Review Article

A Review on In-vitro Antioxidant Methods

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ABSTRACT

Cellular damage or oxidative injury arising from free radicals or reactive oxygen species (ROS) now appears the fundamental mechanism underlying a number of human neurodegenerative disorders,diabetes, inflammation, viral infections, autoimmune pathologies and digestive system disorders. Free radical are generated through normal metabolism of drugs, environmental chemicals and other xenobiotics as well as endogenous chemicals, especially stress hormones (adrenalin and noradrenalin). It emphasizes the method simplicity, time required, instrumentation which makes us to decide which method to be fallowed to perform antioxidant property based on the feasibilities afforded to determine it. The research studies should also be carried mostly on the accurate methods for exact results which also act as a good reference for the further researches. .

INTRODUCTION

The term antioxidant originally was used to refer specifically to a chemical that prevented the consumption of oxygen. In the late 19th and early 20th century, extensive study was devoted to the uses of antioxidants in important industrial processes, such as the prevention of metal corrosion, the vulcanization of rubber, and the polymerization of fuels in the fouling of internal combustion engines (Matill HA et. al,. 1947). Natural antioxidants present in the plants scavenge harmful free radicals from our body. Free radical is any species capable of independent existence that contains one or more unpaired electrons which reacts with other molecule by taking or giving electrons, and involved in many pathological conditions **(**Madhavi et.al,. 1996). Free radical is a chemical compound which contains an unpaired electron spinning on the peripheral layer around the nucleus. The family of free radicals generated from the oxygen is called ROS which cause damage to other molecules by extracting electrons from them in order to attain stability. ROS are ions, atoms or molecules that have the ability to oxidize reduced molecules. ROS are various forms of activated oxygen, which include free radicals such as superoxide anion radicals (.O2-) and hydroxyl radicals (OH·), as well as non-free radicals (H_2O_2) and singlet oxygen (Halliwell et. al,. 1995). Oxidative stress has been implicated in the pathology of many diseases such as inflammatory conditions, cancer, diabetes and aging (Marx et. al,. 1987). Foods rich in antioxidants have been shown to play an essential role in the prevention of cardiovascular diseases, cancer, neurodegenerative diseases, inflammation and problems caused by cell and cutaneous aging (Pratt et. al,. 1992). like Alzheimer's disease, Parkinson's disease, atherosclerosis, cancer, arthritis, immunological incompetence and neurodegenerative disorders, etc. Nutritional antioxidant deficiency also leads to oxidative stress, which signifies the identification of natural anti-oxidative agents present in die consumed by human population (Sies et. al,. 1995), (Parker et. al,. 1980). Currently there has been an increased interest globally to identify antioxidant compounds and low or no side effects for use in preventive medicine and food industry.

Screening Methods of Antioxidant Activity

1. Free Radical Scavenging Assays

α, α--Diphenyl- β-picryl-hydrazyl radical scavenging (DPPH) Assay. The DPPH is a stable free radical and is widely used to assess the radical scavenging activity of

antioxidant compounds. This method is based on thereduction of DPPH in methanol solution in the presence of a hydrogen–donating antioxidant due to the formation of the nonradical form DPPH-H (Blois, 1958). 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 free radical scavenging activity can be measured by using 2, 2-diphenyl-1picryl-hydrazyl or 1, 1- diphenyl-2-picryl-hydrazyl by the method of McCune and Johns (2002). The reaction mixture (3.0 ml) consist of 1.0 ml of DPPH in methanol (0.3 mM), 1.0 ml of the extract and 1.0 ml of methanol. It is incubated for 10 min in dark, then the absorbance is measured at 517 nm. In this assay, the positive controls can be ascorbic acid, gallic acid (Blois, 1958), BHA, α-tocopherol (Shimada et al., 1992), quercetin (Shon et al., 2003), BHT (Liyana- Pathirana and Shahidi, 2005), rutin (Yamasaki et al., 1994), catechin (Astudillo et al., 2000) or glutathione (Kato et al., 1988). The percentage of inhibition can be calculated using the formula:

Inhibition (%) = (Ao – A1 / Ao) × 100

Where; A0 is the absorbance of control and A1 is the absorbance of test.

2. Ferric reducing antioxidant power (FRAP) assay

FRAP assay is based on the ability of antioxidants toreduce Fe^{3+} to Fe^{2+} in the presence of 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ), forming an intense blue Fe^{2+}-TPTZ complex with an absorption maximum at 593 nm. This reaction is pH-dependent (optimum pH 3.6). The absorbance decrease is proportional to the antioxidant content (Benzie and Strain, 1996). 0.2 ml of the extract is added to 3.8 ml of FRAP reagent (10 parts of 300 mM sodium acetate buffer at pH 3.6, 1 part of 10.0 mM TPTZ solution and 1 part of 20.0 mM FeCl3. 6H2O solution) and the reaction mixture is incubated at 37°C for 30 min and the increase in absorbance at 593 nm is measured. FeSO4 is used for calibration. The antioxidant capacity based on the ability to reduce ferric ions of sample is calculated from the linear calibration curve and expressed as mmol FeSO4 equivalents per gram of sample. BHT, BHA, ascorbic acid, quercetin, catechin or trolox (Benzie and Strain, 1996) can be used as a positive control.

3. Hydrogen peroxide radical scavenging (H_2O_2) assay

Hydrogen peroxide occurs naturally at low concentration levels in the air, water, human body, plants, microorganisms, food and beverages. It is widely used as a bleaching agent in the textile, paper and pulp industries. Human beings exposed to H2O2 indirectly via the environment are estimated as 0.28 mg/kg/day with intake from leaf crops contributing most to this exposure. Hydrogen peroxide enters the human body through inhalation of vapor or mist and through eye or skin contact. In the body, H_2O_2 is rapidly decomposed into oxygen and water and this may produce hydroxyl radicals ($OH^{\cdot}$) that can initiate lipid peroxidation and cause DNA damage. The ability of plant extracts to scavenge hydrogen peroxide is determined according to the method of Ruch et al. (1989). A solution of hydrogen peroxide (40 mM) is prepared in phosphate buffer (50mMpH 7.4). The concentration of hydrogen peroxide is determined by absorption at 230 nm using a spectrophotometer. Extract (20 - 60 µg/ml) in distilled water isadded to hydrogen peroxide and absorbance at 230 nm is determined after 10 min against a blank solution containing phosphate buffer without hydrogen peroxide. The percentage of hydrogen peroxide scavenging is calculated as follows:

% Scavenged (H_2O_2) = (A0 – A1 / A0) X 100

Where; A0 is the absorbance of control and A1 is the absorbance of test. Ascorbic acid, rutin BHA (Jayaprakasha et al., 2004), α-tocopherol (Gulcin et al., 2003) or quercetin (Ruch et al., 1989) can be used as a positive control.

4. Hydroxyl radical scavenging (HO) assay

Hydroxyl radical is one of the potent reactive oxygenspecies in the biological system. It reacts with polyunsaturated fatty acid moieties of cell membrane

phospholipids and causes damage to cell (Halliwell and Gutteridge, 1981). The model used is ascorbic acid-iron- EDTA model of $HO^{\cdot}$ generating system. This is a totally aqueous system in which ascorbic acid, iron and EDTA conspire with each other to generate hydroxyl radicals. The scavenging ability for hydroxyl radicals is measured by the method of Kunchandy and Rao (1990). The reaction mixture (1.0 ml) consist of 100 µl of 2-deoxy-Dribose (28 mM in 20 mM KH2PO4 -KOH buffer, pH 7.4), 500 µl of the extract, 200 µl EDTA (1.04 mM) and 200 µM FeCl3 (1:1 v/v), 100 µl of H2O2 (1.0 mM) and 100 µl ascorbic acid (1.0 mM) which is incubated at 37ºC for 1 h. 1.0 ml of thiobarbituric acid (1%) and 1.0 ml of trichloroacetic acid (2.8%) are added and incubated at 100ºC for 20 min. After cooling, absorbance is measured at 532 nm, against a blank sample. Gallic acid, mannitol, catechin (Kunchandy and Rao, 1990), vitamin E (Halliwell et al., 1987), quercetin, BHA (Halliwell and Gutteridge, 1981), α-tocopherol (Klein et al., 1981), rutin or ascorbic acid (Jayaprakasha et al. 2004) can be used as a positive control.

5. Metal chelating activity

Ferrozine can quantitatively chelate with Fe^{2+} and form a complex with a red color. This reaction is limited in the presence of other chelating agents and results in a decrease of the red color of the ferrozine-Fe^{2+} complexes. Measurement of the color reduction estimates the chelating activity to compete with ferrozine for the ferrous ions (Soler-Rivas et al., 2000).The chelation of ferrous ions is estimated using the method of Dinis et al. (1994). 0.1 ml of the extract is added to a solution of 0.5 ml ferrous chloride (0.2 mM). The reaction is initiated by the addition of 0.2 ml of ferrozine (5 mM) and incubated at room temperature for 10 min and then the absorbance is measured at 562 nm. EDTA or citric acid (Dinis et al., 1994) can be used as a positive control.

6. Nitric oxide radical scavenging (NO) assay

Nitric oxide generated from sodium nitroprusside in aqueous solution at physiological pH interact with oxygen to produce nitrite ions, which were measured using the Griess reaction reagent (Green et al., 1982). 3.0 ml of 10 mM sodium nitroprusside in phosphate buffer is added to 2.0 ml of extract and reference compound in different concentrations (20 - 100 µg/ml). The resulting solutions are then incubated at 25°C for 60 min. A similar procedure is repeated with methanol as blank, which serves as control. To 5.0 ml of the incubated sample, 5.0 ml of Griess reagent (1% sulphanilamide, 0.1% naphthyethylene diamine dihydrochloride in 2% H3PO3) is added and absorbance of the chromophore formed is measured at 540 nm. Percent inhibition of the nitrite oxide generated is measured by comparing the absorbance values of control and test preparations. Curcumin, caffeic acid, sodium nitrite (Sreejayan, 1997), BHA, ascorbic acid, rutin (Jayaprakasha et al., 2004), BHT or α-tocopherol (Garrat, 1964) can be used as a positive control.

7. Oxygen radical absorbance capacity (ORAC) assay

The capacity of a compound to scavenge peroxyl radicals, generated by spontaneous decomposition of 2, 2'- azo-bis, 2- amidinopropane dihydrochloride (AAPH), was estimated in terms of standard equivalents, using the ORAC assay (Prior et al., 2005). The method of Ou et al. (2002a, 2002b) is used for the estimation. The reaction mixture (4.0 ml) consist of 0.5 ml extract in phosphate buffer (75 mM, pH 7.2) and 3.0 ml of fluorescein solution both are mixed and pre-incubated for 10 min at 37°C. Then, 0.5 ml of 2, 2'-azo-bis, 2- amidinopropane (AAPH) dihydrochloride solution is added and immediately the loss of fluorescence (FL) is observed at 1 min intervals for 35 min. The final results are calculated using the differences of areas under the FL decay curves between the blank and a sample and are expressed as micromole trolox equivalents (TE) per gram (µmol TE g-1).

8. Reducing power (RP)

Reducing power is associated with antioxidant activity and may serve as a significant reflection of the antioxidant

activity (Oktay et al., 2003). Compounds with reducing power indicate that they are electron donors and can reduce the oxidized intermediates of lipid peroxidation processes, so that they can act as primary and secondary antioxidants (Yen and Chen, 1995). The reducing power can be determined by the method of Athukorala et al. (2006). 1.0 ml extract is mixed with 2.5 ml of phosphate buffer (200 mM, pH 6.6) and 2.5 ml of potassium ferricyanide (30 mM) and incubated at 50°C for 20 min. Thereafter, 2.5 ml of trichloroacetic acid (600 mM) is added to the reaction mixture, centrifuged for 10 min at 3000 rpm. The upper layer of solution (2.5 ml) is mixed with 2.5 ml of distilled water and 0.5 ml of FeCl3 (6 mM) and absorbance is measured at 700 nm. Ascorbic acid, butylated hydroxyanisole (BHA), α-tocopherol, trolox (Oyaizu, 1986) or butylated hydroxytoluene (BHT) (Jayaprakasha et al., 2001) can be used as positive control.

9. Superoxide anion radical scavenging (SO) assay

Although superoxide anion is a weak oxidant, it gives rise to generation of powerful and dangerous hydroxyl radicals as well as singlet oxygen, both of which contribute to oxidative stress (Meyer and Isaken, 1995). Numerous biological reactions generate superoxide anions which are highly toxic species. In the PMS/NADH-NBT system, the superoxide anion derived from dissolved oxygen from PMS/NADH coupling reaction reduces NBT. The decrease of absorbance at 560 nm with antioxidants thus indicates the consumption of superoxide anion in the reaction mixture. The superoxide anion scavenging activity is measured as described by Robak and Gryglewski (1988). The superoxide anion radicals are generated in 3.0 ml of Tris-HCl buffer (16 mM, pH 8.0), containing 0.5 ml of NBT (0.3 mM), 0.5 ml NADH (0.936 mM) solution, 1.0 ml extract and 0.5 ml Tris-HCl buffer (16 mM, pH 8.0). The reaction is started by adding 0.5 ml PMS solution (0.12 mM) to the mixture, incubated at 25°C for 5 min and then the absorbance is measured at 560 nm against a blank sample. Gallic acid (Robak and Gryglewski, 1988), BHA, ascorbic acid, α-tocopherol, curcumin (Nishikimi et al., 1972), quercetin (Beauchamp and Fridovich, 1971) or trolox (Fernandes et al., 2003) can be used as a positive control.

10. Total phenolic content (TPC)

Plant polyphenols, a diverse group of phenolic compounds (flavanols, flavonols, anthocyanins, phenolic acids, etc.) possess an ideal structural chemistry for free radical scavenging activity. Antioxidative properties of polyphenols arise from their high reactivity as hydrogen or electron donors from the ability of the polyphenol derived radical to stabilize and delocalize the unpaired electron (chain-breaking function) and from their potential to chelate metal ions (termination of the Fenton reaction) (Rice-Evans et al., 1997). The amount of total phenol content can be determined by Folin-Ciocateu reagent method (McDonald et al., 2001). 0.5 ml of extract and 0.1 ml of Folin-Ciocalteu reagent (0.5 N) are mixed and incubated at room temperature for 15 min. Then 2.5 ml saturated sodium carbonate is added and further incubated for 30 min at room temperature and absorbance measured at 760 nm. Gallic acid (McDonald et al., 2001), tannic acid (Wolfe et al., 2003), quercetin (Singleton and Rossi, 1965), chlorogenic acid (Singleton et al., 1999), pyrocatechol (Slinkard and Singleton, 1977) or guaiacol (Yildirim et al., 2001) can be used as positive controls. The total phenolic content is expressed in terms of standard equivalent (mgg-1 of extracted compound).

11. Total flavonoid (TF)

The antioxidative properties of flavonoids are due to several different mechanisms, such as scavenging of free radicals, chelation of metal ions, such as iron and copper, and inhibition of enzymes responsible for freeradical generation (Benavente-Garcia, 1997). Depending on their structure, flavonoids are able to scavenge practically all known ROS. The amount of total flavonoid content can be determined by Aluminum chloride method (Chang et al., 2002). The reaction mixture (3.0 ml) comprised of 1.0 ml of extract, 0.5

ml of aluminum chloride (1.2%) and 0.5 ml of potassium acetate (120 mM) is incubated at room temperature for 30 min and absorbance measured at 415 nm. Quercetin (Ordonez et al., 2006) or catechin (Kim et al., 2003) can be used as a positive control. The flavonoid content is expressed in terms of standard equivalent (mgg-1 of extracted compound).

12. Total antioxidant activity

The oxidation of linoleic acid generates peroxyl free radicals due to the abstraction of hydrogen atoms from diallylic methylene groups of linoleic acid (Kumaran and Karunakaran, 2006). The free radicals then will oxidize the highly unsaturated beta carotene. Consequently, the orange coloured chromophore of beta carotene would be degraded and the results can be monitored spectrophotometrically. The antioxidant activity is determined by the conjugated diene method (Lingnert et al., 1979). Each extract (0.1 - 20 mg/ml) in water or ethanol (100 µl) is mixed with 2.0 ml of 10 mM linoleic acid emulsion in 0.2 M sodium phosphate buffer (pH 6.6) in a test tube and kept in dark at 37°C to accelerate oxidation. After incubation for 15 h, 0.1 ml from each tube is mixed with 7.0 ml of 80% methanol in deionized water and the absorbance of the mixture is measured at 234 nm against a blank in a spectrophotometer.

13. Trolox equivalent antioxidant capacity (TEAC) assay

The $ABTS^{\cdot}+$ formed from the reaction $ABTS\text{-}e^{-} \rightarrow ABTS^{\cdot}+$ reacts quickly with ethanol/hydrogen donors to form colourless 2, 2'-azinobis (3-ethyl-benzothiazoline 6- sulfonate (ABTS). The reaction is pH - independent. A decrease of the $ABTS^{\cdot}+$ concentration is linearly dependent on the antioxidant concentration. The ABTS free radical-scavenging activity of plants samples is determined by the method of Stratil et al. (2006). The radical cation ABTS. + is generated by
persulfate oxidantion of ABTS. A mixture (1:1, v/v) of ABTS (7.0 mM) and potassium persulfate (4.95 mM) is allowed to stand overnight at room temperature in dark to form radical cation ABTS +. A working solution is diluted with phosphate buffer solution to absorbance values between 1.0 and 1.5 at 734 nm. An aliquot (0.1 ml) of each sample is mixed with the working solution (3.9 ml) and the decrease of absorbance is measured at 734 nm after 10 min at 37°C in the dark. Aqueous phosphate buffer solution (3.9ml, without ABTS. + solution) is used as a control. The ABTS + scavenging rate is calculated. Trolox, BHT, rutin (Re et al., 1999), ascorbic acid (Alzoreky and Nakahara, 2001) or gallic acid (Auddy et al., 2003) can be used as a positive control.

14. Xanthine oxidase method

To determine superoxide anion-scavenging activity, two different assays can be used: the enzymatic method with cytochrome C (McCord and Fridovich, 1969) and nonenzymatic method with nitroblue tetrazolium (NBT) (Zhang and Lu, 1990). With cytochrome C method, superoxide anions can be generated by xanthine and xanthine oxidase system. The xanthine oxidase activity with xanthine as the sub-substrate is measured spectrophotometrically, by the method of Noro et al. (1983). The extract (500 µl of 0.1 mg/ml) and allopurinol (100 µg/ml) (in methanol) is mixed with 1.3 ml phosphate buffer (0.05M, pH 7.5) and 0.2 ml of 0.2 units/ml xanthine oxidase solution. After 10 min of incubation at room temperature (25°C), 1.5 ml of 0.15 M xanthine substrate solution is added to this mixture. The mixture is again incubated for 30 min at room temperature (25°C) and then the absorbance is measured at 293 nm using a spectrophotometer against blank (0.5 ml methanol, 1.3 ml phosphate buffer, 0.2 ml xanthine oxidase). The solution of 0.5 ml methanol, 1.3 ml phosphate buffer, 0.2 ml xanthine oxidase and 1.5 ml xanthine substrate is used as a control. Percentage of inhibition was calculated using the formula:

Percentage of inhibition = $[1 – (As / Ac)] \times 100$

Where; As and Ac are the absorbance values of the test sample and control, respectively. BHT (Chang et al., 1996) or catechin (Hirschmann et al., 1996) can be used as a positive control.

15. Dye-substrate oxidation method

A novel microtiter plate assay was developed to determine the total peroxyl radical trapping activity of antioxidant extracted from marine organisms by measuring the inhibition rate of dye-substrate oxidation. They compared use of dihydrorhodamine- 123, dihydrofluorescein and dichlorodihydrofluorescein as reduced substrates for oxidation by peroxyl radicals generated from 2,2- azobis (2-amidinopropane) dihydrochloride. The oxidation products of these highly reactive substrates are intensely colored dyes that absorb maximally in the wavelength region, 489 to 512 nm, and their concentrations were determined photometrically using a 96-well, microtiter plate reader. The microtiter plate method provides for concurrent multisample analysis with automated data storage, regression analyses, and calculation of oxidation inhibition rates. Dihydrorhodamine was selected as the preferred substrate for screening crude extracts, and typical assay results are presented. Novel lead antioxidants are selected from active extracts by chromatographic analysis with electrochemical detection. (Walter Dunlap, et.al, 2003)

16. Cupric Ion Reducing antioxidant capacity(CUPRAC)

CUPRAC method is Novel hydroxyl radical scavenging antioxidant activity assay for water-soluble antioxidants. Reactive oxygen species (ROS) may attack biological macromolecules giving rise to oxidative stress-originated diseases. Since OH is very short-lived, secondary products resulting from OH attack to various probes are measured. Although the measurement of aromatic hydroxylation with HPLC / electrochemical detection is more specific than the low-yield TBARS test, it requires sophisticated instrumentation. As a more convenient and less costly alternative, we can use p-aminobenzoate, 2,4- and 3,5-dimethoxybenzoate probes for detecting hydroxyl radicals generated from an equivalent mixture of Fe(II) + EDTA with hydrogen peroxide. The produced hydroxyl radicals attacked both the probe and the water soluble antioxidants in 370C incubated solutions for 2 h. The CUPRAC absorbance of the ethylacetate extract due to the reduction of Cu (II)-neocuproine reagent by the hydroxylated probe decreased in the presence of OH scavengers, the difference being proportional to the scavenging ability of the tested compound. A rate constant for the reaction of the scavenger with hydroxyl radical can be deduced from the inhibition of color formation. The second-order rate constants of the scavengers were determined with competition kinetics by means of a linear plot a A0/A as a function of C scavenger / C probe where A0 and A are the CUPRAC absorbences of the system in the absence and presence of scavenger, respectively and C is the molar concentration of relevant species. The 2,4 – and 3, 5-dimethoxybenzoates were the best probes in terms of linearity and sensitivity. Iodide, metabisulfite, hexacyanoferrate (II), thiourea, formate, and dimethyl sulfoxide were shown by the modified CUPRAC assay to be more effective scavengers than mannitol, glucose, lysine, and simple alcohols as in the TBARS assay. The developed method is less lengthy, more specific, and of a higher yield than the classical TBARS assay. The hydroxyl radical scavenging rate constants of ascorbic acid, formate and hexacyanoferrate(II) that caused interference in other assays could be easily found with the proposed procedure. (Burcu Bektasoglu, et.al, 2003) Apricots as five varieties of Malatya region are screened for antioxidant capacity by using CUPRAC. The novel reagent for the CUPRAC total antioxidant capacity assay, bis(neocuproine) copper(II) chloride, was easily accessible, stable, selective and responding to all antioxidants. Sulphite (normally contributing to the colour formed in the CUPRAC assay) was removed prior to assay on a strongly basic anion exchanger at pH 3 in the form of HSO. The CUPRAC findings correlated well with the results of ABTS /TEAC and Folin assays. This work reports for the first time the use of a novel spectrophotometric

method (CUPRAC) for the assay of both total antioxidant capacity and sulphite levels of diverse apricot samples.(Guclu and Kubilay, et.al,2006)

17. Cellular antioxidant activity

This method contains different principles to detect antioxidant property. It is based on solubilisation of the oils in aqueous buffer, labeling of the resulting emulsions with a suitable reporter fluorophore, which reflects lipid oxidation, and continuous monitoring of the decomposition process. Antioxidant capacity of number of non-refined seed oils is compared with that of refined oils by using this simple technique. And it was found that, most of the antioxidative components are removed from edible oils during refining process.(Gilbert Otto, et.al,) A novel fluorometric method has been developed to evaluate hydroxyl radical is generated by a Co(II) – mediated Fenton-like reaction, and the hydroxyl radical formation under the experimental condition is indirectly confirmed by the hydroxylation of phydroxybenzoic acid. The fluorescence decay curve of FL is monitored in the absence or presence of antioxidant, the area under the fluorescence decay curve (AUC) is integrated, and the net AUC, which is an index of the hydroxyl radical prevention capacity, is calculated by subtracting the AUC of the blank from that of the antioxidant. Gallic acid is chosen as a reference standard, and the activity of sample is expressed as gallic acid equivalents. The method is rigorously validated through linearity, precision, accuracy and ruggedness. A wide range of phenolic antioxidants is analyzed and the hydroxyl radical prevention capacity is mainly due to the metalchelating capability of the compounds.(Ou,. et.al, 2002)The hydroxyl radical scavenging capacity and efficacy of a novel organosiliceous anionic hydride compound, silica hydride, were quantified by a recently developed method. The method measures a direct relationship between the hydroxyl radical scavenging capability of the antioxidant compound and the linear decrease in signal from a fluorescent 2-hydroxyterephthalate product created by reacting a Fe2+-EDTA complex in the presence of a potential radical scavenger. A fluorescence signal half-inhibition, IC50, value of 1.4 ± 0.1 µm was obtained for silica hydride compounds. The validity of the analysis was verified by electron spin resonance spectroscopy, spectrophotometric analysis of NAD+ / NADH ratios, mitochondrial membrane potential measurements and assays of reductions of both cytochrome C (Fe3+) to cytochrome c (Fe2+) and epinephrine to adenochrome reductions.(cory,. et.al, 2003).

18. Enhanced chemiluminescence (ECL)

ECL has been used to measure antioxidant capacity in biological fluids. The assay involves the chemiluminescent substrate luminal. Light emission occurs when the luminal is oxidized by hydrogen peroxide that is generated in a reaction catalyzed by horseradish peroxidase (fluid because the reaction HRP). This method can quantify the antioxidant capacity of a is sensitive to radical scavenging antioxidants that reduce the light output. A method of assay of the antioxidant activity of biological sample suspected of having such activity, is under patent and this method comprises the steps of (a) initiating a chemiluminescent reaction and allowing said reaction to progress, thereby to generate a level of luminescence, said level being selected from the group consisting of (i) A rising level between 90 to 100 % of maximum, (ii) The maximum (iii) A postmaximum substantially constant plateau level: (b) Adding said sample to said progressing chemiluminescent reaction, said sample causing said level of luminescence generated by said reaction to change when said sample has antioxidant activity: (c)Monitoring said change in the level of luminescence: and (d) Determining the antioxidant activity of said sample assayed by reference to that of samples of known antioxidant activity subjected to steps (a) to (c) above. The principle behind the enhanced chemiluminescent assay for TAC measurement is best described in the work by (Whitehead,. et.al, 1992). To perform the enhanced chemiluminescence assay, a signal

reagent (luminal plus para-iodophenol), which is a source of chemiluminescence, is mixed with horseradish peroxidase (HRP)-linked immunoglobulin to produce ROS, which in turn is mixed with a substrate, hydrogen peroxide (H2O2). The power of the antioxidants in the seminal plasma to reduce the chemiluminescence of the signal reagent is compared with that of Trolox (6-hydroxy-2,5,7,8-tetramethyl chroman-2-carboxylic acid), a water-soluble tocopherol analogue, and is measured as molar Trolox equivalents. Although accurate, this method is cumbersome and time consuming, because fresh signaling reagent solution must be prepared each time the assay is performed standardized with Trolox. Moreover, the signal reagent may reduce in intensity, adding another technical problem. Finally, expensive instrumentation (eg, luminometer) is needed to measure the chemiuluminescence, which means that this assay is often not readily available in a physician's office. (Whitehead,. et.al, 1992).

19. Total radical trapping antioxidant parameter (TRAP)

Another assay which has been applied in human plasma is the total radical trapping antioxidant parameter (TRAP). In this assay, the rate of peroxidation induced byAAPH(2'-azobis(2-amidinopropane) hydrochloride) is monitored through the loss of fluorescence of the protein R-phycoerythrin (R-PE). In the TRAP assay the lag-phase induced by plasma is compared with that induced by Trolox in the same plasma sample.(Huang,.et.al, 2005)

20. ABTS {2,2' – azinobis-(3-ethyl-benzothiazoline-6-sulphonic acid)}

Miller et al (1993) described another technique for TAC measurement based on colorimetry. This assay is based on the principle that when 2,2'-azinobis-(3- ethyl-benzothiazoline-6-sulphonic acid) {ABTS} is incubated with a peroxidase (such as metmyoglobin and H2O2, a relatively stable radical cation, ABTS+ , is formed (see equation below). The formation of ABTS+ on Interaction With Ferryl Myoglobin produces a relatively stable blue-green color, Measured at 600nm. Antioxidants in the fluid sample suppress this color production to a degree that is proportional to their concentrations. In this equation, HX-FeIII = metmyoglobin, X–[FeIV = 0] = ferrylmyoglobin,
ABTS = 2,2' –azino-di-[3-ethylbensthiazoline sulphonate].(Miller,. et.al,1993)

21. Conjugated diene assay

This method allows dynamic quantification of conjugated dienes as a result of initial PUFA (Poly unsaturated fatty acids) oxidation by measuring UV absorbance at 234 nm. The principle of this assay is that during linoleic acid oxidation, the double bonds are converted into conjugated double bonds, which are characterized by a strong UV absorption at 234 nm. The activity is expressed in terms of Inhibitory concentration (IC50). (Jacob,. et.al,1999), (David,. et.al, 2000).

22. DPPH Method (1, 1 diphenyl 2, picryl hydrazyl)

This is the most widely reported method for screening of antioxidant activity of many plant drugs. DPPH assay method is based on the reduction of methanolic solution of colored free radical DPPH by free radical scavenger. The procedure involves measurement of decrease in absorbance of DPPH at its absorption maxima of 516 nm, which is proportional to concentration of free radical scavenger added to DPPH reagent solution. The activity is expressed as effective concentration EC50. (vani,. et.al, 1997), (Navarro,. Et.al, 1993).

23. Phospho molybdenum Method

It is a spectroscopic method for the quantitative determination of antioxidant capacity, through the formation of phospho molybdenum complex. The assay is based on the reduction of Mo (VI) to Mo (V) by the sample analyte and subsequent formation of a green phosphate Mo (V) complex at acidic pH. (Kanner,. et.al, 1994)

24. Peroxynitrite radical scavenging activity

Peroxynitrite is now recognized by researchers as the culprit in many toxic reactions that were previously ascribed to its chemical precursors, superoxide and nitric oxide. Hence, an in vitro method for scavenging of peroxy radical has been developed to measure antioxidant activity. The scavenging activity is measured by monitoring the oxidation of dihydrorhodamine on a microplate fluorescence spectro-photometer at 485 nm. (Hye,. et.al, 2002)

25. DMPD (N, N-dimethyl-p-phenylene diamine dihydrochloride) Method:

This assay is based on the reduction of buffered solution of colored DMPD in acetate buffer and ferric chloride. The procedure involves measurement of decrease in absorbance of DMPD in presence of scavengers at its absorption maxima of 505nm. The antioxidant activity of wines was measured by using this method. The activity was expressed as percentage reduction of DMPD. (Rice EC,. et.al, 1994), (Vinson,. et.al, 1995)

26. b-Carotene Linoleate model:

This is one of the rapid method to screen antioxidants, which is mainly based on the principle that Linoleic acid, which is an unsaturated fatty acid, gets oxidized by "Reactive Oxygen Species" (ROS) produced by oxygenated water. The products formed will initiate the b carotene oxidation, which will lead to discoloration. Antioxidants decrease the extent of discoloration, which is measured at 434nm and the activity is measured. (Joseph,. et.al, 1994)

27. FRAP Method

FRAP (Ferric Reducing Ability of Plasma) is one of the most rapid test and very useful for routine analysis. The antioxidative activity is estimated by measuring the increase in absorbance caused by the formation of ferrous ions from FRAP reagent containing TPTZ (2, 4, 6 – tri (2 – pyridyl) – s – triazine) and FeCl36H2O. The absorbance is measured spectrophotometrically at 595nm. (Benzie,. et.al, 1996)

28. Cytochrome C-test

Superoxide anions were assayed spectrophotometrically by a cytochrome reduction method described by McCord and Fridovich (1969). Xanthine oxidase converts xanthine to uric acid and yields superoxide anions and these radicals directly reduce ferri- cytochrome C to ferro- cytochrome C, having an absorbance change at 550 nm. When test compounds showed superoxide scavenger activity, there was a decrease in the reduction of ferri-cytochrome C. (Ho KY,. et.al, 1999)

29. Erythrocyte ghost system

This method involves isolation of erythrocytes ghost cells and the induction of lipid peroxidation using erythrocyte ghosts and the induction of tetra-butyl hydroxyl peroxide (t-BHP). TBARS (thio barbituric acid reactive substance) produced during the reaction is measured at 535 nm. (Chiaki,. et.al, 1988)

30. Microsomal lipid peroxidation or Thiobarbituric acid (TBA) assay

TBA test is one of the most frequently used tests for measuring the peroxidation of lipids. Method involves isolation of microsomes from rat liver and induction of lipid peroxides with ferric ions leading to the production of small amount of Malonaldehyde (MDA). TBA reacts with MDA to form a pink chromagen, which can be detected spectrophotometricaly at 532 nm. (Kimura,. et.al, 1984), (Gutteridge,. et.al, 1986)

CONCLUSION

This review provides information on a number of plants which show promising antioxidant activity. It lists various methods for evaluating antioxidant activity along with different standards so it will be easy for the experimenter. It is also recommended to use at least two different types of assays for antioxidant activity. It emphasizes that *in vitro* antioxidant assays have been carried out for most of the plants, but *in vivo* remains to be done in majority of them. Methanol as a solvent has priority for extraction of plants for evaluating their antioxidant activity.

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