ISSN: 2320 - 4230

Journal of Drug Discovery and Therapeutics 1 (9) 2013, 65-72

REVIEW ARTICLE

REVIEW ON *IN-VITRO* ANTIOXIDANT SCREENING ASSAYS

Maryam Bincy Thomas*, Lalit Singh, S.K.Sharma

Department of Pharmacology, Sunder Deep Pharmacy College, Ghaziabad, UP, India- 201001.

Received 10 September 2013; Revised 15 September 2013; Accepted 18 September 2013

ABSTRACT

Oxygen, an element indispensable for life, can under certain circumstances, adversely affect the human body. The oxygen consumption inherent in cell growth leads to the generation of a series of reactive oxygen species (ROS). The role and beneficial effects of antioxidants against various disorders and diseases induced by oxidative stress have therefore received much attention. The free radical scavenging antioxidants are one of the important classes of antioxidants and the assessment of their capacity has been the subject of extensive studies and argument. This stimuli article reviews the different *in-vitro* methods for the estimation of the antioxidant capacity. All the models are described along with the different standards that can be used for the estimation.

Keywords: *In-vitro* screening models, Antioxidant assay.

INTRODUCTION:

Antioxidants are substances that may protect cells from the damage caused by unstable molecules known as free radicals. Antioxidants interact with and stabilize free radicals and may prevent some of the damage free radicals might otherwise cause. An antioxidant is a molecule capable of slowing or preventing the oxidation of other molecules. Oxidation is a chemical reaction that transfers electrons from a substance to an oxidizing agent. Oxidation reactions can produce free radicals, which can start chain reactions that damage cells[1].

Reactive oxygen species (ROS) is a term which encompasses highly reactive, oxygen-containing molecules, including free radicals. Aerobic organisms produce a number of reactive free radicals (molecules or atoms having unpaired electrons) continuously in cells during respiration, metabolism and phagocytosis. Out of these, the most important source of free radicals being the respiratory chain where ~ 1 to 2% oxygen is converted into superoxide radicals (O2 •−). While superoxide radical is not so reactive and may not be able to cause any direct damage to cells, its reaction product hydrogen peroxide in presence of trace metal ions, is converted to more powerful hydroxyl radicals ($^{\bullet}OH$), which can oxidize most of the biomolecules. Organic substrates (RH) or lipids (LH) on reaction with hydroxyl radicals in presence of oxygen are converted in to peroxyl radicals ($ROO^{\bullet}$)/ ($LOO^{\bullet}$), which are known to undergo chain reactions, and thereby multiplying the damage. Thus, free radicals formed within the cells can induce multiple chemical changes in cellular organelles like membrane lipids, DNA and proteins, which can eventually lead to cell death. The collective terms "reactive oxygen species (ROS) and reactive nitrogen species (RNS)" have been applied for a variety of free radicals such as superoxide, hydroxyl, peroxyl, nitric oxide, nitrogen dioxide radicals as well as for non-radical reactive intermediates like hydrogen peroxide (H_2O_2) and peroxynitrite ($ONOO^-$) etc. and their excessive production, termed as "oxidative stress" has been implicated in many pathological disorders like heart disease, cancer and ageing. Environmental factors such as pollution, radiation, cigarette smoking and herbicides can also spawn free radicals in the body but, if antioxidants are not available to check the free radical production, it becomes excessive ad cause damage to the body and any substance in which oxidation occurs.[2-5]

Free radicals are very unstable and react quickly with other compound trying to gain stability. Generally, free radicals attack the nearest stable molecules abstracting its electron to attain stability. When the attacked molecule loses its electron, it becomes a free radical itself, these formations of free radicals continue on and on. In general, the antioxidants act by the following routes:

✓Chain breaking mechanism by which the antioxidants donate electrons to the free radicals present in the system, ex: lipid radicals.

✓Removal of ROS and RNS initiator by quenching chain initiator catalyst.

*Corresponding author: Maryam Bincy Thomas | Email: maryambincythomas@gmail.com

✓By chelating transition metal catalyst: a group of compound which act by sequestration of transition metals that are well established proxidants. In this way transferring, lactoferrin and ferritin function to keep iron induced oxidant stress in check and ceroluplasmin and albumin as copper sequestrants.

To protect the cells and organ systems of the body against reactive species, humans have evolved a highly sophisticated and complex antioxidant protection system.[6] Natural antioxidant enzymes manufactured in the body provide an important defense against free radicals. Glutathione peroxidase, glutathione reductase, catalase, thioredoxin reductase, superoxide dismutase, heme oxygenase, methionine sulfoxide reductase, and biliverdin reductase, are the most important antioxidant enzymes. Nutrient-derived antioxidants like ascorbic acid (Vitamin C), tocopherols and tocotrienols (Vitamin E), carotenoids and other low molecular weight compounds such as glutathione and lipoic acid are capable of neutralizing ROS. Synthetic antioxidants like butylated hydroxyl anisole (BHA), butylated hydroxyl toluene (BHT), propyl gallate (PG), metal chelating agent (EDTA), tertiary butyl hydroquinone (TBHQ), nordihydro guaretic acid (NDGA) are phenolic compounds that also perform the function of capturing the free radicals and stopping the chain reaction.[7]

IN-VITRO SCREENING ASSAYS:

1) DPPH Scavenging Assay:[8] The DPPH is a stable organic nitrogen radical, which bears a deep purple color and is widely used to assess the radical scavenging activity of antioxidant compounds. This method is based on the reduction of DPPH in methanol solution in the presence of a hydrogen–donating antioxidant due to the formation of the nonradical form DPPH-H. This transformation results in a color change from purple to yellow, which is measured spectrophotometrically. The disappearance of the purple color is monitored at 517nm. The DPPH assay is considered to be mainly based on the ET reaction, and hydrogen-atom abstraction is a marginal reaction pathway. This assay is simple and rapid and uses only a UV-visible spectrophotometer and thus explains its widespread use in the antioxidant screening.

The reaction mixture consists of 4 ml DPPH in methanol (0.1mM), 1 ml sample solution in methanol at different concentrations. It is incubated in the dark for 30 minutes and then the absorbance measured at 517 nm. In this assay, positive controls may be quercetin, gallic acid, ascorbic acid, BHA, rutin, trolox, catechin, BHT, α-tocopherol. Lower absorbance of the reaction mixture indicates higher free radical scavenging activity. The percentage of inhibition can be calculated from the formula:

$$\% \text{ Inhibition } = (A_0 - A_1 / A_0) \times 100$$

where; A_0 is the absorbance of control and A_1 is the absorbance in the presence of sample and standard.

2) Hydrogen Peroxide Scavenging Activity:[9,10] Hydrogen peroxide is an intermediate during endogenous oxidative metabolism and mediates radical oxygen formation such as OH, which may be used to predict the scavenging capability of antioxidants in biological systems. Hydrogen peroxide has only a weak activity to initiate lipid peroxidation, but its activity as an active oxygen species comes from its potential to produce the highly reactive hydroxyl radical through Fenton reaction.

Solution of hydrogen peroxide (20mM) is prepared in phosphate buffer saline (PBS at pH 7.4). Different concentrations of the extract and standard in the particular solvent (1 ml) are added to 2 ml of hydrogen peroxide in PBS. After 10 minutes, the absorbance is measured at 230 nm against a blank solution containing phosphate buffer without hydrogen peroxide. The standards used in this assay include ascorbic acid, BHA, α-tocopherol, quercetin, gallic acid. The percentage of hydrogen peroxide scavenging is calculated from the formula:

$$\% \text{ Scavenged } (H_2O_2) = (A_0 - A_1 / A_0) \times 100$$

where; A_0 is the absorbance of control and A_1 is the absorbance in the presence of sample and standard.

3) Hydroxyl Radical Scavenging Assay:[11-13] Hydrogen peroxide is the most reactive chemical species known. The hydroxyl radical is known to react with the components of the DNA molecule, damaging both the purine and pyrimidine bases and also the deoxyribose backbone. When the hydroxyl radical reacts with polyunsaturated fatty acid moieties of the cell membrane phospholipids, ultimately yields numerous carbonyl products such as malonialdehyde (MDA) which is responsible for DNA damage. The hydroxyl radical scavenging can be assayed by the 2-deoxyribose oxidation method. In this method, 2-deoxyribose is oxidized by the hydroxyl radical that is formed by Fenton reaction and degraded to malondialdehyde.

The reaction mixture (1 ml) consists of 500µl of sample (different concentrations), 100µl each of 2-deoxyribose (28 mM), EDTA (1.04 mM), $FeCl_3$ (0.2 mM), H_2O_2 (1 mM) and ascorbic acid (1 mM), which is incubated at 37ºC for 1 hour. After incubation, 1 ml of thiobarbituric acid (1%) and 1 ml of trichloroacetic acid (2.8%) are added and placed in boiling water bath for 20 minutes. The reaction mixture is allowed to cool and the absorbance is measured at 532 nm against blank. Trolox, gallic acid, mannitol, BHA, BHT, quercetin, catechin, rutin,

α-tocopherol, ascorbic acid may be used as standards. Percentage radical scavenging can be obtained from the formula:

$$\% \text{ Scavenged (DPPH)} = (A_0 - A_1 / A_0) \times 100$$

where; A_0 is the absorbance of control and A_1 is the absorbance in the presence of sample and standard.

4) Reducing Power Assay:[14,15] In the reducing power assay, the presence of reductants (antioxidants) in sample / standard resulted in the reduction of the ferricyanide (Fe^{3+}) complex into ferrous form (Fe^{2+}). The amount of Fe^{2+} complex can be estimated by measuring formation of Perl's Prussian blue at 700nm.

Different concentrations of the sample and standard in 1 ml of distilled water are mixed with 2.5 ml phosphate buffer (0.2M, pH 6.6) and 2.5 ml of potassium ferricyanide (1% w/v). This mixture is then incubated at 50ºC for 30 minutes. Following incubation, 2.5 ml of trichloroacetic acid (10% w/v) is added and centrifuged at 3000rpm for 10 minutes. Finally, 2.5 ml of the supernatant is mixed with 2.5 ml of distilled water and 0.5 ml of ferric chloride (0.1% w/v). The absorbance is measured at 700 nm. Ascorbic acid, rutin, quercetin, gallic acid, BHT may be used as the reference compounds. Higher the absorbance of the reaction mixture indicates higher reducing power.

5) Superoxide Radical Scavenging Activity:[16] Superoxide radical, a precursor of the reactive oxygen species is very harmful to cellular components and thus contributes to tissue damage and various diseases. The superoxide anions generated in a non-enzymatic phenazine methoxy sulphate- nicotinamide adenine dinucleotide (PMS-NADH) by the oxidation of NADH can be assayed by reduction of nitro blue tetrazolium (NBT) into purple colored formazan measured spectrophotometrically at 560 nm.

In this experiment, all the solutions are prepared in 100mM phosphate buffer (pH 7.4). 1 ml of NBT (156µM), 1 ml of NADH (456µM), and 0.1 ml of sample extracts are mixed. The reaction mixture is initiated by the addition of 1 ml of PMS (60µM). It is then incubated at 25ºC for 5 minutes followed by the measurement of absorbance at 560 nm against blank samples. BHA, ascorbic acid, curcumin, gallic acid, quercetin, trolox, α-tocopherol, and superoxide dismutase may be used as standards. Decreased absorbance at 560 nm indicates increased scavenging of the superoxide anions in the reaction mixture. The percentage inhibition of superoxide anion generation can be evaluated from the following equation:

$$\% \text{ Inhibition} = (A_0 - A_1 / A_0) \times 100$$

where; A_0 is the absorbance of control and A_1 is the absorbance in the presence of sample and standard.

6) Nitric Oxide Scavenging Activity:[17,18] Nitric oxide is a potent pleiotropic mediator of smooth muscle relaxation, neuronal signaling, inhibition of platelet aggregation and regulation of cell mediated toxicity. It is a diffusible free radical which plays many roles as an effector molecule in diverse biological system including neuronal messenger, vasodilation, antimicrobial and anti-tumor activities. This assay is based on the principle that sodium nitroprusside in aqueous solution at physiological pH generates nitric oxide spontaneously which interacts with oxygen to produce nitrite ions that can be measured by using Griess reagent.

The reaction mixture consists of 4 ml of sodium nitroprusside (10mM) in 1 ml of phosphate buffer (pH 7.4), and 1 ml of extract or reference compound in different concentrations which are incubated at 25ºC for 150 minutes. Following incubation, 0.5 ml of the reaction mixture is removed and mixed with 1 ml of sulphanilic acid (0.33% in 20% glacial acetic acid) and kept for 5 minutes for diazotization reaction to complete. Then, 1 ml of naphthylethylenediamine dihydrchloride (0.1%) is added and maintained at room temperature for 30 minutes. The absorbance is measured at 540nm against the corresponding blank solution. The compounds that can be used as positive controls include curcumin, gallic acid, sodium nitrite, caffeic acid, BHT, BHA, rutin, α-tocopherol, ascorbic acid. The percentage inhibition of nitric oxide radical generation can be calculated from the following equation:

$$\% \text{ Inhibition} = (A_0 - A_1 / A_0) \times 100$$

where; A_0 is the absorbance of control and A_1 is the absorbance in the presence of sample and standard.

7) Metal Chelating Assay:[19,20] Ferrozine quantitatively chelates with Fe^{2+} to form a red colored complex. But in the presence of other cheating agents, the formation of ferrozine- Fe^{2+} complex is disrupted and hence the intensity of red color also decreases. The chelating activity of a compound to compete with ferrozine for the ferrous ions can be evidenced by the reduction in the color. Metal chelating activity is one of the significant antioxidant mechanisms as it reduces the concentration of the catalyzing transition metal in the lipid peroxidation.

In this assay, 1 m1 of ferric chloride (2 mM; diluted 20 times) is mixed with different dilutions of the extract (1 ml). The reaction is initiated by the addition of 1 ml of ferrozine (5 mM; diluted 20 times). The absorbance is measured at 562 nm after 10 minutes. The positive controls that can be used in this assay are EDTA, citric acid. The ability of the sample to chelate ferrous ions can from the following equation:

$$\text{Chelating Effect (\%)} = (A_0 - A_1 / A_0) \times 100$$

where; A_0 is the absorbance of control and A_1 is the absorbance in the presence of sample

8) Total Phenolic Content:[21,22] Natural antioxidants, particularly phenolics, has been under very close scrutiny as potential therapeutic agents against a wide range of ailments including neurodegenerative diseases, cancer, diabetes, cardiovascular dysfunctions, inflammatory diseases and also aging. The medicinal actions of phenolics is mostly ascribed to their antioxidant capacity, free radical scavenging, chelation of redox active metal ions, modulation of gene expression and interaction with the cell signaling pathways. The total phenolic content can be determined by Folin-Ciocalteau (FC) method. For the FC assay, the oxidant is molybdotungstophosphoric heteropolyanion and the absorbance increase is measured. This assay measures the change in color when metal oxides are reduced by polyphenolic antioxidants, resulting in a blue solution which shows maximal absorption at 765 nm.

The samples (1 ml of different concentrations) are mixed with Folin-Ciocalteau reagent (5 ml, diluted 10 times) and allowed to stand for 5 minutes. Aqueous sodium carbonate (4 ml, 1 M) is then added to the above mixture. The calibration curve is prepared by mixing solution of standard (100, 200, 300, 400, 500 µg/ml) with the above reagents. The absorbance is measured after 2 hours at 765 nm. The total phenolic content in the sample is expressed in terms of standard equivalent (mg/g of the extracted compound). Gallic acid, tannin acid, caffeic acid, ferrulic acid, vanillic acid, pyrocatechol, D-catechin, chlorogenic acid may be used as positive controls.

9) Total Flavonoids Content:[23] Flavonoids play an important role in the antioxidant system. The antioxidant properties of flavonoids may be due to several mechanisms such as scavenging of active oxygen species i.e. hydroxyl radical, superoxide radical, superoxide anion radical, singlet oxygen, and nitrogen oxide; inhibition of hydrolytic and oxidative enzymes, chelation of metal ions. The antioxidant activity of flavonoids depends mainly on their ability to donate electrons or hydrogen atoms. The total flavonoids content may be determined by Aluminium chloride colorimetric method. This method is based on the principle that aluminium forms stable complex between the keto and the hydroxyl groups of flavones and flavonoids.

In this assay, quercetin or catechin may be used as the standard flavonoid compound. The calibration curve is prepared by dissolving 10 mg of the standard in 10 ml of methanol/ ethanol and then diluting to 20, 40, 60, 80, 100 µg/ml. the diluted standard solution (0.5 ml) is mixed separately with 1.5 ml of methanol/ ethanol, 0.1ml of 10% aluminium chloride, 0.1ml of 1 M potassium acetate and 2.8 ml of distilled water. The reaction mixture is incubated at room temperature for 30 minutes and the absorbance measured at 415 nm with a UV-spectrophotometer. Similarly, 0.5 ml of ethanolic/methanolic extracts is reacted with aluminium chloride for the determination of flavonoids content. In blank, the amount of 10% aluminium chloride is substituted by the same amount of distilled water. The total flavonoid content in the sample is expressed in terms of standard equivalent (mg/g of the extracted compound).

10) Ferric Reducing Antioxidant Power (FRAP) Assay:[24] FRAP assay was developed by Benzie and Strain to measure the reducing power in plasma, but now it has been adapted for use in the assay of antioxidants in botanicals. Reducing power is related to the degree of hydroxylation and extent of conjugation in polyphenols. In this assay, the ability of the antioxidants to reduce ferric ion is measured. It is based on the reduction of the complex of ferric ion and 2,3,5-triphenyl-1, 3, 4-triaza-2-azoniacyclopenta-1,4-diene chloride (TPTZ) to the ferrous ion at low pH. The absorbance is measured at 593 nm using a diode-array spectrophotometer.

The reaction mixture consists of 3.8 ml of FRAP reagent (300 mM acetate buffer at pH 3.6; 10 mM TPTZ in 40 mM HCl; 20 mM $FeCl_3.6H_2O$ in proportions of 10:1:1) and 0.2ml of extract which is incubated at 37ºC for 30 minutes. After incubation, the absorbance is measured at 593 nm. The standard antioxidants used in the assay include BHT, BHA, gallic acid, Trolox, ascorbic acid, quercetin, and catechin. The calibration curve is plotted by using $FeSO_4$. The ability of the antioxidant to reduce the ferric ion is expressed in mmol $FeSO_4$ equivalents per gram of sample.

11) Trolox Equivalent Antioxidant Capacity (TEAC) Assay:[25-27] This assay uses a diode-array spectrophotometer to measure the loss of color when an antioxidant is added to the blue-green chromophore ABTS [2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)]. The antioxidant reduces ABTS to $ABTS^{\bullet+}$ and decolorizes it.

The TEAC original assay was developed by Miller and Rice Evans (ABTS assay). This method utilized metmyoglobin and H_2O_2 to generate ferrylmyoglobin (a free radical species, $OH^{\bullet}$), which then reacted with ABTS to form a relatively stable radical cation $ABTS^{\bullet+.}$ This radical cation produces a stable blue-green color which is measured at 600 nm. The sample to be tested is added into the reaction medium before the radical was formed. Calibration of the assay is done using Trolox and the results are expressed in mmol/l of Trolox equivalent. This

order of addition of reagents in the TEAC assay however, leads to the overestimation of antioxidant capacity, because antioxidants can itself react with the radical oxidant as well as $ABTS^{\bullet+}$. Thus the assay has been revised to clearly generate $ABTS^{\bullet+}$ by first using oxidizing agents such as potassium persulfate , manganese dioxide, horseradish peroxide and peroxyl radicals, then adding antioxidants and measuring the direct reaction on the radical.

In the improved version of the TEAC assay, the radical cation $ABTS^{\bullet+}$, is pregenerated by the persulfate oxidation of ABTS. The reaction mixture consists of ABTS (7 mM) and potassium persulfate (2.45 mM) incubated at room temperature in the dark for 12-16 hours to give a deep blue solution. This solution is diluted with phosphate buffer (pH 7.4) until the absorbance values reached 0.7 at 734 nm. 10µl of sample solution is mixed with 1 ml of the above solution and the decrease in absorbance is measured at 734 nm after 6 minutes. Phosphate buffer solution without $ABTS^{\bullet+}$ solution is taken as control. The concentration of antioxidant giving the same percentage change of absorbance of the $ABTS^{\bullet+}$ as that of 1 mM Trolox is regarded as TEAC. Trolox, ascorbic acid, BHT, rutin or gallic acid may be used as standards.

12) Total Antioxidant Activity by Phosphomolybdenum complex method:[28] In the phosphomolybdenum complex method, the reduction of Mo (VI) to Mo (V) is detected at 695 nm by spectrophotometer due to the formation of green phosphate Mo (V) compounds at acidic pH.

For the total antioxidant capacity assay, 0.1 ml of extract is mixed with 1 ml of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) in eppendorff tube. The tubes are then caped and incubated at 95ºC for 90 minutes in a thermal block. After incubation, the reaction mixture is cooled to room temperature and the absorbance is measured at 695 nm against reagent blank. Ascorbic acid may be used as the standard antioxidant. The total antioxidant capacity is expressed as equivalents of ascorbic acid.

13) Oxygen radical Absorbance Capacity (ORAC) Assay:[29] ORAC assay measures antioxidant inhibition of peroxyl radical induced oxidations and hence reflects the classical radical chain breaking antioxidant activity by H atom transfer. In this assay, when free radical generators like azo-initiator compound (AAPH) is added to fluorescent molecules like beta-phicoerythrin or fluorescein and allowed to heat; the azo-initiator (AAPH) produces peroxyl free radicals that damages the fluorescent molecule and hence result in the loss of fluorescence. The antioxidant capacity is therefore determined by the decreased rate and amount of product formed over the time. The standard used in this assay may be Trolox.

The reaction mixture (4 ml) consists of 0.5 ml sample in phosphate buffer (75mM, pH 7.2) and 3 ml fluorescein solution incubated at 37ºC for 10 minutes. Then 0.5 ml of AAPH solution is added to initiate the reaction. The fluorescence intensity [485 nm (ex) / 525 nm (em)] is measured at 1 minute interval for 35 minutes at ambient conditions (pH 7.4, 37ºC). As the reaction progresses, fluorescence intensity decreases as fluorescein is consumed. Trolox of different concentration is used to construct the standard calibration curve. The final results are calculated using the differences of areas under the FL decay curves between the blank and sample and are expressed as micromole trolox equivalents (TE) per gram (µmol TE/g).

14) Total Radical Trapping Antioxidant Parameter (TRAP) Assay:[30,31] The TRAP assay is most often used for measurements of *in-vivo* antioxidant capacity in the serum or plasma because it measures the non-enzymatic antioxidants, such as glutathione, β-carotene, ascorbic acid and α-tocopherol. The TRAP may be either directly measured by a fluorescence based method or calculated by a mathematical formula, taking into account the serum levels of natural antioxidants: protein- bound SH (thiol) groups, uric acid, vitamin E and vitamin C. The assay involves initiation of lipid peroxidation by the generation of water-soluble peroxyl radicals and is sensitive to all known chain breaking antioxidants. This assay determines the ability of antioxidant compounds to interfere with the reaction between peroxyl radicals generated by AAPH [2,2'-azobis (2-amidinopropane) dihydrochloride] and R-phycoerythrin (probe). The fluorescence decay of R-phycoerythrin during a controlled peroxidation is measured using a luminescence spectrometer. The TRAP values are calculated from the length of the lag phase caused by the antioxidant and compared with standard Trolox.

15) Total Oxyradical Scavenging Capacity (TOSC) Assay:[32,33] TOSC assay permits the quantification of absorbance capacity of antioxidants specifically to three potent oxidants i.e. hydroxyl radicals, peroxyl radicals and peroxynitrite. This method is based on the ethylene yielding reaction of α-keto-ɣ-methiolbutyric acid (KMBA) with peroxyl radicals, hydroxyl radicals and peroxynitrite. Peroxyl radicals are generated by the thermal homolysis of ABAP. Hydroxyl radical are formed during iron-ascorbate driven Fenton reaction. Peroxynitrite is produced by the decomposition of SIN-1. Added antioxidant competes with KMBA for the radicals, reducing the production of ethylene, which is generally measured by gas chromatography. Linear dose-response

curves of antioxidants can be generated from the kinetics of the reaction.

16) Ferric Thiocyanate (FTC) Assay:[34,35] Membranes lipids are rich in unsaturated fatty acids that are most susceptible to oxidative processes. Specially, the linoleic acid and arachidonic acid are targets of lipid peroxidation. The FTC assay is used to measure the amount of peroxide in lipid peroxidation. Here, the peroxide will react with ferrous chloride and form ferric ions. These ferric ions combine with ammonium thiocyanate and produce a reddish pigment ferric thiocyanate.

In this assay, 4 mg of sample in 4 ml of absolute ethanol is mixed with 4.1 ml of linoleic acid (2.5%) in absolute ethanol, 8 ml of phosphate buffer (0.05M, pH 7) and 3.9 ml of distilled water and placed in screw cap containers incubated at 40ºC in the dark. To 0.1 ml of this solution, 9.7 ml of ethanol (75%v/v), 0.1 ml of ammonium thiocyanate (30%) and 0.1 ml of ferrous chloride (20 mM) in HCl (3.5%v/v) are added to allow any peroxides resulting from the oxidation of linoleic acid to react, forming a red color complex that can be detected spectrophotometrically at 500 nm. The measurement is taken every 24 hours until the absorbance of the control reached its maximum value. High absorbance value indicated high levels of linoleic acid oxidation. Phosphate buffer is used as the reaction blank. BHT, BHA, ascorbic acid and α-tocopherol may be used as standards n this assay. The percentage inhibition of lipid peroxidation can be calculated as:

$$\% \text{ inhibition} = (A_0 - A_1 / A_0) \times 100$$

where; A_0 is the absorbance of control (linoleic acid alone) and A_1 is the absorbance in the presence of sample or standard.

17) Thiobarbituric Acid Reactive Substances (TBARS):[36-38] 2-Thiobarbituric acid reactive substances are naturally present in the biological systems and include lipid hydroperoxides and aldehydes which increase in concentration as a response to oxidative stress. TBARS assay uses a fluorometric microplate assay method and the values are usually reported in malondialdehyde (MDA) equivalents, a compound that results from the decomposition of polyunsaturated fatty acid lipid peroxides. The TBARS assay is a well recognized, established method for quantifying MDA in biological samples. This assay is based in the reaction of a chromogenic reagent, 2-thiobarbituric acid (TBA) with MDA at 25ºC. Here, one molecule of MDA reacts with 2 molecules of 2-thiobarbituric acid via a Knoevenagel type condensation to yield a chromophore with an absorbance maximum at 532 nm.

18) Cupric Reducing Antioxidant Capacity (CUPRAC):[39] The CUPRAC assay is usually based on the reduction of Cu (II) to Cu (I) by the action of antioxidants in the sample. This assay uses neocuproine (2,9-dimethyl-1,10-phenanthroline) which forms complex with Cu (I), yielding a chromophore with a maximum absorbance at 450 nm. A dilution curve generated by the uric acid standards is used to convert the sample absorbance to uric acid equivalents. Copper has advantages over iron for the antioxidant assays in that all classes of antioxidants, including thiols, are detected with little interference from the reactive radicals and the copper reaction kinetics are faster than iron. The standards that may be used for the assay include ascorbic acid, uric acid, gallic acid, and quercetin.

19) Peroxynitrite Scavenging Assay:[40,41] Superoxide and nitric oxide react under diffusion control rate to form peroxynitrite ($ONOO^-$). It is a short lived oxidant species that is a potent inducer of cell death. A fundamental reaction of $ONOO^-$ in biological systems is its fast reaction with carbon dioxide (in equilibrium with physiological levels of bicarbonate anion), which leads to the formation of carbonate ($CO_3^{\bullet -}$) and nitrogen dioxide ($^{\bullet}NO_2$) radicals.

Peroxynitrite is synthesized 12 hours prior to the assay according to a standard procedure. An acidic solution (0.6M HCl) of 5 ml H_2O_2 (0.7M) is mixed with 5 ml of KNO_2 (0.6M) on an ice bath and 5 ml of ice-cold NaOH (1.2M) is added to the mixture. The mixture is treated with granular MnO_2 to adsorb the excess H_2O_2. The reaction mixture is left overnight at -20ºC. The peroxynitrite concentration is measured spectrophotometrically at 302 nm.

The peroxynitrite scavenging activity is determined by Evans blue bleaching assay. In a final volume of 1ml, the reaction mixture consists of phosphate buffer (50mM, pH 7.4), NaCl (90mM), DTPA (0.1mM), KCl (5mM), Evans blue (12.5µM), various concentrations of the sample and peroxynitrite (1mM). the mixture is incubated at 25C for 30 minutes. the absorbance is measured at 611 nm. The standard used may be gallic acid. The percentage of peroxynitrite scavenging can be obtained from the formula:

$$\% \text{ Scavenging of peroxynitrite} = (A_0 - A_1 / A_0) \times 100$$

where; A_0 is the absorbance of control and A_1 is the absorbance in the presence of sample and standard.

20) Chemiluminescence:[42,43] In the Chemiluminescence analysis, radical oxidants react with marker compound to produce excited state species which emit chemiluminescence (chemically induced light). Chemiluminescence is usually characterized by low emission intensity. The radical oxidant sources include horseradish peroxidase, and H_2O_2-hemin. The most widely used marker compounds to trap oxidants are

luminol, lucigenin and bioluminescent proteins such as pholasin. Continuous light output depends on constant production of free radical intermediates derived from p-iodophenol, luminol and oxygen, and this light emission is sensitive to interference by radical scavenging antioxidants. The antioxidant capacity is determined as the time of depressed light emission, which is wantonly measured at 10% recovery of light output.

21) Photochemiluminescence:[44]

Photochemiluminescence (PCL) is an advanced technique for the estimation of the total radical scavenging activity. The PCL method is based on a photo-induced chemiluminescence accompanied by antioxidant inhabitable auto-oxidation of luminol. The luminol is a photosensitizer generating superoxide radicals and also a chemiluminogenic probe for free radicals. The integral antioxidative capacity of the extract and fractions are reported as ascorbic acid equivalents.

CONCLUSION:

There has been abundant evidence showing the involvement of oxidative stress in various disorders and diseases. It is reasonable therefore to expect the beneficial effects of antioxidants in maintain the health and lowering the risk of diseases. Antioxidants are compounds that prevent the oxidation of essential biological macromolecules by inhibiting the propagation of oxidizing chain reactions. The researchers have channeled their interest in isolation of antioxidants from natural sources, keeping in view the adverse effects of synthetic antioxidants. Various methods have been developed and applied in different systems, but most of the methods result in inconsistent results. Therefore, it is recommended that the capacity of antioxidant compounds and their mixtures should be assessed from their effect on the levels of plasma lipid peroxidation *in-vitro* and biomarkers of oxidative stress *in-vivo.*

REFERENCES:

1. Sies H. Oxidative stress: oxidants and antioxidants. Exp Physiol. 1997; 82(2): 291-295.
2. Finkel T, Holbrook N. J. Nature 408, 2000, 239-247.
3. Halliwell, B. and Gutteridge, J. M. C. Free radicals in biology and medicine. Clarendon Press Oxford, 1993. 22-81.
4. R. H. Bisby, and A. W. Parker, in Free radicals : from basic science to medicine, Eds G. Poli, E. Albano and M. U. Dianzani, Birkhauser Verlag, Berlin, 1993, 31-37.
5. Borek C. Antioxidants and cancer, science and medicine. The baby-boomers guide New Canaan connecticus keats publishing 1991; 4: 51-61.
6. Jacob RA. The Integrated Antioxidant System. Nutr Res. 1995; 15(5): 755-766.
7. Hurrell R. Influence of vegetable protein sources on trace element and mineral bioavailability. J Nutr. 2003; 133(9): 2973S-2977S.
8. Blois MS. Antioxidant determinations by the use of a stable free radical. Nature 1958,181: 1199-1150.
9. Juntachote, T. and Berghofer, E. Antioxidative properties and stability of ethanolic extracts of Holy basil and Galangal. Food Chem. 2005;92:193-202.
10. G.K. Jayaprakasha, L.J. Rao and K.K. Sakariah, Bioorg. Med. Chem., 2004,12, 5141.
11. Halliwell, B. and J.M.C. Gutteridge. Free Radicals, Aging and Disease. In: Free Radicals in Biology and Medicine, Halliwell, B. and J.M.C. Gutteridge (Eds.). Clarendon Press, Oxford, UK., 1989, 416-508.
12. Valentao, P., E. Fernandes, F. Carvalho, P.B. Andrade, R.M. Seabra and M.D.L. Bastos. Antioxidant activity of *Hypericum androsaemum* infusion: Scavenging activity against superoxide radical, hydroxyl radical and hypochlorous acid. Biol. Pharm. Bull., 2002,22: 1320-1323.
13. Kunchandy E, Rao MNA. Oxygen radical scavenging activity of curcumin. Int. J. Pharm. 1990,58: 237-240.
14. G.C. Yen and H.Y. Chen, J. Agric. Food Chem., 1995,43, 27.
15. Gulcin I, Oktay M, Kufrevioglu O, Aslan A. Determination of antioxidant activity of lichen Cetraria islandica (L) Ach. J Ethnopharmacol 2002;79:325–329.
16. M. Nishikimi, N. Appaji and K. Yagi, Biochem. Biophys. Res. Commun.,1972, 46, 849.
17. Miller, N.J., C. Rice-Evans, M.J. Davies, V. Gopinathan and A. Milner. A novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonates. Clin. Sci., 1993,84: 407-412.
18. L. Marcocci, L. Packer, M.T. Droy-Lefaix, A. Sekaki and M. Gardes- Albert, Methods Enzymol., 1994,234, 462.
19. N. Singh and P.S. Rajini, Food Chem., 2004,85, 611.
20. T.C.P. Dinis, V.M.C. Maderia and L.M. Almeida, Archives Biochem. Biophys., 1994,315, 161.
21. G. Miliauskas. P.R. Venskutonis and T.A. Venskutonis, Food Chem., 2004,85, 231.
22. A. Shekher Pannala, T.S. Chan, P.J. O’Brien, C.A. Rice-Evans, Flavonoids B-ring chemistry and antioxidant activity: fast reaction kinetics, Biochem. Biophys. Res. Commun. 2001,282,1161–1168.
23. Nabavi SM, Ebrahimzadeh MA, Nabavi SF, Hamidinia A, Bekhradnia AR, 2008. Determination of antioxidant

activity, phenol and flavonoids content of Parrotia persica Mey. Pharmacol, 2008,2: 560-567.

24. Benzie IF, Strain JJ. The ferric reducing ability of plasma (FRAP) as a measure of “antioxidant power” the FRAP assay. Anal. Biochem. 1996,239: 70-76.

25. Miller, N. J.; Diplock, A. T.; Rice-Evans, C.; Davies, M. J.; Gopinathan, V.; Milner, A. A novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonates. *Clin. Sci.* 1993, *84*, 407-412.

26. Strube, M.; Haenen, G. R. M. M.; van den Berg, H.; Bast, A Pitfalls in a method for assessment of total antioxidant capacity. *Free Radical Res.* 1997, *26*, 515-521.

27. Stratil P, Klejdus B, Kuban V. Determination of total content of phenolic compounds and their antioxidant activity in vegetables – Evaluation of spectrophotometric methods. J. Agric. Food Chem. 2006,54: 607-616.

28. Kumaran A. and J.R. Karunakaran. In-vitro antioxidant activities of methanol extracts of five Phyllanthus species from India, LWT-Food Science and Technology, 2007,40(2), 344- 352.

29. Ou, B., Huang, D., Hampsch-Woodill, M., Flanagan, J.A., Deemer, E.K. Analysis of antioxidant activities of common vegetables employing oxygen radical absorbance capacity (ORAC) and ferric reducing antioxidant power (FRAP) assays: a comparative study. Journal of Agricultural and Food Chemistry 2002,50, 3122–3128.

30. Huang D, Ou B, Prior RL. The chemistry behind antioxidants capacity assays. J Agric food chem., 2005,53(6), 1841 – 1856.

31. Wayner, D. D. M.; Burton, G. W.; Ingold, K. U.; Barclay, L. R. C.; Locke, S. J. The relative contribution of vitamin E, urate, ascorbate and protiens to the total peroxyl radical-trapping antioxidant activity of human blood plasma. Biochim. Biophys. Acta 1987, 924, 408-419.

32. Winston, G. W.; Regoli, F.; Dugas, A. J., Jr.; Fong, J. H.; Blanchard, K. A. A rapid gas chromatographic assay for determining oxyradical scavenging capacity of antioxidants and biological fluids. Free Radical Biol. Med. 1998, 24, 480-493.

33. Regoli, F.; Winston, G. W. Quantification of total oxidant scavenging capacity of antioxidants for peroxynitrite, peroxyl radicals, and hydroxyl radicals. Toxicol. Appl. Pharmacol. 1999, 156, 96-105.

34. Abbasi, M. A., S. Arshad, T. Riaz, A. Zafar, Aziz-ur-Rehman, S.Z. Siddiqui, T. Shahzadi, and M. Ajaib. In vitro Assessment of Relief to Oxidative Injury by different samples of Xanthoxylum armatum. Asian J. Chemistry, 2011,23(8): 3353- 3356.

35. Kikuzaki H, Nakatani N. Antioxidant effects of some ginger constituents. J Food Sci 1993,58: 1407-1410.

36. Armstrong, D.; Browne, R. Free Rad. Diag. Med. 1994,366; 43-58.

37. Botsoglou, N.A., et al. J. Agric. Food Chem. 1994,42; 1931-1937.

38. Yagi, K. Free Rad. Antiox. Prot., 1998,108; 101-106.

39. Apak, R.; Gu¨cülu¨, K. G.; Ozyu¨rek, M.; Karademir, S. E. Novel total antioxidant capacity index for dietary polyphenols and vitamins C and E, using their cupric iron reducing capability in the presence of neocuproine: CUPRAC method. J. Agric. Food Chem. 2004, 52, 7970-7981.

40. Beckman JS, Chen H, Ischiropulos H, Crow JP: Oxidative chemistry of peroxynitrite**.** Methods Enzymol 1994, 233:229-240.

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

42. Whitehead, T. P.; Thorpe, G. H. G.; Maxwell, S. R. J. Enhanced chemiluminescent assay for antioxidant capacity in biological fluids. Anal. Chim. Acta 1992, 266, 265-277.

43. Bastos, E. L.; Romoff, P.; Eckert, C. R.; Baader, W. J. Evaluation of antiradical capacity of H2O2-hemin induced luminol chemiluminescence. J. Agric. Food Chem. 2003, 51, 7481-7488.

44. Govindarajan, R., M. Vijaykumar, C.V. Rao, A. Shirwaikar, A.K.S. Rawat, S. Mehrotra and P. Pushpangadan. Antioxidant potential of Anogeissus latifolia. Biol. Pharm. Bull., 2004,27: 1266-1269.