Pharmacognosy Reviews
Vol 1, Issue 2, Jul-Dec, 2007

PHCOG REV.
An official Publication of Phcog.Net

Phcog Rev. : Review Article
Screening Methods of Antioxidant Activity: An Overview

Ajay Sharma, Sudhir Bhardwaj, A.S.Mann, Amit Jain and M. D. Kharya*

Pharmacognosy Research Laboratory, Department of Pharmaceutical Sciences,
Dr. Hari Singh Gour University, Sagar-470 003, INDIA.
**Author for correspondence : Telephone:- +917582-265457, +917582-264381Fax:- +917582-264236 e-mail:- mdkharya_dops@yahoo.co.in*

ABSTRACT

This study was intentional to assess the antioxidant activity of drugs. The antioxidants divulge an gigantic ability to trim down DPPH, superoxide, peroxide and nitric oxide radical scavenging activity. Antioxidants restrain OH^- radical induced oxidation of protein (BSA) and LPO in hepatic microsomes. The fortitude of metal chelating competence of antioxidant indicates chelation of metal ions (Fe^{2+}) to be a putative means concerned in the inhibition of OH^- radical induced BSA oxidation and LPO. Antioxidants also exhibited a noteworthy activity in discriminating oxidative tissue grievance animal model constituted by CCl_4 induced hepatotoxicity. The administration of the antioxidant significantly defend CCl_4 induced elevation in AST and ALT in the serum, elevation in hepatic LPO, diminution of hepatic GSH and decrease in the activities of hepatic antioxidant enzymes: SOD, CAT and GPX. Antioxidant gives fortification against histopathological changes produced by CCl_4 such as necrosis, fatty changes, ballooning degeneration, etc.

KEY WORDS

Antioxidant, Free radicals, 2,2-Diphenyl-1-picrylhydrazyl, Nitroblue tetrazolium;

ABBREVIATIONS

DPPH, 2,2-diphenyl-1-picrylhydrazyl; NBT, nitroblue tetrazolium; ROS, reactive oxygen species; DNA,deoxyribo-nucleicacid; NO,nitric oxide; H_2O_2,Hydrogen peroxide; O^{2-},superoxide radical;LPO,lipid peroxidation;ORAC,oxygen radical anti-oxidant capacity;TEAC, trolox equivalent antioxidant capacity;NADP, nicotinamide adenine dinucleotide phosphate; SOD, superoxide dismutase; GSH, Glutathione; AST,Aspartate amino transferase; ALT; Alanine amino transferase; GPX,Glutathione peroxidase; TCA, trichloro acetic acid; TBA,thiobarbituric acid; BSA,bovine serum albumin; PBS, phosphate buffer saline.

INTRODUCTION

The potentially imprudent derivatives of oxygen, endorsed as ROS such as O^{2-}, H_2O_2 and OH radical are incessantly generated within the human body as a consequences of revelation to a superfluity of exogenous chemicals in our ambient milieu and/or a number of endogenous metabolic processes linking redox enzymes and bioenergetic electron transmit. Under normal state of affairs, the ROS generated are detoxified by the antioxidants nearby in the body and there is symmetry between the ROS generated and the antioxidants present. However due to ROS overproduction and/or derisory antioxidant argument, this equilibrium is hindered favouring the ROS gain that culminates in oxidative hassle. The ROS readily attack and induce oxidative damage to various biomolecules including proteins, lipids, lipoproteins and DNA (1). This oxidative damage is a decisive etiological factor concerned in quite a lot of chronic human diseases such as diabetes mellitus, cancer, atherosclerosis, arthritis, and neurodegenerative diseases and also in the ageing course (2). Based on growing interest in free radical biology and the lack of effective therapies for most chronic diseases, the expediency of antioxidants in protection against these diseases is defensible. Epidemiological studies have brought into being that the intake of antioxidants such as Vitamin C reduces the risk of coronary heart disease and cancer (3). The antioxidants may reconcile their upshot by directly reacting with ROS, quenching them and/or chelating the catalytic metal ions (4). Several synthetic antioxidants, *e.g.*, BHA and BHT are commercially accessible but are quite perilous and their toxicity is a problem of disquiet (5). Natural antioxidants, especially phenolics and flavonoids, are safe and also bioactive. Therefore, in current years, substantial attention has been directed towards credentials of plants with antioxidant ability that may be used for human expenditure. The task of free radicals in many disease conditions has been well customary. Several biochemical reactions in our body generate reactive oxygen species and these are capable of damaging critical bio-molecules. If they are not effectiv, which attracted a great treaty of attention, lead to disease conditions (6,7). In recent years one of the areas, which attracted a great treaty of attention, is antioxidant in the control of degenerative diseases in which oxidative dent has been implicated. Several plant extracts and different lessons of phytochemicals have been shown to have antioxidant activity (8-10). This paper report models for evaluating antioxidant activity of various radicals (implicated in oxidative damage in cellular systems), *viz.*, superoxide, hydroxyl and nitric oxide radicals, that are generated in several *in vitro* and *ex vivo* models.

METHODS OF EVALUATION

Determination of total phenolic content

Total soluble phenolics in the ethanolic extracts were determined with Folin–Ciocalteu reagent,according to the method of Slinkard using pyrocatechol as a standard (11). Briefly, 1 ml of extract solution (contains 2000 lg) in a

volumetric flask was diluted glass-distilled water (46 ml). Folin–Ciocalteu reagent (1 ml) was added and the contents of the flask were mixed thoroughly. After 3 min, 3 ml of Na_2CO_3 (2%) was added, then the mixture was allowed to stand for 2 h with intermittent shaking. The absorbance was measured at 760 nm. The concentration of total phenolic compounds in the ethanolic extract, determined as microgrammes of pyrocatechol equivalents, by using an equation that was obtained from the standard pyrocatechol graph, is given as:

$$\text{Absorbance} = 0.00246\ \mu\text{g pyrocatechol} + 0.00325 \times (R^2 = 0.9996)$$

Determination of reducing power

The reducing power of the compound was evaluated according to Oyaizu (12). Different amounts of the extract were perched in distilled water and diverse with 2.5 ml of 0.2M phosphate buffer (pH 6.6), and 2.5 ml of 1% $K_3Fe(CN)_6$. This mixture was incubated at 50 ◦C for 20 min, 2.5 ml of 10% TCA was added to the blend and centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5 ml) was assorted with distilled water (2.5 ml) and $FeCl_3$ (0.5 ml, 0.1%), and the absorbance was measured at 700 nm. Increase in absorbance of the reaction mixture indicated reducing power.

Free radical scavenging assays

DPPH assay

The hydrogen atom or electron donating abilities of the resultant compounds and some untainted compounds was measured from the bleaching of the purple-coloured methanol solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH). This spectrophotometric assay uses the stable radical, DPPH as a reagent (13,14). One thousand microlitre of diverse concentrations of the extracts in ethanol were added to 4 ml of 0.004% methanol solution of DPPH. After a 30 min incubation period at room temperature, the absorbance was read against a blank at 517 nm. Inhibition of free radical by DPPH in percent (I%) was calculated in following way:

$$I\% = (A_{\text{blank}} - A_{\text{sample}}/A_{\text{blank}}) \times 100,$$

where, A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound) and Asample is the absorbance of the test compound. The values of inhibition were calculated for the various concentrations of extract. Tests were conceded out in triplicate.

Determination of super oxide anion ($O^{2\cdot-}$) radical scavenging activity

The method was adapted from (15). The reaction mixture consist of 1ml sample solution in distilled water, 1 ml of phenazine methosulphate (60 µM) in phosphate buffer (0.1 M, pH 7.4), 1ml NADH (450µM) in phosphate buffer, was incubated at 25 ◦C for 5 min. The absorbance was then examine at 560 nm against blank samples. The superoxide radical scavenging activity is calculated as

$$I\% = (A_{\text{blank}} - A_{\text{sample}}/A_{\text{blank}}) \times 100,$$

Where Ablank is the absorbance of control and Asample is the absorbance of test.

Determination of peroxide (H_2O_2) radical scavenging activity

The scavenging ability of H_2O_2 radical by the sample extract was dogged by the scheme of (16). One millilitre of extract solution [prepared in phosphate buffered saline (PBS)] was incubated with 0.6 ml of 4mM H_2O_2 solution (prepared in PBS) for 10 min. The absorbance of the solution was considered at 230 nm against a blank solution containing the extract without H2O2. The concentration of H_2O_2 was spectrophotometrically stubborn from absorption at 230 nm using the molar absorptivity of 81 M^{-1} cm^{-1}. The H_2O_2 radical scavenging activity is calculated as

$$I\% = (A_{\text{blank}} - A_{\text{sample}}/A_{\text{blank}}) \times 100,$$

Where A_{blank} is the absorbance of control and Asample is the absorbance of test.

Determination of nitric oxide (NO) radical scavenging activity

This procedure is based on the principle that sodium nitropruside in aqueous solution at physiological pH instinctively generates nitric oxide which interacts with oxygen to produce nitrite ions that can be anticipated using Greiss reagent. Scavengers of nitric oxide contend with oxygen leading to reduced production of nitrite ions. For the experimentation sodium nitroprusside (10 mM) in phosphate buffered saline was diverse with different concentrations of extract dissolved in suitable solvent and incubated at room temperature for 150 min. The same reaction mixture without the methanolic extract of sample but with equivalent amount of methanol served as control. After the incubation period, 0.5 ml of Griess reagent (1% sulfanilamide, 2% H_3PO_4 and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride was added. The absorbance of the chromophore formed was read at 546 nm. Curcumin was used as positive control (17). The NO radical scavenging activity is calculated as

$$I\% = (A_{\text{blank}} - A_{\text{sample}}/A_{\text{blank}}) \times 100,$$

Where A_{blank} is the absorbance of control and Asample is the absorbance of test.

Determination of hydroxyl radical (OH^-) scavenging activity

The scavenging ability for hydroxyl radical was measured by (18). Stock solution of EDTA (1mM), $FeCl_3$ (10mM), ascorbic acid (1mM) H_2O_2 (10mM) and deoxyribose (10mM) was prepared in distilled de- ionized water. The attempt was performed by adding up 0.1ml EDTA, 0.01ml of $FeCl_3$, 0.1ml H_2O_2, 0.36 ml deoxyribose, 1ml of sample extract (10-100µg/ml) dissolved in distilled water, 0.33ml of phosphate buffer (50mM,pH 7.4) and 0.1ml of ascorbic acid added. The mixture was incubated at 37oC for 1 hour.A1.0 ml of incubated mixture was mixed with 1.0 ml of 10% trichloro acetic acid and 1.0ml of 0.5% thiobarbituric acid (in 0.025M NaOH containing 0.025% BHA) to urbanized the pink color measured at 532nm. The hydroxyl radical scavenging activity is reported as percent inhibition of deoxyribose sugar dilapidation and is calculated as

$$I\% = (A_{\text{blank}} - A_{\text{sample}}/A_{\text{blank}}) \times 100,$$

Where A_{blank} is the absorbance of control and Asample is the absorbance of test.

Determination of protein oxidation

The protein (BSA) was oxidized by a Fenton-type reaction. BSA (1 mg/ml) was incubated at 25 ◦C in solution with 2.5mM H_2O_2, 1.0mM FeCl3, 1.0mM ascorbate and 3.0mM EDTA in presence or absence of extract. After incubation for 45 min, protein was precipitated with 10% TCA, centrifuged (5000 rpm, 4 ◦C, 10 min) and the supernatants decanted. Protein pellets were dissolved in 1ml of 50mM potassium phosphate buffer, pH 7.5. Total sulphydryl (−SH) group determinations were performed according to the method of Sedlak and Lin**tion of metal ion chelating activity**dsay (1968) using Ellman's reagent (19).

Determina

The chelation of ferrous ions by the extract was anticipated by the method of (20). Briefly, 50µl of 2mM $FeCl_2$ was added to the extract (1 ml). The reaction was initiated by the addition of 0.2 ml of 5mM ferrozine solution. The mixture was enthusiastically shaken and left to rest at room temperature for 10 min. The absorbance of the solution was subsequently measured spectrophotometrically at 562 nm.

Determination of total flavonoid concentration

Flavonoid concentration was determined as follows: Drug extract solution (1 ml) was dilutedwith 4.3 ml of 80% aqueous ethanol containing 0.1 ml of10% aluminium nitrate and 0.1 ml of 1 M aqueous potassium acetate. After 40 min at room temperature, the absorbance was determined spectrophotometrically at 415 nm.Total flavonoid concentration was calculated using quercetin as standard (21):

$$\text{Absorbance} = 0.002108\ \mu\text{g quercetin} - 0.01089 \times (R^2 : 0.9999)$$

ORAC assay

The ORAC assay used fluorescein as fluorescent probe and was an amendment of the protocols premeditated by Huang (22). Fluorescein sodium salt (16.7 mg) was dissolved in 10 ml of phosphate buffer solution (PBS) (75 mM, pH 7.0) to get a stash solution. The operational solution (60 nM) was obtained by ensuing dilution in PBS.A10 ml solution of AAPH (2,2-azinobis (2-amidinopropan) dihydrochloride) was prepared each day at a concentration of 153 mM and maintained in ice earlier than automatic injection. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) solutions used for the calibration curve (8, 16, 24, 32 and 40 lM) were prepared each day by dilution in PBS of a 1 mM stock solution made in the same solvent and stored at 80 °C. The scrutiny was performed using microplates (96-well, opaque white) and a fluorimeter . Sample (25 µl) was mixed with 250 µl of fluorescein (60 nM) and incubated for 10 min at 37°C in the microplate. AAPH (25 µl) solution was then robotically injected and the microplate was traumatized. The fluorescence (kexcitation = 485 nm, kemission = 520 nm) was registered each minute over 50 min. All samples were analyzed at three dilutions and the mean value was taken for ORAC . The quantification of the antioxidant activity was based on the calculation of the area under the curve, as proposed by Cao and Prior (23). The antioxidant activity by ORAC was articulated as l mol of trolox equivalents (TE) per gram of FW.

TEAC assay

The antioxidant activity was determined by the TEAC assay via the radical cation ABTS+ (24). The analysis was adapted to be conducted in a microwell plate (96-well,transparent) with a spectrophotometer λMax 190. The ABTS+ stock solution (7 mM) was prepared using $K_2S_2O_8$ as the oxidant agent. The working solution of ABTS+ was obtained by diluting the stock solution in ethanol to confer an absorption of 0.70 ± 0.02 at k = 734 nm. Extract or trolox (10 µl) diluted in ethanol was lay in the well and the reaction began with the addition of 290 µl of the ABTS+ working solution. The trolox solution (50 mM) was prepared in ethanol and was stored at 20 °C under an atmosphere of nitrogen. For each measurements, a standard curve with trolox was plotted. The TEAC value was calculated by the dimension of the area under the curve, consequential from plotting the percentage reticence of the absorbance as a function of time. The absorbance was recorded every 10 s for 6 min, to allow a close monitoring of the reaction rate, which is important for those antioxidants that show a time dependency when submitted to this assay. The calculation of the area under the curve was performed for one sample dilution, which had a final percentage inhibition between 20% and 80%. Each extract was analysed in triplicate. The antioxidant activity for the plant extracts was articulated as l mol of TE per litre and was calculated by the equation (25).

$$\text{TEAC (TE, µmol/L)} = 30 \times k \times \frac{\text{AUC}_{\text{sample}}}{\text{r.c.}_{\text{trolox}}},$$

where k is the dilution of the sample, 30 is a dilution factor originated from the protocol, r.c.trolox is the regression coefficient calculated from the calibration curve:

$$\text{AUC}_{\text{trolox}} = \text{r.c.}_{\text{trolox}} \times [\text{trolox}]$$

AUCsample and AUCtrolox are the areas produced correspondingly by the sample and trolox and are calculated by the following equation:

$$\text{AUC} = \left(\%\text{Inh}_{(t=0)} \times 0.5 + \sum_{i=1}^{36} \%\text{Inh}_{(t=10\times i)} \right) \times 10$$

where % Inh(t) is the inhibition percentage at t seconds.

Lipid peroxidation (LPO) assay

LPO was induced and assayed in rat liver microsomes (26). The reaction muddle, in a total volume of 1.0 ml, contained 0.58 ml phosphate buffer (0.1 M, pH 7.4), 0.2 ml of hepatic microsome (10%, w/v), 0.2 ml ascorbic acid (100 mM) and 0.02 ml ferric chloride (100 mM) and was incubated at 37 ◦C in a shaking water bath for 1 h. The reaction was clogged by the addition of 1.0 ml TCA (10%, w/v). subsequent which of 1.0 ml TBA (0.67%, w/v) was added and all the tubes were to be found in a boiling water bath for 20 min. At the end, the tubes were shifted to an ice-bath and centrifuged at 2500×*g* for 10 min. The amount of malonaldehyde formed in each of the samples was assessed by measuring the optical density of the supernatant at 535 nm allied with a reagent blank. The molar extinction coefficient for MDA was taken to be $1.56\times105\ M^{-1}\ cm^{-1}$.

Animals and treatment protocol

Male or female rats/mice used to evaluate this study. All the experimental procedures were carried out in peace with internationally accepted guiding principle for the care and use of laboratory animals. Rats were divided into five groups containing six rats each. Group 1 served as control, group 2 was administered CCl_4 (negative control), and groups 3–5 were administered sample extract for 7 days. The rats of the groups 1 and 2 were simultaneously administered saline until the seventh day. On the seventh day, the rats of the groups 2–5 were given a single oral dose of CCl_4 (1:1) in olive oil at 2.0 g/kg of body weight 6 h after the last dose of extract/saline. After 24 h of CCl_4 administration, rats were sacrificed, the livers were secluded and hepatic PMS prepared.

Determination of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activity

AST and ALT were assayed by the means adapted from Reitman and Frankel (1957). Briefly, 1 ml of the substrate (2mM α-ketoglutarate, 0.2M d,l-aspartate for ALT and 2mM α -ketoglutarate, 0.2Md, l-alanine for AST) was incubated with 0.2 ml serum for 30 min (for AST) or 60 min (for ALT) in a water bath at 40 ◦C. subsequent incubation, the reaction was clogged by addition of 1ml of 1mM DNPH. After 20 min, 10 ml 0.4N NaOH was added to each tube. The absorbance of the solution was measured at 505 nm after 30 min in opponent to water as the blank (27).

Determination of glutathione (GSH) activity

GSH was assayed by the method of Jollow et al. (1974). An aliquot of 1.0 ml of hepatic PMS (10%, w/v) was precipitated with 1.0 ml of sulphosalicylic acid (4%, w/v). The samples were detained in reserve at 4 ◦C for 1 h and then centrifuged at 3500 rpm for 15 min. The analyze mixture contained 0.1 ml filtered aliquot, 2.7 ml phosphate buffer (0.1 M, pH 7.4) and 0.2 ml DTNB (40 mg/10 ml of phosphate buffer 0.1 M, pH 7.4) in a total volume of 3.0 ml. The yellow color developed was comprehend at 412 nm on a spectrophotometer (28).

Determination of superoxide dismutase (SOD) activity

SOD activity was estimated by Beauchamp and Fridovich (1971). The reaction mixture consisted of 0.5 ml of hepatic PMS, 1ml 50mM sodium carbonate, 0.4 ml of 25μM NBT and 0.2 ml 0.1mM EDTA. The reaction was initiated by addition of 0.4 ml of 1mM hydroxylamine-hydrochloride. The change in absorbance was recorded at 560 nm. The control was simultaneously run without liver homogenate. Units of SOD activity were expressed as the amount of enzyme required inhibiting the reduction of NBT by 50% (29).

Determination of catalase (CAT) activity

CAT activity was assayed by the method of Claiborne (1985). The assay mixture consisted of 1.95 ml phosphate buffer (0.05 M, pH 7.0), 1.0 ml H_2O_2 (0.019 M), 0.05 ml of hepatic PMS (10%, w/v). Changes in absorbance were recorded at 240 nm. CAT activity was calculated in terms of nmol H_2O_2 consumed/min/mg of protein (30).

Determination of glutathione peroxidase (GPX) activity

GPX activity was measured according to course of action of Mohandas et al. (1984) (31). The reaction mixture consisted of 1.44 ml 0.05M phosphate buffer, pH 7.0, 0.1 ml 1mM EDTA, 0.1mM sodium azide, 0.05 ml 1 U/ml glutathione reductase, 0.10 ml 1mM GSH, 0.1 ml 2mM NADPH, 0.01 ml 0.25mM H_2O_2 and 0.1 ml 10% PMS in a total volume of 2 ml. Disappearance of NADPH at 340 nm was recorded at 25 ◦C. Enzyme activity was calculated as nmol NADP reduced/min/mg protein using a molar extinction coefficient of $6.22\times103\ M^{-1}\ cm^{-1}$.

Histopathological observation

For histological studies, the liver tissues were predetermined with 10% phosphate-buffered neutral formalin, dehydrated in graded (50–100%) alcohol and embedded in paraffin. Thin sections (4–5μM) were cut and stained with hematoxylin and eosin (H&E) stain. The preliminary assessment was qualitative, with rationale of determining histopathological lesions and their distributions in liver. This was followed by a semiquantitative analysis to resolve the relentlessness of morphological component in each compartment. The parameters related to microarchitecture configuration (disorganization of parenchymal structure and parameters related to hepatic grievance (ballooning degeneration, steatosis and necrosis) were analyzed.

Measurement of effect on lipid peroxidation in rat liver homogenate

Rat liver homogenate (10% w/v) was described by Tripathi et al. (1996). In liver tissue peroxidation was induced by Iron-ADP complex in the presence of ascorbic acid. The incubation medium constituted 0.5 ml of liver homogenate (10% w/v), 100 μM $FeCl_3$, 1.7 μM ADP, 500 μM of ascorbate and different concentrations of extract in 2 ml of total incubation medium. The medium was incubated for 20 min at 37 °C. Extent of lipid peroxidation was measured by assessment of malondialdehyde (MDA) content (Vani et al., 1997). Results were articulated in terms of decrease in MDA formation by the sample extract ά-tocopherol acetate was used as positive control (32, 33).

Assay for phenylhydrazine induced hemolysis of erythrocytes (membrane stabilization study)

20% PCV (packed cell volume) of erythrocyte suspension (from human blood) was prepared according to the procedure described by Hill and Thornalley (1983). The assay was carried out according to the procedure described by Cazana et al. (1990), with certain modifications. In brief the method is as follows: the incubation mixture comprises of 1 ml of phenylhydrazine hydrochloride (0.5 mM), different concentrations of sample extract and 0.1 ml of 20% erythrocyte suspension made to a total volume of 3 ml with phosphate buffered saline (PBS) solution. The mixture was incubated at 37 °C for 1 hour and centrifuged at 1000 g for 10 min. The extent of haemolysis was measured by recording the absorbance of the supernatant at 540 nm. Suitable controls were kept to nullify the effect of solvents and inherent haemolysis.ά- tocopherol acetate was used as a positive control for the inhibition of phenylhydrazine induced haemolysis of erythrocytes (34,35).

CONCLUSION

Since polyphenols are responsible for the antioxidant activity, the obtained amount of total polyphenols in the extract indicated the extract to possess a high antioxidant activity.The quantity of polyphenols in the extract was expressed as pyrocatechol equivalent. The reducing ability of

the compound may serve as significant indicator of its potential antioxidant activity.

Free radical scavenging activity

The capacity of sample to scavenge DPPH, O^{2-}, OH^- and NO was a measure of antioxidant activity. The antioxidants react with DPPH, a purple colored stable free radical and convert it into a colorless á-á-diphenyl-picryl hydrazine. The amount of DPPH reduced could be quantified by measuring a decrease in absorbance at 517 nm. The DPPH scavenging ability of the extract and may be attributed to its hydrogen donating ability. The primary free radical in most biological systems is $O2^-$. Although O^{2-}. itself is quite unreactive compared to the other radicals, the biological systems convert it into more reactive species, *e.g.* OH^- radicals (36). The O^{2-} scavenging activity was determined by Phenazine methosulphate/NADH-NBT system wherein O^{2-} derived from dissolved oxygen by Phenazine methosulphate/NADHcoupling reaction reduces NBT. The spontaneous or catalytic dismutation of O^{2-} leads to the formation of H_2O_2, which in the presence of a transition metal ion like Fe^{3+}, decomposes into OH^- radicals, a highly damaging species in free radical pathology (37). The extract also scavenged H_2O_2. Incubation of SNP solution in PBS at $^{25}\circ$C for 120 min resulted in the release of NO. The antioxidants decreased the release of NO. ROS like O^{2-} may react with NO and give rise to various other reactive nitrogen species (RNS) such as NO_2, N_2O_4, and peroxynitrite. Both ROS and RNS together attack and damage various cellular molecules.Virtually all cellular components including lipids, proteins, nucleic acids, carbohydrates are susceptible to oxidative damage (38). Antioxidants owing to its radical scavenging ability may provide protection against oxidative damage induced to the biomolecules: proteins and lipids.

Protein oxidation and LPO assay

Antioxidants effectively inhibited BSA oxidation induced by Fe–ascorbate–H_2O_2 system that generates •OH radicals. BSA oxidation was determined in terms of –SH group loss by Ellman reagent [5,5, -dithiobis-(2- nitrobenzoic acid)]. Ellman reagent rapidly forms a disulfide bond with the SH group and releases a thiolate ion which is yellow colored and absorbs at 412 nm. The oxidized thiols are unable to bind with Ellman reagent. Thus, the oxidation of a protein is monitored by measurement of a reduction in the absorbance at 412 nm. The incubation of BSA with Fe–ascorbate–H_2O_2 system caused the oxidation of 57.3% of the SH groups. Antioxidants SH OXIDATION .The SH groups of proteins are crucial for several important functions. These groups maintain the functional conformation of proteins and also participate in catalytic activity of several enzymes. SH groups, due to their ability to be reversibly oxidized, are recognized as key components involved in the maintenance of redox balance. Most DNA binding proteins are also redox sensitive, performing their functions by virtue of the SH groups. These proteins are involved in the regulation of cellular processes such as replication, recombination, viral integration and transcription. Therefore, oxidation of SH groups by oxidants may thus lead to disruption of various cellular functions and even lead to cell death. Antioxidants inhibit protein oxidation (39). The lipids in membrane are continuously subjected to oxidant challenges. Oxidant induced abstraction of a hydrogen atom from an unsaturated fatty acyl chain of membrane lipids initiates the process of LPO, which propagates as a chain reaction. In the process, cyclic peroxides, lipid peroxides and cyclic endoperoxides are generated which ultimately fragment into aldehydes like-MDA. MDAforms a pink chromogen with TBA that absorbs at 535 nm. Incubation with Fe–ascorbate system caused a significant increase in MDA formation liver microsomes . Antioxidants inhibit the amount of MDA generated (and thus lipid peroxidation) in liver microsome . The normal cellular physiology is reliant on the intactness of the plasma membrane. Oxidative damage to membrane by peroxidation of membrane lipids may modulate the signal transduction pathways that may consequently affect various downstream processes. Oxidative damage to membrane may also disrupt the ionic channels, membrane transport proteins and inactivate membrane-associated enzymes, the lipid bilayer itself may become more permeable due to oxidative damage. LPO associated damage to membrane may therefore trigger/propagate various diseases. A number of chronic diseases are indeed characterized by an intense increase in LPO products (40). The inhibition of protein oxidation and LPO is thus a crucial property of antioxidant compounds by virtue of which they can inhibit/impede the induction/progression of a number of diseases implicating oxidative stress. Thus intake of antioxidant may be helpful in protection from numerous diseases.

The chelating of Fe^{2+} by antioxidant was estimated by the method of Dinis et al. (1994). Ferrozine can quantitatively form complexes with Fe^{2+}. However, in the presence of chelating agents, the complex formation is disrupted with the result that the red colour of the complex is decreased. Measurement of colour reduction, therefore, allows the estimation of the chelating activity of the coexisting chelator. Antioxidants interfered with the formation of ferrous and ferrozine complex, suggesting that it has chelating activity and captures ferrous ion before ferrozine. The transition metal ion, Fe2+ possess the ability to move single electrons by virtue of which it can allow the formation and propagation of many radical reactions, even starting with relatively non-reactive radicals. For instance, $O^{2\bullet-}$, which is moderately reactive in aqueous solution, gets converted to extremely reactively •OH through H_2O_2 in the presence of a transition metal such as Fe3+ by the following reactions (41):

$Fe^{3+} + O^{2\bullet-} \rightarrow Fe^{2+} + O_2$ (1)

$Fe^{2+} + H_2O_2 \rightarrow \bullet OH + OH^- + Fe^{3+}$ (2)

Net:

$O^{2\bullet-} + H_2O_2 \rightarrow O_2 + \bullet OH + OH^-$

This reaction mechanism (Eqs. (1) and (2) is a type of Fenton chemistry known as the iron-catalyzed Haber-Weiss reaction. Reactions (1) and (2) propagate in the presence of Fe yielding a substantial amount of extremely reactive •OH radicals, which can induce oxidative damage to almost all cellular molecules. The main strategy to avoid ROS generation that is associated with redox active metal catalysis involves

chelating of the metal ions. In the present study •OH radicals used to induce BSA oxidation and LPO in microsomal system were generated by Fe-ascorbate-H_2O_2 system that is based on Fenton type reaction mechanism. Ascorbate, in this reaction aids in the reduction of Fe^{3+} to Fe^{2+}, which is oxidized byH_2O_2 forming •OH by Fenton reaction and also in the generation of $O^{2\bullet-}$ during the Fe catalyzed oxidation of ascorbate. Indeed the presence of Fe generates $O^{2\bullet-}$, H_2O_2 and ultimately •OH (42). Thus the inhibition of •OH mediated BSA oxidation and LPO apparently implicates chelating of catalytic metal ion (Fe^{2+}) by Antioxidants. Several plant extract/constituents have been found to exert their antioxidant activity by chelating the catalytic metals (43,44).

***ORAC (oxygen radical antioxidant* l**

ORAC Fluorescein was the only method adapted to measure the scavenging activity of peroxyl radicals in this study. In fact, the ORAC b-PE, which uses the protein b-phycoerythrin (b-PE) as fluorescent probe (23) did not work appropriately with several of the samples (results not shown) since the b-PE fluorescence was completely inactivated, which might be explained by the capacity of flavanol polymers (proanthocyanidins or condensed tannins) to precipitate proteins (45). Furthermore, an absence of linearity was observed between extract concentrations nand initial relative fluorescences, which can be attributed to interactions between b-PE and the polyphenols for grape seed extracts. Fortunately, the ORAC FL which uses the fluorescein (FL) as fluorescent probe overcame all these troubles, and all samples could be measured properly.

TEAC measures

TEAC assay is considered a method that only measures the redox power of the antioxidant mixture (as an extract) in relation to the radical cation ABTS+. The increase in the correlation between ORAC values and polyphenol contents by removing samples from the regression analysis suggests that ORAC measures the activity of antioxidants other than the phenolics.

REFERENCES

1. J.L. Farber. Mechanisms of cell injury by activated oxygen species. *Environ. Health Pers.* 102:17–24 (1994).
2. N. Hogg. Free radicals in disease. *Semin. In Reprod. Endocrin.* 16: 241–88 (1998).
3. R. Marchioli, C. Schweiger, G. Levantesi, L. Tavazzi and F. Valagussa. Antioxidant vitamins and prevention of cardiovascular disease:epidemiological and clinical trial data. *Lipid.* 36: 53–63 (2001).J
4. J. Robak and E. Marcinkiewicz. Scavenging of reactive oxygen species as the mechanism of drug action. *Pol. J. Of Pharmacol.* 47: 89–98 (1995).
5. D.L. Madhavi and D.K. Salunkhe. Toxicological aspects of food antioxidants. In: D.L. Madavi, S.S. Deshpande and D.K. Salunkhe. (Eds). Food antioxidants. New York: Dekker; 267 (1995).
6. B. Halliwell and J.M.C. Gutteridge. In: Free radicals, ageing and disease; Free radicals in Biology and Medicine. Oxford: Clarendron Press; 2, 279–315 (1985).
7. H. Halliwell. Free radicals, antioxidants, and human disease:Curiosity, cause or consequence. *Lancet.* 344:721–24 (1994).
8. R.A.Larson. The antioxidants of higher plants. *Phytochem.* 27:969–78 (1998).
9. Y.B.Tripathi, S. Chaurasia, E. Tripathi, A.Upadhyay and G.P. Dubey. *Bacopa monniera* Linn. As an antioxidant: Mechanism of action. *Ind. J. of Exp. Biol.* 34:523–26 (1996).
10. R.M.N.A. Sreejayan. Nitric oxide scavenging by curcuminoids. *J. Pharm. and Pharmacol.* 49:105–7 (1997).
11. K. Slinkard and V.L. Singleton . Total phenol analyses: automation and comparison with manual methods. *Am. J. of Ecol. and Viticul.* 28:49–55 (1977).
12. M. Oyaizu. Studies on products of browning reaction prepared from glucosamine. *Jpn. J. of Nutr.* 44: 307–15 (1986).
13. M. Burits and F. Bucar. Antioxidant activity of *Nigella sativa* essential oil. *Phytother. Res.* 14:323–28 (2000).
14. M. Cuendet, K. Hostettmann and O. Potterat. Iridoid glucosides with free radical-scavenging properties from *Fagraea blumei*. *Hel. Chim. Act.* 80:1144–52 (1997).
15. G.Yen and H. Chen. Antioxidant activity of various tea extract in relation to their antimutagenicity. *J. of Agri. and food chem.* 43: 7–32 (1995).
16. R.J. Ruch, S.J. Cheng and J.E. Klaunig. Prevention of cytotoxicity and inhibition of intercellular communication by antioxidant catechins isolated from Chinese green tea. *Carcinogen.* 10:1003–8 (1989).
17. R. Sreejayan. Nitric oxide scavenging by curcuminoids. *J.Pharm. and Pharmacol.* 49:104–7 (1997).
18. B. Halliwell, J.M.C. Gutteridge and O. Amoma. The deoxy ribose method: A simple test tube assay for the determination of rate constant for reaction of hydroxyl radical. *Anal. Biochem.* 165: 215-19 (1987).
19. J. Sedlak and R.H. Lindsay. Estimation of total, protein-bound, and nonprotein sulfhydryl groups in tissue with Ellman's reagent. *Anal. Biochem.* 25:192–205 (1968).
20. T.C.P. Dinis, V.M.C. Madeir and M.L.M. Almeida. Action of phenolic derivatives (acetoaminophen, salycilate, and 5-aminosalycilate) as inhibitors of membrane lipid peroxidation and as peroxyl radical scavengers. *Arch.of Biochem. Biophys.* 315:161–9 (1994).
21. Y.K. Park, M.H. Koo, M. Ikegaki and J.L.Contado. Comparison of the flavonoid aglycone contents of *Apis mellifera propolis* from various regions of Brazil. Arquivos de *Biol. Technol.* 40:97–106 (1997).
22. D. Huang, B. Ou, M.F. Hampsch-Woodill, A. Judith and R.L. Prior. High-throughput assay of oxygen radical absorbance capacity using a multichannel liquid handling system coupled with a microplate fluorescence reader in 96-well format. *J.of Agri. and Food Chem.* 50: 4437–44 (2002).
23. G. Cao and R.L. Prior. Measurement of oxygen radical absorbance capacity in biological samples. *Methods in Enzymology.* 299:50–62 (1999).
24. R. Re, N. Pelligrini, A. Proteggente, A. Pannala, M.Yang and C.A. Evans. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biol. and Med.* 26:1231–37 (1999).
25. B.R. Vanden, G.R. Haenen, B.H. Vanden and A.A. Bast. Applicability of an improved trolox equivalent antioxidant capacity (TEAC) assay for evaluation of antioxidant capacity measurements of mixtures. *Food Chem.* 66: 511–17 (1999).
26. J.R. Wright, H.D. Colby and P.R. Miles. Cytosolic factors which affect microsomal lipid peroxidation in lung and liver. *Arch.of Biochem. and Biophys.* 206: 296–304(1981).
27. S. Reitman and S. Frankel. A colorimetric method for the determination of serum glutamic oxalacetic and glutamic pyruvic transaminases. *Am. J. of Clin.Path.* 28: 56–63 (1957).
28. D.J. Jollow, J.R. Mitchell, N. Zampaglione and J.R. Gillete. Bromobenzene induced liver necrosis: protective role of glutathione and evidence for 3,4 bromobenzeneoxide as the hepatotoxic intermediate. *Pharmacol.* 11:151–69 (1974).
29. C. Beauchamp and I. Fridovich. Superoxide dismutase: improved assays and an assay applicable to acrylamide gel. *Anal.Biochem.*44: 276–87 (1971).
30. A. Claiborne. Catalase activity. In: R.A. Greenwald. (Ed.). CRC Handbook of Methods for Oxygen Radical Research. Boca Raton: CRC Press; 283–84 (1985).
31. J. Mohandas, J.J. Marshall, G.G. Duggin, J.S. Horvath and D. Tiller. Differential distribution of glutathione and glutathione related enzymes in rabbit kidney: possible implications in analgesic neuropathy. *Cancer Res.* 44: 5086–91 (1984).
32. Y.B. Tripathi, S. Chaurasia, E. Tripathi, A. Upadhyay and G.P. Dubey. *Bacopa monniera* Linn. As an antioxidant: Mechanism of action. *Ind. J. Exp. Biol.* 34: 523–26 (1996).
33. T. Vani, M. Rajani, S. Sarkar and C.J .Shishoo. Antioxidant properties of the Ayurvedic formulation Triphala and its constituents. *Int. J. Pharmacog.* 35:313–17 (1997).
34. H.A.O. Hill and P.J. Thornalley. The effect of spin traps on phenylhydrazine induced haemolysis. *Biochem. Biophys. Acta.* 762: 44–51 (1983).
35. L.F.J. Cazana, D.M.L. Marques, A. Jimenez and R. Puyol. *In vitro* effect of plasma during phenylhydrazine induced erythrocyte oxidative damage. *Biomed. Biochem. Acta.* 495: 425–28(1990).
36. C.C. Winterbourn and A.J. Kettle. Radical-radical reactions of superoxide: a potential route to toxicity. *Biochem. and Biophys. Res. Commun.* 305: 729–36 (2003).
37. R.S. Pardini. Toxicity of oxygen from naturally occurring redox active pro-oxidants. *Arch .of Insect Biochem. and Physiol.* 29: 101–18 (1995).
38. R.E. Pacifici and K.J. Davies. Protein, lipid and DNA repair systems in oxidative stress: the free-radical theory of aging revisited. *Gerontol.* 37:166–80 (1991).
39. G. Martinez, A. Giuliani, O.S. Leon, G. Perez, A.J. Nunez and A.J. Selles. Effect of *Mangifera indica* L. extract (QF808) on protein and hepatic microsome peroxidation. *Phytother. Res.* 15: 581–85 (2001).
40. N. Santanam, S. Ramachandran and S. Parthasarathy. Oxygen radicals, antioxidants, and lipid peroxidation. *Semin. in Reprod. Endocrinol.*16: 275–80 (1998).
1. 41. T.C.P. Dinis, V.M.C. Madeira and M.L.M. Almeida. Action of phenolic derivates (acetoaminophen, salycilate, and 5-aminosalycilate) as inhibitors of membrane lipid peroxidation and as peroxyl radical scavengers. *Arch. of Biochem. and Biophys.* 315:161–69 (1994).
42. D.M. Miller and S.D. Aust. Studies of ascorbate-dependent, iron catalyzed lipid peroxidation. *Arch. of Biochem. and Biophys.* 271:113–19 (1989).

43. S.A. Dillon, R.S. Burmi, G.M. Lowe, D. Billington and K. Rahman. Antioxidant properties of aged garlic extract: an in vitro study incorporating human low density lipoprotein. *Life Sci*. 72:1583–94 (2003).
44. X.L. Niu, K. Ichimori, X. Yang, Y. Hirota, K. Hoshiai, M. Li and H. Nakazawa .Tanshinone II-A inhibits low density lipoprotein oxidation in vitro. *Free Radical Res.* 33:305–12 (2000).
45. A.E. Hagerman and L.G. Butler. The specificity of proanthocyanidin-protein interactions. *J. of Biol. Chem*. 256: 4494–97 (1981).