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REVIEW ON INVIVO AND INVITRO METHODS OF EVALUATIONOF ANTIOXIDANT ACTIVITY

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ABSTRACT

In present scenario number of abstracts and research articles were published for evaluating antioxidant activity of several samples. Where researches gone through 407 methods which are repeated from 29 different methods. These methods are classified into invitro and in vivo methods. These methods are described in review article. In next part of review article invitro and in vivo methods are analyzed. Solvents are commonly used to extract antioxidants from natural sources. In later part of this article results of above methods are described. Currently for the evaluation of antioxidant activity of the sample 19 *in vivo* methods 10*invitro* methods are used, some of them are discussed in this article. LPO is mostly used in vivo antioxidant assay while DPPH method is used for invitro antioxidant activity evaluation. Ethanol is highly used as solvent for extraction purpose.

KEYWORDS: In Vivo, In Vitro, Dpph, Lpo, Ethanol.

INTRODUCTION

Antioxidants act like defence mechanism that protect against inimical effects of oxidative reactions raised from reactive oxygen species in living system. Oxygen species not only are produced naturally by cell functions but also have been produced by radiation, viral toxins andbacteria, alcohol, and psychological or emotional stress. Excessive production of insufficient antioxidants has been implicated in the pathogenesis of some disease conditions like cancer, arthritis, alziemers, neurodegenerative disease, and aging process. Antioxidants are used to prevent oxidative damage caused by reactive oxidative species by reacting with free chelating, free radicals and catalytic metals and also by oxygen scavengers. The antioxidants in living system can be either enzymatic or non-enzymatic. The enzymatic antioxidants include superoxide dismutase, and glutathione peroxidase which catalyse neutralization of many types of free radicles, while the nonenzymatic antioxidants include Vitamin E, polyphenols, vitamin C, carotenoids, and selenium. There is an increasingly evidence that antioxidants play a important role in the prevention of heart disease, cancer, DNA degeneration, pulmonary disease, and neurological disorder. Recently, there has been an increase in interest in the therapeutic potential of plants as antioxidants in reducing oxidative tissue injuries. Plants, herbs, and spice, rich in phenolic compounds like flavonoids, have been indicated to have anti- inflammatory, antiallergenic, antiviral, antiaging, and anticarcinogenic activities which can be assign to their antioxidant properties.[1]

Different methods are used to determine the antioxidant property of samples (plant extracts, artificial antioxidants etc.). The aim of this review article is to gather all probable methods thatare used to evaluate the antioxidant property of various samples. A complete information of all available *invitro* and *in vivo* antioxidant models provide advantages to the researches of this area by reducing the time for literature review and method development.[2]

In vitro Methods

In vitro studies are carried with microorganisms, cells, or biological molecules outside normal biological environment. To perform these methods labware such as test tubes, flasks, Petri dishes, and microtiter plates are used. Studies are conducted by using biological parts of an organism that have been separated from their usual biological environment. However, results acquired from *in vitro* experiments may not predict effects on organism fullyor accurately. In divergence to *in vitro* experiments, *in vivo* studies are conducted in living organisms, including humans and plants.[3]

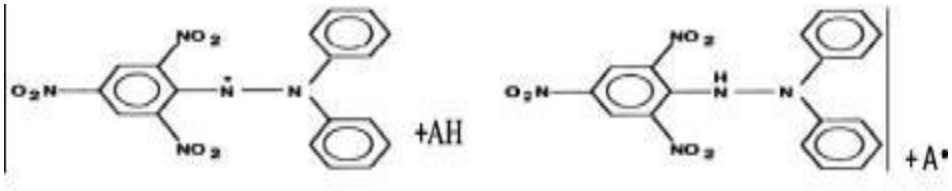
Methods

A. Dpph radical Scavenging Activity

DPPH is stable free radical; its assay method is based on the reduction of DPPH. The free radical DPPH with an odd electron gives absorption maximum at 517 nm (purple color). When Antioxidants react with DPPH, it becomes paired off in the presence of a hydrogen donor and is reduced to the DPPHH and as a result the absorbance's decreased from the DPPH. Radical of DPPH-H form, results in fading of color (yellow color).

More the decolorization more is the reducing ability which is a result of number of electrons captured. This method has been the most accepted one for evaluating the free radical scavenging activity of any new drug. When a DPPH solution is mixed with the substance that can donate a hydrogen atom, then this gives rise to the reduced form (Diphenyl picrylhydrazine; non radical) with the loss of this violet color.

The scavenging reaction between (DPPH) and an antioxidant (H-A) takes place as shown in figure 1. To evaluate the antioxidation potential.



Diphenylpicrylhydrazyl (free radical) Diphenylpicrylhydrazine (nonradical)

3 mg of DPPH (1, 1-Diphenyl –2-picrylhydrazyl) was dissolved in 3.3 ml of methanol; the sample containing test tubes are covered by aluminum foil as it should be protected from light.150 µl DPPH solution was added to 3ml methanol and absorbance is measured at 517nm after 15min.At 517nm using methanol as blank on UV-visible spectrometer Shimadzu, UV-1601, Japan calculated the IC50 values for each drug compounds as well as standard preparation. Theformula used to calculate the DPPH free radical antioxidant activity, % scavenging = [Absorbance of control - Absorbance of test sample/Absorbance of control] X100.[4]

B. Hydrogen Peroxide Scavenging Assay

This method was determined by the Barros *et al* [5]. The plant extracts were dissolved in 3.4 mL of 0.1 M phosphate buffer (pH 7.4) and mixed with 0.6 ml of H_2O_2 (40 mM) prepared in 0.1M phosphate buffer (pH 7.4). Using BHA as standard the absorbance value of the H_2O_2 was determined at 230 nm. Percentage of H_2O_2 scavenging was calculated from the formula: % Scavenged $[H_2O_2] = [(A_S - A_C)/A_S] \times 100$[6]

C. Nitric Oxide Scavenging Activity

The scavenging activity this was determined by using the improved method of Sreeja Yan andRao.[7] and Kidarn et al.[8] 800 µL of sodium nitroprusside in phosphate buffer saline (PBS) pH 7.4 was mixed with 200 µL of several concentrations of tested samples in the final concentration range from 10–200 µg/mL. The mixture solutions were incubated for 150 min at37 °C. 100 µL of Griess reagent was prepared by equal mixing of 0.1% (*w*/*v*) naphthyl ethylenediamine dihydrochloride (NEDA) with 1% (*w*/*v*) sulfanilamide in phosphoric acid. 96-wells plate are used to mix with 150µL of the mixture solutions. The absorbance of the formedcolor measured at 540 nm after 5 min of reaction time by spectrophotometry.[9] The results were calculated using following equation**:** %Scavenged $[NO] = [(A_O - A_S)/A_O] \times 100$.[2]

D. Peroxynitrite Radical Scavanging Activity

By the examined substances this method was based on the prevention of peroxynitrite– mediated oxidation of Evans blue dye. Their antioxidant abilities of $ONOO^-$ were measuredindirectly, by estimation of the hindrance of Evans blue bleaching (at 608 nm). Using the below equation % Of sample bleaching = 100 × (A0–A1)/A0.

A decrease of the Evans dye color, induced by 1 mM $ONOO^-$, was estimated. The absorbance for control samples was presumed as A0 value. After 30 min of incubation ofreaction mixtures absorbance noted was A1. Results obtained for samples treated with $ONOO^-$– in the absence of the antioxidants were then assumed as 100% of bleaching.[10]

E. Trolox Equivalent Antioxidant Capacity (Teac) Method/Abts Radical Cation Decolorization Assay

At 734 nm TEAC assay method was analyzed spectrophotometrically.[11] With potassium persulfate the radical cation ABTS + (2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid) was generated by ABTS oxidation. To obtain an initial absorbance value at 734 nm the ABTS• + solution was diluted with 5 mM sodium- phosphate buffer at pH 7.4. The assay contained 1.0 mL of the ABTS• + diluted solution is mixed in 96 well plates [2], to obtain a final volume of 1.1 mL assay sample absorbance at 734 nm was read after 5 min. By using a concentration–response Trolox curve 10–150 µM, the decrease of absorbance (%) with respect to the blank was used to quantify antioxidant capacity.

F. Cuprac Assay (Cupric Ion Reducing AntioxidantCapacity)

CUPRAC is based on ET-assay which is mostly used method to estimate the complete total antioxidant capacity of a compound. This method is estimated by simple redox reaction linking antioxidant and the free radicals. The assay is done by Adding, 1 mL 7.5 mM neocuproine 1 mL of 10 mM CuCl2 solution, 1 mL 1 M NH4 Ac, and x mL of antioxidant neutral solution now makeup the final volume to 4.1 mL by adding distilled water. Incubate the contents under normal condition (room temperature) after incubation the absorbance was read at 450 nm after30min.

CUPRAC method found to be important method to

analyze fluids of biological system. This method is also applicable to both lipophilic serum antioxidants as well as hydrophilic serum antioxidants. Hence, this procedure is useful in estimating the total antioxidant capacity of thefluids of living system.[12]

G. Reducing Power Method

The RP assay was raised to determine the ability of the extracts tested to reduce ferric (Fe^{3+}) existing in the potassium ferricyanide complex to ferrous (Fe^{2+}).[13] Based on the concord developed by Oyaizu in 1986[14] and as described in publication,[15] results are expressed in μg ascorbic acid equivalent per milligram of extract (μg AAE/mg E). The complex having maximum absorbance at 700nm.[11]

H. Metal Chelating Activity

Some antioxidants contain potent metal-chelating potentials. The principle of this assay is based on the ability of the sample to compete for ferrozine, that is a chemical which bind to Fe^{2+} and produces colour red. The Fe^{2+} chelating potential of the sample (Gao et al., 2007)[16] isindicated by reduced red colour at 562 nm. To perform the analysis, 0.1 ml of various concentrations of the sample extract are mixed with 0.5 ml of 0.2 mM $FeCl_2$ solution followed by the addition of 0.2 ml of 5 mM ferrozine. The mixture is incubated and the absorbance is read at 562 nm after10 min at room temperature. The metal chelating activity of the extract (%) is as = [(A *control* –A *sample (standard))/*A *control* × 100].[17]

I. Oxygen Radical Absorbance Capacity (ORAC) Assay

The ORAC assay was performed by a previous report of Huang.[18] A 25-µL diluted sample was added to 200 µL of fluorescein and mixed on a black 96-well fluorescence microplate for10 min at 37 °C and then 50 µL of AAPH was added to each well fluorescence microplate. Byusing a fluorescence microplate reader, the fluorescence raised was read at 535 nm emission and 485 nm excitation for every 1.5 min for 2 hrs. The standard used was Trolox (10–100 µg/mL). the compound used as a negative control was Methanol and positive controls was Vc and BHT. The results of ORAC values were estimated using the area under the curve (AUC) along with the Trolox standard curve. They are expressed as mg of Trolox equivalents (TE)/gdry extract (mg TE/g DE).[19]

J. Hydroxyl Radical Averting Capacity(HORAC) Method

The scavenging capacity for hydroxyl radical was estimated according to the modified method.This method was performed by adding 0.1 ml of EDTA, 0.1 ml of hydrogen peroxide,0.01 ml of ferric chloride, 0.36 ml of deoxyribose, 1.0 ml of test solutions (5-100 µg/ml) in distilled water, 0.33 ml of phosphate buffer (50 mM, pH 7.4), and 0.1 ml of ascorbic acid were dissolved in order. Then, the contents were incubated at 37°C for 1hr and 1.0 ml portion of the incubated contents was mixed with 1.0 ml of 0.5% TBA and10% TCA to develop the chromogen in pinkand measured at 532 nm.[13]

K. Superoxide Radical Scavenging Activity(SOD)

This method depends on the ability of an antioxidant to search the O2 radical in a test tube based on the NADH oxidation and the concomitant assay of nitro blue tetrazolium (NBT) (Robak and Gurgles, 1988; Chun et al., 2003)[20].The reaction mixture contains 1 ml Tris-HCl buffer (pH = 8) 1 ml of the extract, 0.5 ml NBT and 0.5 ml of 0.936 ml NADH. The reaction is begined by the addition of 0.5 ml of 0.12 mM phenazine methosulfate. The contents are incubated and the absorbance is read at 560 nm against the blank for 5 min at room temperature. Superoxide radical scavenging activity (%) is estimated using the following equation = [(A *control* –A *sample (standard))/*A *control* × 100].[18]

L. Phosphomolybdenum Method

This method is based on the reduction of Mo (VI) to Mo (V) by the sample analyte and later on formation of a green phosphate Mo (V) complex at acidic ph. Total antioxidant capacity can be determined by the method described by Prieto et al. (1999)[21]. 0.1 mL of sample (100 µg) solution is added to 1 mL of reagent (0.5 M sulfuric acid, 29 mM sodium phosphate and 5mM ammonium molybdate).The tube contain mixture is closed and incubated in a heating water bath for 90min at 95°C. now the sample is allowed to cool at room temperature. In UV spectrophotometer the absorbance of the aqueous solution is measured at 695 nm against blank solution. The blank solution contained 1 mL of reagent and the approximate volume of the similar solvent used for the sample and it is incubated under similar conditions as rest of the sample. For samples of unknown elements, antioxidant capacity can be determined as equivalents of α-tocopherol.[2]

M. Assay of Malondialdehyde (MDA)

Lipid peroxidation was determined in terms of Thiobarbituric acid reactive species (TBARS), using standard as Malondialdehyde (MDA). The homogenized liver tissue (400 µl) was added to 10% TCA and incubated at 4°C for 15 min and then centrifuged 200 g at 4°C for15 min.1 mL of protein-free supernatant is mixed with 1 mL of fresh TBA reagent and incubated for 1 hr in water bath at 60°C. now the optical density was determined at 532 nm for the assay of MDA. Lipid peroxide is determined in terms of nm. of MDA mg-1 of liver tissue.[22]

In Vivo Methods

In vivo methods are carried out in biological environment i.e., oversamples that should be tested are directly administered to selected animals (rats, rabbits, mice, etc.) in particular dose as authorized by various methods.

After some time, the animals are slaughtered and blood are tissues are collected for assay.[2]

N. Ferric Reducing Ability of Plasma (FRAP)

The FRAP method was introduced by Benzie and Strain to determine the antioxidant capacityof plasma and it has been modified with the use of plant extracts. The procedure of the assay is based on the ability of the test compound to transfer single electron to reduce ferric 2,4,6- tripyridyl-s-triazine (TPTZ) to ferrous TPTZ.[23]

The method involves the use of blood samples that are collected from a rat retro-orbital venous plexus into heparinized glass tubes at and kept under observation for 14 days. 3ml of freshly prepared and boiled FRAP reagent (1ml) of 2,4,6 TPTZ solution in 40Mm Hcl, 1ml Fecl2-6-H2O, 10ml of 0.3M acetate buffer(pH36) is mixed with 0.375ml distilled water and 0.925 ml of test sample. The absorbance of increased color in organic layer is estimated at 593nm.The temperature is maintained up to 37^0C.The readings at 180 s are selected for calculation of FRAP values.[2]

O. Reduced Glutathione (GHS) Estimation

Glutathione exists in two states i.e., reduced (GSH) and oxidized (GSSG) states. cellular oxidative stress measures the ratio of reduced glutathione to oxidized glutathione in cells where increased GSSG-to-GSH ratio indicates greater oxidative stress. NADPH convert oxidized state to the reduced state. To estimate the reduced glutathione (GSH) content add 1.5 ml cuvette600 µl of reaction buffer, 40 µl of 0.4% (w/v) DTNB, 10-100 µl of CE and Milli-Q water to afinal volume of 1,140 µl, and mix gently.[24]

P. Glutathione Peroxidase Estimation

Gpx (Glutathione peroxidase) is an enzyme category with peroxidase activity whose main role is to protect the organism from oxidative degradation. The role of GPx is to bring lipid hydroperoxides to their corresponding alcohols and to bring free hydrogen peroxide to water. GPx is assayed by 3mL cuvette containing 2.0 mL of 75 mM/L phosphate buffer which has pH 7.0. Following solutions are added 50lL of 60 mM/L glutathione reductase solution (30 U/mL), 50 lL of 0.12 M/L NaN3,100 lLof 3.0 mM/L NADPH, 50 lL of 0.12 M/L NaN3, 0.10 of 0.15mM/L Na2EDTA, and 100 lL of cytosolic part to gpx assay obtained after centrifugation for 25 min to 20,000g. Water was added to makeup total volume of 2.9 mL. The reaction is initiatedby the adding 100 lL of 7.5 mM/L H2O2, and by converting NADPH to NADP which is monitored by a continuous recording of the change in absorbance at 340 nm for 5min at 1 min interval. Enzyme activity of GSHPx was expressed in terms of mg of proteins.[25]

Q. Catalase Assay

Catalase (CAT) activity was estimated by observing degradation of H2O2. The reaction was started by adding 50 µL of homogenized sample of liver to the reaction mixture of 250 mM PBS along with 12 M CH3OH and 44 mM H2O2 and reaction mixture was incubated for 20 min at room temperature. The reaction was finished by the addition of Purpled (22.8 mM) and again incubated for 20 min at room temperature. The absorbance of the sample was measured at 550 nm after adding potassium periodate (65.2 mM). Catalase concentration was determined by constructing standard graph using the known concentrations HCHO and results expressed in IU mg1Protein.[22]

R. Assay of Total Tissue Sulfhydryl Group (reducedglutathione level)

The determination of soluble sulfhydryl content in liver was done according to the method ofGrunert and Phillips [26]. liver containing metaphosphoric acid extract was saturated with NaCl and allowed to stand for 15-30 min and centrifuged for 10 min at 4°C at 3000 rpm. To 3 mL saturation NaCl solution add1 mL of the aliquot of the supernatant and allow it to stand at 25°C for 10 min. The nonspecific absorption in the sample was removed by determining the sample against a blank tube containing 2% Sodium nitroprusside and metaphosphoric acid. The colored complex developed is estimated at 520 nm on a colorimeter by using blank tube.[22]

S. Lipid Peroxidation (LPO) Assay

LPO is an autocatalytic process. This method was explained by Okhawa (1979) is as follows: The tissues are homogenized with a Teflon-glass homogenizer in 0.1 M buffer. LPO in this homogenate is determined by determining the amounts of malondialdehyde (MDA) produced.Tissue homogenate (0.2ml), 1.5 mL of 8% TBA homogenate, 0.2 mL of 8.1% sodium dodecyl sulphate, 1.5 mL of 20% acetic acid are added. The volume of the contents is made up to 4 mLby adding distilled water and then heated for 60 min using glass balls as condenser at 95^0c. After incubation the test tubes are allowed to cool at room temperature and 5ml final volume was made in each tube. 5mL of butanol: pyridine (15:1) mixture is added and the mixture is swirled thoroughly for 2 min. After centrifugation for 10min at 3000 rpm, the upper organic layer is collected and its OD is observed at 532 nm against an appropriate blank without the sample. The stages of lipid peroxides might be measured as n moles of TBARS/mg protein using coefficient of 1.56×10^5 ML cm^{-1}.[27]

DISCUSSION

Out of all the in vitro strategies, DPPH is that the most straightforward, straightforward and fairly expensive technique and therefore it'd are used principally for the inhibitor activity analysis of a sample. If one appearance into it like a shot seems that the frequency of use is higher for LPO assay and it absolutely was followed by CAT and GSHPx. supermolecule maybe a major element of cell wall and therefore its peroxidation nearly directly co-relates peroxidative harm of cell in vivo and therefore it'd are found to possess the very best frequency in vivo inhibitor activity assay.

CONCLUSION

This review article gives information about in-vitro and in-vivo methods of antioxidant activity evaluation. DPPH method is mostly used for in-vitro antioxidant activity evaluation whereas for in-vivo antioxidant activity evaluation LPO method is mostly used and the IC50value was also measured for some methods. Ethanol is highly used solvent to study the antioxidant activity.

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