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ANTIOXIDANT ACTIVITY EVALUATION METHODS: *IN VITRO* AND *IN VIVO*

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ABSTRACT

Free radicals are molecules or molecular fragments containing one or more unpaired electrons. Oxidative stress occurs due to free radical generation and deficiency of antioxidant defence mechanism. Oxygen consumption inherent in cell growth leads to the generation of a series of reactive oxygen species (ROS). Oxidative damage caused by free radicals may be related to aging and diseases, such as atherosclerosis, diabetes, cancer and cirrhosis. The growing interest in the substitution of synthetic food antioxidants by natural ones has fostered research on vegetable sources and the screening of raw materials for identifying new antioxidants. Oxidation reactions are not an exclusive concern for the food industry, and antioxidants are widely needed to prevent deterioration of other oxidisable goods, such as cosmetics, pharmaceuticals and plastics. The aim of this review is to focus on the different in *vitro* and *in vivo* methods for evaluation of antioxidant activities.

Key word: Antioxidant, DPPH assay, FRAP assay, Reducing power, TBARS

INTRODUCTION

Free radicals can be defined as molecules or molecular fragments containing one or more unpaired electrons in atomic or molecular orbitals[1]. Unpaired electrons (s) are responsible for reactivity of free radicals. Radicals derived from oxygen represent the most important class of radical species generated in living systems[2].

There are a number of evidence of an association between the oxidative stress resulting from radical generation and antioxidant insufficiency and tissue damage. Lipid oxidation as a result of radical chain reaction causes quality loss in flavour, colour and nutritive values of food. Therefore many methods for determination of antioxidant activity in food samples and biological fluids have been proposed during the past decade. They are mainly based on reaction between a chromogen compound and an antioxidant. After reaction, the residual concentration of chromogen compound is determined spectrophotometrically or colorimetrically[3].

Reactive intermediates in oxidation processes, particularly free radicals, are receiving increased attention in biology, medicine and food chemistry, and as well as in environmental areas[4]. Free radicals are highly reactive and are capable of damaging almost all type of bio molecules the fact is that free radical generate free radicals from normal compounds which continue as a chain reaction[5].

Oxygen consumption inherent in cell growth leads to the generation of a series of reactive oxygen species (ROS). They are continuously produced by the body's normal use of oxygen such as respiration and some cell-mediated immune functions. ROS include free radicals such as superoxide anion radicals ($O2^{\bullet -}$), hydroxyl radicals ($OH^{\bullet}$) and non-free radical species such as hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$)[6].

The most common forms of ROS include superoxide radical, hydrogen peroxide, hydroxyl free radical, singlet oxygen and nitric oxide, which have

significantly high biological activities *in vivo* and *in vitro*. They can directly lead to DNA mutation, alteration of gene expression, modification of cell signal transduction, cell apoptosis, lipid peroxidation and protein degradation[7]. Oxidation is essential to many living organisms for the production of energy to fuel in biological processes. However, oxygen-centered free radicals and other reactive oxygen species, which are continuously, produced *in vivo*, result in cell death and tissue damage. Oxidative damage caused by free radicals may be related to aging and diseases, such as atherosclerosis, diabetes, cancer and cirrhosis[8]. Cooperative defence systems that protect the body from free radical damage include the antioxidant nutrients and enzymes. The antioxidant enzymes include superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx). Their role as protective enzymes is well-known and has been investigated extensively with *in vivo* models[9].

In general, the methods to determine the total antioxidant capacity were divided into two major groups: assays based on the single electron transfer (SET) reaction, displayed through a change in colour as the oxidant is reduced, and assays based on a hydrogen atom transfer (HAT) [10]. The ferric reducing antioxidant power (FRAP), the α-tocopherol/ Trolox equivalent antioxidant capacity (α-TEAC/TEAC) and the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assays include electron transfer reaction[11].

Enzymatic antioxidants include superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), etc. Antioxidant enzymes, SOD and CAT, are not consumed and have high affinity and rate of reaction with ROS. Therefore, it may be hypothesized that the enzymes afford more effective protection against acute massive oxidative insults, such as hyperoxia or inflammation. Antioxidant enzymes are more potential agents in treating severe acute insults due to oxidative stress[12].

I. DPPH assay

The molecule 1, 1-diphenyl-2-picrylhydrazyl (DPPH) is characterized as a stable free radical by virtue of the delocalisation of the spare electron over the molecule as a whole, so that the molecule does not dimerize, as would be the case with most other free radicals. The delocalization of electron also gives rise to the deep violet colour, characterized by an absorption band in ethanol solution centered at about 515 nm. When a solution of DPPH is mixed with that of a substrate (AH) that can donate a hydrogen atom, then this gives rise to the reduced form with the loss of this violet colour.

The antioxidant activity of sample and BHA can be measured in terms of electron transfer/hydrogen donating ability, using the stable radical, $DPPH^-$ method. Prepare methanol solution (0.1 ml) of the sample extracts at various concentrations was added to a 3.9 ml (0.025 g/L) of $DPPH^-$ solution. The decrease in absorbance at 515 nm was determined continuously at every 1 min with a UV–Visible Spectrophotometer until the reaction reached a plateau. The remaining concentration of $DPPH^-$ in the reaction medium was calculated from a calibration curve obtained with $DPPH^-$ at 515 nm[13, 14]. The percentage of remaining $DPPH^-$ calculated as follows:

$DPPH^\bullet$ scavenging effect (%) = (1-As/Ac) ×100

Where A_C is the absorbance of the control (0.5 ml, containing $DPPH^\bullet$ solution without test sample), and A_S is the absorbance in the presence of sample.

II. Hydroxyl radical scavenging assay

Hydroxyl radical is one of the potent reactive oxygen species in the biological system that reacts with polyunsaturated fatty acid moieties of cell membrane phospholipids and causes damage to cell. The scavenging activity of the raw and dry-heated horse gram seed extracts on the hydroxyl radical (OH^-) was measured by the deoxyribose method (Aruoma, 1994) modified by (Hagerman 1998). The reactions were performed in 10 mM phosphate buffer, pH 7.4, containing 2.8 mM deoxyribose, 2.8 mM H_2O_2, 25 µM $FeCl_3$, 100 µM EDTA, and the test sample (200 µg). The reaction was started by adding ascorbic acid to a final concentration of 100 µM and the reaction mixture was incubated for 1 h at 37 ºC in a water bath. After incubation, the colour was developed by addition of 1% thiobarbituric acid followed by ice-cold 2.8% trichloroacetic acid and heating in a boiling water bath (95–100 ºC) for 20 min. The sample was cooled, and the chromophore was extracted into n-butanol and the absorbance was measured at 532 nm against n-butanol (as blank) [15, 16]. The reaction mixture not containing test sample was used as control. The scavenging activity on hydroxyl radicals (HRSA) was expressed as:

Percentage (%) scavenging of $OH^\cdot$ = $[(A_0 - A_1)/A_0]$ ×100

Where A_0 absorbance of the control. A_1 is the absorbance in presence of sample.

III. FRAP assay

The antioxidant capacity of phenolic extracts of raw and processed horse gram seed samples was estimated according to the procedure described by (Benzie et al.,1996) with slight modifications made

by (Pulido et al., 2000). FRAP reagent (900 µL), prepared freshly and incubated at 37 ºC, was mixed with 90 µL of distilled water and 30 µL of test sample, BHA and Trolox or methanol (for the reagent blank). The test samples and reagent blank were incubated at 37 ºC for 30 min in water bath. The final dilution of the test sample in the reaction mixture was 1/34. The FRAP reagent contained 2.5 mL of 20 mmol/L TPTZ solution in 40 mmol/L HCl plus 2.5 mL of 20 mmol/L $FeCl_3$.$6H_2O$ and 25 mL of 0.3 mol/L acetate buffer, pH 3.6. At the end of incubation, the absorbance readings were taken immediately at 593 nm using a Spectrophotometer. Methanolic solutions of known Fe (II) concentration ranging from 100 to 2000 µmol/L ($FeSO_4.7H_2O$) were used for the preparation of the calibration curve. The parameter Equivalent Concentration (EC_1) was defined as the concentration of antioxidant having a ferric-TPTZ reducing ability equivalent to that of 1 mmol/L $FeSO_4.7H_2O$. EC_1 was calculated as the concentration of antioxidant giving an absorbance increase in the FRAP assay equivalent to the theoretical absorbance value of a 1 mmol/L concentration of Fe (II) solution determined using the corresponding regression equation[17,18].

FRAP value of sample (µM) = (Change in abs. of sample from 0 to 4 minute / Change in abs. of std. from 0 to 4 minute) X FRAP value of std. (1000 µM). FRAP value of Ascorbic acid is 2 µM.

IV. Nitric oxide (NO·) scavenging assay

NO^- is generated in biological tissues by specific nitric oxide synthases, which metabolizes arginine to citrulline with the formation of NO^- via a five electron oxidative reaction[19]. The compound sodium nitroprusside is known to decompose in aqueous solution at physiological pH (7.2) producing NO^- Under aerobic conditions, NO^- reacts with oxygen to produce stable products (nitrate and nitrite), and the quantities of which can be determined using Griess reagent. Two (2) mL of 10 mM sodium nitroprusside dissolved in 0.5 mL phosphate buffer saline (pH 7.4) is mixed with 0.5 ml of sample at various concentrations (0.2–0.8 mg/ml). The mixture is then incubated at 25 ºC. After 150 min of incubation, 0.5 mL of the incubated solution is withdrawn and mixed with 0.5 mL of Griess reagent [(1.0 ml sulfanilic acid reagent (0.33% in 20% glacial acetic acid at room temperature for 5 min with 1 mL of naphthyl ethylene diamine dichloride (0.1% w/v)]. The mixture is then incubated at room temperature for 30 min and its absorbance pouring into a cuvette is measured at 546 nm[20].

Percentage (%) inhibition of $NO^·$ Radical= $[A_0 - A_1]/A_0 \times 100$

Where A_0 is the absorbance before reaction and A_1 is the absorbance after reaction has taken place with Griess reagent

Total Phenolic content

The amount of total phenol content can be determined by Folin-Ciocateu reagent method. 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[21]. Gallic acid can be used as a positive control.

Superoxide anion scavenging activity

The superoxide anion scavenging activity is measured described by (Robak et al., 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 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[22], BHA, ascorbic acid, α-tocopherol and curcumin[23] can be used as a positive control.

Xanthine oxidase method

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 0.1mg/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[24]. BHT[25] can be used as a positive control.

Reducing power (RP)

The reducing power can be determined by the method of 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 $FeCl_3$ (6 mM) and absorbance measured at 700 nm[26]. Ascorbic acid, butylated hydroxyanisole (BHA), α-tocopherol, trolox[27] butylated hydroxytoluene (BHT) [28] can be used as positive control.

Hydrogen peroxide radical scavenging (H_2O_2) assay

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 (50 Mm pH 7.4). The concentration of hydrogen peroxide is determined by absorption at 230 nm using a spectrophotometer. Dissolve sample (20 - 60 µg/ml) in distilled water and added hydrogen peroxide. Absorbance measured at 230 nm is determined after 10 min against a blank solution containing phosphate buffer without hydrogen peroxide[29]. Hydrogen peroxide scavenging is calculated as follows:

Percentage (%) inhibition $H_2O_2 = (A_0 - A_1 / A_0)$ X 100

Where; A_0 is the absorbance of control and A_1 is the absorbance of test.

Ascorbic acid, rutin BHA[30], α-tocopherol[31] or quercetin[29] can be used as a positive control.

Oxygen radical absorbance capacity (ORAC) Method

The test can be performed using Trolox (a water-soluble analogue of Vitamin E) as a standard to determine the Trolox Equivalent (TE). The ORAC value is then calculated from the Trolox Equivalent and expressed as ORAC units or value. The higher the ORAC value, the greater the ''Antioxidant Power''. This assay is based on generation of free radical using AAPH (2, 2-azobis 2-amidopropane di-hydrochloride) and measurement of decrease in fluorescence in the presence of free radical scavengers[32]. (Prior et al. 2003) have reported an automated ORAC assay. In this assay β-phycoerythrin (β-PE) was used as target free radical damage, AAPH as a peroxy radical generator and Trolox as a standard control. After addition of AAPH to the test solution, the fluorescence is recorded and the antioxidant activity is expressed as trolox equivalent.

The assay can be carried out according to (Prior et al. 2003) in 96-well polypropylene fluorescence plates with a final volume of 200 µL. Assays are conducted at pH 7.0 with Trolox (6.25, 12.5, 25, and 50 µmol/L for lipophilic assays; 12.5, 25, 50 and 100 µmol/L hydrophilic assays) as the standard and 75 mM/L phosphate buffer as the blank. After the addition of AAPH, the plate is placed immediately in a multilabel counter preheated to 37 ºC. The plate is shaken in an orbital manner for 10 s and the fluorescence is read at 1 min intervals for 35 min at the excitation wavelength of 485 nm and emission wavelength of 520 nm. Area-under-the-curve is calculated for each sample using Wallac Workout 1.5 software. Final computation of results is made by taking the difference of areas-under- the-decay curves between blank and sample and/or standard (Trolox) and expressing this in µM of Trolox equivalents(TE) per g dry weight of sample (µM TE/g)[33,34,35].

Metal chelating activity

Ferrozine can quantitatively chelate with Fe^{2+} and form a complex with a red colour. This reaction is limited in the presence of other chelating agents and results in a decrease of the red colour of the ferrozine-Fe^{2+} complexes. Measurement of the colour reduction estimates the chelating activity to compete with ferrozine for the ferrous ions[36]. 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[37] can be used as a positive control.

Trolox equivalent antioxidant capacity (TEAC) assay

In fact, both the ORAC and TEAC assays are inhibition methods: a sample is added to a free radical- generating system, and the inhibition of the free radical action is measured. This inhibition is related to the antioxidant capacity of the sample. In addition, both assay methods measure antioxidants in serum or plasma proteins, including albumin[38].

The $ABTS^{\cdot+}$ formed from the reaction $ABTS^{-e-}$ $ABTS^{\cdot+}$ 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 $ABTS^{+}$ 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 $ABTS^{\cdot+}$ 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[39]. Aqueous phosphate buffer solution (3.9ml, without $ABTS^{\cdot}$ + Solution) is used as a control. The $ABTS^{+}$ scavenging rate is calculated. Trolox, BHT, rutin, ascorbic acid[40] or gallic acid[41] can be used as a positive control.

***In Vivo* Antioxidant activity assays methods**

Determination of LPO /TBARS method

According to this method the tissues are homogenized in 0.1 M buffer pH 7.4 with a Teflon glass homogenizer. LPO in this homogenate is determined by measuring the amounts of malondialdehyde (MDA) produced primarily. Tissue homogenate (0.2 mL), 0.2 mL of 8.1% sodium dodecyl sulfate (SDS), 1.5 ml of 20% acetic acid and 1.5 ml of 8% TBA are added. The volume of the mixture is made up to 4 ml with distilled water and then heated at 95 ºC on a water bath for 60 min using glass balls as condenser. After incubation the tubes are cooled to room temperature and final volume was made to 5 ml in each tube. Five ml of butanol: pyridine (15:1) mixture is added and the contents are vortexed thoroughly for 2 min. After centrifugation at 3000 rpm for 10 min, the upper organic layer is taken and its OD is taken at 532 nm against an appropriate blank without the sample[42]. The levels of lipid peroxides can be expressed as n moles of thiobarbituric acid reactive substances (TBARS)/mg protein using an extinction coefficient of $1.56x10^{5}$ ML cm^{-1}.

II. Determination Superoxide dismutase (SOD)

SOD and CAT are among the most potent antioxidants known in nature. There are three types of SODs in humans namely cytosolic CuZn-SOD, mitochondrial Mn- SOD and extracellular SOD. SOD catalyzes dismutation of superoxide into oxygen and hydrogen peroxide and it is widespread in nature in eukaryotic and prokaryotic organisms[43].

Superoxide dismutase (SOD) activity was determined by the method of *Kakkar et al*. The assay mixture contained 0.1ml of sample, 1.2ml of sodium pyrophosphate buffer (pH 8.3, 0.052 M), 0.1 ml of phenazine methosulphate (186µm), 0.3 ml of nitro blue tetrazolium (300µ m), 0.2 ml of NADH (750 µm). Reaction was started by addition of NADH. After incubation at 30 ^{o}C for 90 sec, the reaction was stopped by the addition of 0.1 ml of glacial acetic acid. The reaction mixture was stirred vigorously with 4.0 ml of n-butanol. The mixture was allowed to stand for 10 min, centrifuged and butanol layer was separated. The colour intensity of the chromogen in butanol layer was measured at 560 nm against n-butanol and concentration of SOD was expressed as units/g of liver tissue. Absorbance values were compared with a standard curve generated from known SOD[44].

III. Determination of Catalase (CAT)

CAT occurs abundantly in the body, with the highest activity in the liver, followed by erythrocytes, then the lungs. CAT protects cells by catalyzing hydrogen peroxide decomposition into molecular oxygen and water with no free radical production. In addition, CAT acts on toxic compounds such as phenols, formic acid, formaldehyde and alcohols by peroxidative reaction. These free radical scavenging enzymes have been found to change qualitatively and quantitatively in various tissues and cells of patients with mitochondrial diseases and elderly subjects[45].

Catalase activity can measure based on the ability of the enzyme to break down H_2O_2. Homogenized the tissues in 50 M phosphate buffer (pH 7.0) at 1 – 4 °C and centrifuged at 3,000-5,000 rpm. The reaction mixture contained 0.01 M phosphate buffer (pH 7.0), 2 mM H_2O_2 and the enzyme extract. Measure the spectrophotometric estimation decrease in absorbance at 240 nm. Catalase activity was calculated from the turnover time of hydrogen peroxide (H_2O_2) resulting in an absorbance decrease at 240 nm**.** The specific activity of catalase is expressed in terms of units/mg of protein[46].

IV. Determination of reduced glutathione GSH

Reduced Glutathione (GSH) is the smallest intracellular thiol (-SH) molecule. Its high electron donating capacity (high negative redox potential) combined with high intracellular concentration (mmol) generate great reducing power. This characteristic underlies its potent antioxidant action and enzyme cofactor properties and supports a complex thiol exchange system which hierarchically regulates cell activity[47].

The method illustrated by can be used for determination of antioxidant activity. Take tissue homogenate (in 0.1 M phosphate buffer pH 7.4) and added with equal volume of 20% trichloroacetic acid (TCA) containing 1 mM EDTA to precipitate the tissue proteins. The mixture is allowed to stand for 5 min prior to centrifugation for 10 min at 2000 rpm. The supernatant(200 µL) is then transferred to a new set of test tubes and added with 1.8 ml of the Ellman's reagent (5,50-dithiobis-2- nitrobenzoic acid (0.1 mM) prepared in 0.3 M phosphate buffer with 1% of sodium citrate solution). Then all the test tubes are made up to the volume of 2 ml. After completion of the total reaction, solutions are measured at 412 nm against blank. Absorbance values were compared with a standard curve generated from known GSH[48, 49].

γ–Glutamyl transpeptidase activity (GGT) assay
According to this method the serum sample is added to a substrate solution containing glycylglycine, $MgCl_2$ and γ- Glutamyl-p-nitroanilide in 0.05 M tris (free base), pH 8.2. The mixture is incubated at 37 ºC for 1 min and the absorbance read at 405 nm at 1 m interval for 5 m. The activity of GGT is calculated from the absorbance values[50].

Glutathione-S-transferase (GSt)
Glutathione-S-transferase is thought to play a physiological role in initiating the detoxication of potential alkylating agents, including pharmacologically active compounds. These enzymes catalyze the reaction of such compounds with the -SH group of glutathione, thereby neutralizing their electrophilic sites and rendering the products more water-soluble. The reaction mixture (1 ml) consisted of 0.1 N potassium phosphate (pH 6.5), 1 nM/L GSt, 1 M/L l-chloro-2, 4-dinitrobenzene as substrate and a suitable amount of cytosol(6 mg protein/ml). The reaction mixture is incubated at 37 ºC for 5 min and the reaction is initiated by the addition of the substrate. The increase in absorbance at 340 nm was measured spectrophotometrically[51].

Ferric reducing ability of plasma
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-tripyridyl-s-triazine) and $FeCl_2.6H_2O$. The absorbance is measured spectrophotometrically at 593 nm. It involves the use of blood samples that are collected from the rat retroorbital venous plexus into heparinized glass tubes at 0, 7 and 14 days of treatment. Three ml of freshly prepared and warm (37 ºC) FRAP reagent [1 ml (10 mM) of 2,4,6 tripyridyl-s-triazine (TPTZ) solution in 40 mM HCl, 1 ml 20 mM $FeCl_2.6\ H_2O$, 10 ml of 0.3 M acetate buffer (pH 3.6)] is mixed with 0.375 ml distilled water and 0.025 ml of test samples. The absorbance of developed colour in organic layer is measured at 593 nm. The temperature is maintained at 37 ºC[52].

CONCLUSION

This review provides information about different methods for evaluation of *in vivo* and *in vitro* antioxidant activities. It is also recommended that use at least two different type of assay for evaluating antioxidant activity. This article will be helpful for those who are performing antioxidant assay DPPH method is the most frequently used one for *in vitro* antioxidant activity evaluation while LPO was found as the mostly used *in vivo* antioxidant assay.

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