeked by the researcher, suitable in vitro tests described above can be used. For example H_2O_2 can directly nick the DNA and sulfhydryl groups in addition to participating in hydroxyl radical generation. It is also a substrate for catalase and glutathione peroxidases. While the superoxide anion is normally involved in inflammatory conditions. The pathological effects of superoxide anion are indirect in the sense that it is the subsequently formed hydroxyl and peroxynitrite radicals which are involved in different pathological conditions like cancer, cardiac and renal ischemia, atherosclerosis, diabetes etc. Thus, if superoxide anion production is controlled or scavenged, the formation of hydroxyl and peroxynitrite radicals can be limited, such that, the diseases caused by them can be sufficiently reduced. The hydroxyl radical derived from hydrogen peroxide and superoxide anion is also involved in similar pathological conditions. Thus, the nicking of hydrogen peroxide and superoxide anion is a preliminary requirement for the prevention of hydroxyl radical mediated pathologies. Hydroxyl radical cleaves covalent bonds in proteins and carbohydrates causing lipid peroxidation and destroying the cell membranes.

It may thus be said that the evaluation of the scavenging ability of such a wide variety of free radicals/ ROS as described in the present review, can give an estimate of the expected biological activity of the test compound. Overall DPPH radical scavenging test is simple and reliable, considering that DPPH is a fairly stable free radical. Though not naturally present in the body it can give an appraisal of the antioxidant capacity of the agent under test. It is presently being used widely as a suitable indicator of antioxidant activity. Similarly the *in vivo/ex vivo* lipid peroxidation (LPO) and its inhibition is a universal indicator of the antioxidant status. Since lipid peroxidation is involved in a wide variety of disease states, right from ageing to cancer to other cardiovascular and CNS related diseases; hence a measure of the extent of inhibition of LPO can be taken as a significant parameter for evaluating the antioxidant activity. This is a more reliable method than the other *in vitro* tests; because, in this case the test compound has to first permeate inside the biological cells to elicit its antioxidant action. So the test evaluates the biological suitability of the test molecule in addition to its antioxidant action.

ACKNOWLEDGEMENTS

The authors wish to thank the university Grants commission, New Delhi, India for the financial grant.

ABBREVIATIONS

ROS	=	Reactive oxygen species
DNA	=	Deoxyribonucleic acid
DPPH	=	1,1-Diphenyl-2-picryl hydrazyl
NBT	=	Nitroblue tetrazolium
ABTS	=	2,2-α-Azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid)
ADP	=	Adenosine di phosphate
EDTA	=	Ethylene diamine tetra acetic acid
TBARS	=	Thiobarbituric acid reactive substances
AAPH	=	2,2'-Azo-bis-(2-amidinopropane) hydrochloride
NADH	=	Nicotinamide adenine dinucleiotide
DMSO	=	Dimethyl sulphoxide

pNDA = p-Nitroso dimethylaniline

H_2O_2 = Hydrogen peroxide

$CuSO_4$ = Copper sulphate

MDA = Malondialdehyde

DMPO = 5,5-Dimethyl-1-pyrrroline N-oxide

SANA = N-succinyl (Ala)-3-p-nitroanilide

HPLC = High performance liquid chromatography

PUFA = Polyunsaturated fatty acids

LOOH = Lipid hydroperoxides

MTT = 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

ELISA = Enzyme linked immuno sorbant assay

UV = Ultraviolet

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Received: April 29, 2005 Revised: August 13, 2005 Accepted: August 14, 2005

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