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REVIEW ON ANTIOXIDANTS ACTIVITY AND ITS EVALUATION

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ABSTRACT

Oxygen free radical induce damage due to peroxidation to biomembranes and also to DNA, which lead to tissue damage, thus cause occurrence of a number of diseases. There is extensive evidence to involve ROS in the development of degenerative diseases. Antioxidants neutralize the effect of free radicals through different ways and may prevent the body from various diseases. Evidence suggests that Compounds especially from natural sources are capable of providing protection against free radicals. This has attracted a great deal of research interest in natural antioxidants. It is necessary to Screen out medicinal plants for their antioxidant potential. Therefore an attempt has been made to review different *in vitro and in vivo* models for estimating antioxidant properties of natural products from medicinal plants. All the models are described along with the different standards that can be used for estimation.

INTRODUCTION

Antioxidant is, any substance that, when present at low concentration significantly delays or prevent oxidation of cell content like proteins, lipids, carbohydrates and DNA. Antioxidants can be classified into three main types: first line defence antioxidants, second line defence antioxidants and third line defense antioxidants.[1,11]

SOD, CAT, GTx, glutathione reductase and some minerals Se,Mn, Cu, Zn come under first line defence antioxidants. SOD mainly acts by quenching of superoxide (O_2^-), catalase by

catalyzing the decomposition of hydrogen peroxide (H_2O_2) to water and oxygen. Glutathion peroxidase is selenium containing enzyme which catalyse the reduction of hydrogen peroxide and lipid peroxide, generated during lipid peroxidation, to water using redused glutathione as substrate. Selenium and cytosol and cell membrane, respectively. Cu exerts its antioxidant activity through the cytosolic superoxide dismutase. Zinc is an element essential for normal growth, reproduction and other different functions of the body. It is a component of several enzymes like cytosolic superoxide dismutase, alcohol dehydrogenase, alkaline phosphatase, carbonic anhydrase etc.

Glutahion (GSH), Vitamin C, Uric acid, albumin, bilirubin, vitamin E (mainly α-tocopherol), carotenoids, flavonoids, etc., Comes under second line defence antioxidants. B-carotene is an excellent scavenger of singlet oxygen. Vitamin C directly interacts with radicals like O_2^-, OH^- (hydroxyl). OH^- and various lipid hydroperoxides and may help to detoxify many inhaled oxidizing air pollutants like ozone, NO_2 and free radicals in cigarette smoke in the respiratory tract. Vitamin E scavenges peroxyl radicals intermediates in lipid peroxidation and is responsible for proctecting PUFA (Poly unsaturated fatty acid) present in cell membrane and low density lipoprotein (LDL) against lipid peroxidation.

The most important chain breaking antioxaidants is α-tocapherol, present in human membranes. Vitamin C and α-tocopherol both help to minimize the consequences of lipid peroxidation in membrane. A major antioxidants defence of human body is to prevent O_2^- and H_2O_2 from reacting to form dangerous species such as hydroxyl ions, by binding transition metal ions in forms that will not stimulat free radical reactions.

Third line antioxidants are a complex group of enzyme for repair of damaged DNA, damaged protein, oxidized lipid and peroxides and also to stop chain propagation of peroxyl lipid radical. These enzymes repair the damage to biomolecules and reconstitute the damage cell membrane, e.g. lipase, proteases, DNA repair enzymes, transferase, methionine sulphoxide reductase, etc.

Natural sources of antioxidants

Medicinal plants are nature gift to mankind. These plants have been used by man for centuries in various traditional system of medicine like Ayurveda, Siddha, Unani etc.[2,12] Nowadays herbal drugs are prescribed widely even when their biologically active compounds are unknown because of their effectiveness, minimum side effects and relatively low cost.[2,13]

Medicinal plants are the important source of life saving drugs for the majority of the world's population[2].

Medicinal plants are an important source of antioxidants.[3,10] Natural antioxidants increase the antioxidant capacity of the plasma and reduce the risk of certain diseases such as cancer, heart diseases and stroke.[4,10] The secondary metabolites
like phenolics and flavonoids from plants have been reported to be potent free radical scavengers. They are found in all parts of plants such as leaves, fruits, seeds, roots and bark.[5,10] There are many synthetic antioxidants in use. It is reported, however, they have several side effects[6, 10], such as risk of liver damage and carcinogenesis in laboratory animals.[7-10] There is therefore a need for more effective, less toxic and cost effective antioxidants. Medicinal plants appear to have these desired comparative advantages, hence the growing interest in natural antioxidants from plants[10].

Evaluation of antioxidant activity

A great number of *in vitro* methods have been developed to measure the efficiency of natural antioxidants either as pure compounds or as plant extracts. *In vitro* methods can be divided into two major groups: 1) Hydrogen atom transfer reactions like Oxygen Radical Absorbance Capacity (ORAC), Total radical trapping antioxidant potential (TRAP) and β carotene bleaching; 2) Electron transfer reactions like trolox equivalent antioxidant capacity (TEAC), Ferric reducing antioxidant power (FRAP), α, α- diphenyl-β-picryl-hydrazyl radical scavenging assay (DPPH), Superoxide anion radical scavenging assay, Hydroxyl radical scavenging assay, Nitric oxide radical scavenging assay and Total phenol assay.[10,14] These methods are popular due to their high speed and sensitivity. However, it is essential to us more than one method to evaluate antioxidant capacity of plant materials because of the complex nature of phytochemicals.[10,15] The most commonly and uncommonly used antioxidant assays along with various standards that can be used as positive control are described below[10].

SCREENING METHODS OF ANTIOXIDANT ACTIVITY

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).[10,16]

The amount of total phenol content can be determined by Folin-Ciocateu reagent method.[10,17] 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[17], tannic acid[18], quercetin[19], chlorogenic acid [20], pyrocatechol [21] or guaiacol[22] can be used as positive controls. The total phenolic content is expressed in terms of standard equivalent (mgg-1 of extracted compound).

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.[10,23] Depending on their structure, flavonoids are able to scavenge practically all known ROS.[10]

The amount of total flavonoid content can be determined by Aluminum chloride method.[10,24] 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[25] or catechin[26] can be used as a positive control. The flavonoid content is expressed in terms of standard equivalent (mgg-1 of extracted compound).

Reducing power (RP)

Reducing power is associated with antioxidant activity and may serve as a significant reflection of the antioxidant activity.[10,27] 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.[10,28]

The reducing power can be determined by the method[29], 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[30] or butylated hydroxytoluene (BHT)[31] can be used as a positive control.

Free radicals scavenging assay (DPPH radical scavenging activity)

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 the reduction of DPPH in methanol solution in the presence of a hydrogen–donating antioxidant due to the formation of the nonradical form DPPH-H.[10,32] The disappearance of the purple color is monitored at 517 nm. The free radical scavenging activity can be measured by using 2, 2-diphenyl-1-picryl-hydrazyl or 1, 1-diphenyl-2-picryl-hydrazyl by the method.[10,33] 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[32], BHA, α-tocopherol[34], quercetin[35], BHT[36], rutin[37], catechin[38] or glutathione[39]. The percentage of inhibition can be calculated using the formula:

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

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

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[10, 40]. 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 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[41]. Gallic acid[41], BHA, ascorbic acid, α-tocopherol, curcumin[42], quercetin[43], or trolox[44] can be used as a positive control.

Xanthine oxidase method

To determine superoxide anion-scavenging activity, two different assays can be used: the enzymatic method with cytochrome C[45] and nonenzymatic method with nitroblue tetrazolium (NBT).[46] With cytochrome C method, superoxide anions can be generated by xanthine and xanthine oxidase system.

The xanthine oxidase activity with xanthine as the subsubstrate is measured spectrophotometrically[47], 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.[10] Percentage of inhibition was calculated using the formula: Percentage of inhibition = [1 – (As / Ac)] × 100

Where; As and Ac are the absorbance values of the test sample and control, respectively. BHT[48] or catechin[49] can be used as a positive control.

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 H_2O_2 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.[10]

The ability of plant extracts to scavenge hydrogen peroxide is determined according to the method.[50] 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 is added 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:

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

Where; A0 is the absorbance of control and A1 is the absorbance of test. Ascorbic acid, rutin BHA[51], α-tocopherol[52] or quercetin[50] can be used as a positive control.

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.[53] 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 _g/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[54], BHA, ascorbic acid, Rutin[51] can be used as a positive control.

Hydroxyl radical scavenging (HO) assay

The hydroxyl radical scavenging activity was determined according to the method[55]. One milliliter of iron-EDTA solution (0.13% ferrous ammonium sulphate and 0.26% EDTA), 0.5 ml of EDTA (0.018%), and 1 mL of DMSO (0.85% v/v in 0.1 M phosphate buffer, pH 7.4) were added, and the reaction was initiated by adding 0.5 mL of 0.22% ascorbic acid. Test tubes were capped tightly and heated on a water bath at 80-90^{0}C for 15 min. The reaction was terminatedby the addition of 1 mL of of ice-cold TCA (17.5% w/v). three mL of Nash reagent (75.0 g of ammonium acetate, 3 mL of glacial acetic acid and 2 mL of acetyl acetone were mixed and raised to 1 L with distilled water) was added to all of the tubes and left at room temperature for 15 min for colour development. The intensity of the yellow colour formed was measured spectrophotometrically at 412 nm against reagent blank. The percentage hydroxyl radical scavenging is calculated by the following formula:

% hydroxyl radical scavenging activity = 1- (difference in absorbance of sample/difference in absorbance of blank) X 100

Ferric reducing antioxidant power (FRAP) assay

Reducing power of crude extract was determined by the method.[57,58] 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL potassium ferricynide (1%). Reaction was incubated at 50 ^{0}C for 20 min. After incubation, 2.5 mL of trichloroacetic acid (10%) was added and centrifuged (650 g) for 10 min. From the upper layer, 2.5 mL solution was mixed with 2.5 mL distilled water and 0.5 mL $FeCl_3$ (0.1%). Absorbance of all the sample solution was measured at 700 nm. Ascorbic acid is used as a positive control. Frap value is expressed as the number of equivalence of gallic acid.

Total antioxidant activity

The total antioxidant activity is determined according to the method.[59] 3.0 mL of regent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). Reaction mixture was incubated at 95 ^{0}C for 90 min in a water bath. Absorbance of all the sample mixture was measured at 695 nm. Total antioxidant activity is expressed as the number of equivalence of ascorbic acid. A calibration curve of ascorbic acid was prepared and the total antioxidant activity was standardized against ascorbic acid and was expressed as mg ascorbic acid equivalents per gram of sample on a dry weight (DW) basis.

Oxygen radical absorbance capacity (ORAC) assay[10]

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[60].

The method 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).[61, 62].

Antioxidant assay by β-carotene-linoleate model system[63]

The antioxidant activity was evaluated by the β-carotene-linoleate model[64] 0.2 mg of the β-carotene in 0.5 ml of chloroform, 20 mg of linoleic acid and 200 mg of Tween-40 (polyoxyethylene sorbitan monopalmitate) were mixed together. The chloroform was removed at 40 ^{0}C under vacuum using a rotary evaporator. The resulting solution was immediately diluted with 10 ml of triple-distilled water and the emulsion was mixed well for 1 min. The emulsion was further diluted with 40 ml of oxygenated water before use. 4 ml aliquots of this mixture were transferred into different tubes containing 0.2 ml of samples at 100 µg/ml concentrations in ethanol, butylated hydroxyanisole (BHA) was used for comparative purposes. A control containing 0.2 ml of ethanol and 4 ml of the above mixture was prepared. Optical density (OD) at 470 nm were taken for the all extracts and pure compounds immediately (t = 0) at 15 min intervals for 1.5 h (t = 90). The tubes were incubated at 50 ^{0}C in a water bath. All determinations were performed in triplicate. Measurement of OD was continued until the colour of β-carotene disappeared in the control. The antioxidant activities (AA) of the samples were evaluated in terms of bleaching the _-carotene using the following formula.

$$AA = 100[1-(A_0/A_t)/(A^0_0/A^0_t)]$$

where Ao and A^0_0 are the OD measured at zero time of the incubation for test sample and control, respectively. At and A^0_t are the OD measured in the test sample and control, respectively, after incubation for 90 min.

Total antioxidant activity assay by radical cation ($ABTS^{\bullet+}$)[2]

ABTS was dissolved in water to a 7 mM concentration. ABTS radical cation ($ABTS^{\bullet+}$) was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate (final concentration) and allowing the mixture to stand in the dark at room temperature for 12-16 hr before use. Prior to assay, the solution was diluted in ethanol (about 1:89 v/v) and equilibrated to 30ºC to give an absorbance at 734 nm of 0.70±0.02 in a 1-cm cuvette.[65]
The concentration of the extracts that produced between 20-80% inhibitions of the blank absorbance was determined and adapted. After the addition of 1 mL of diluted $ABTS^{\bullet+}$ solution to 10 µL of plant extract or Trolox standards (final concentration 0-15 µM) in ethanol, optical density (OD) was taken at 30ºC exactly 30 min after the initial mixing. The

unit of total antioxidant activity (TAA) is defined as the concentration of Trolox having equivalent antioxidant activity expressed as µmol/g sample extracts on dry matter.

Assay For Phenyl Hydrazine Induced Haemolysis of Erythrocytes (Membrane Stabilization Study) [66]

The erythrocyte suspension 20% PCV (packed cell volume) of human blood was prepared and assay was carried out according to the procedure described by Cazana et al. The method involves the incubation of mixture containing 1 ml of phenyl hydrazine hydrochloride (0.5 mM), different concentration of compounds and 0.1 ml of 20 % erythrocyte suspension and final volume made to 3.0 ml by phosphate buffer solution. The mixture was incubated at 370 C for 1 hour and then centrifuged at 1000 g for 10 min. The absorbance of supernatant was measured at 540 nm. Suitable blank was also carried out to nullify the effect of solvents and inherent haemolysis. α-tocopherol was used as a positive control. Percent inhibition was calculated using following formula.[67, 68]

$$\% \text{ Inhibition} = [\text{Blank} - \text{Test}] / \text{Blank} \times 100$$

Antihemolytic activity[2]

The antihemolytic activity was determined according to the method described by.[69] The erythrocytes from cow blood were separated by centrifugation and washed with phosphate buffer (pH 7.4) until the supernatant become colorless. The erythrocytes were diluted with saline phosphate buffer to give 4 % suspension. 500 µg of sample and 1 mL saline buffer were added to 2 mL of the suspension of erythrocytes and the volume was made up to 3.5 mL with saline buffer. This mixture was preincubated for 5 min and then 0.5 mL H2O2 solution of appropriate concentration in saline buffer was added .The concentration of H2O2 in the reaction mixture was adjusted so as to bring about 90% haemolysis of blood cells after 240 min. After the incubation time the reaction mixture was centrifuged at 1500 rpm for 10 min and the extent of haemolysis was determined by measurement of the absorbance at (540 nm) corresponding to haemoglobin liberation.

Inhibition of copper – catalyzed human plasma Oxidation[70]

The oxidizability activity of blood plasma was described by the method[71]. The Inhibitory effect of different concentrations of silymarin (0.01-1.0 mg/ml) on Cu2+ (50 µM) - induced conjugated diene (CD) formation in plasma (diluted 1:150) were evaluated. Plasma oxidation

was initiated by adding CuSO4 at 37oC and absorbance was continuously monitored at 240nm for 8 h.

Antioxidant activity on human low-density lipoprotein (LDL) oxidation[56]

Plasma was prepared from blood drawn from human volunteers. The plasma collected was stored at 0-4 ^{0} C. The LDL was prepared from theplasma according to the method using differential ultracentrifugation metod[72]. Various concentration (25, 50 and 100 ppm) of extracts were taken. 40 µL of copper sulphate (2 mM) was added, and volume was made to 1.5 mL with phosphate buffer (50 mM, pH 7.4). A tube without extract and copper sulphate served as a negative control, and another tube without copper sulphate served as positive control. All tubes were incubated at 37^{0} C. Aliquots of 0.5 mL from each tubes were drawn at 2 h intervals, and 0.25 mL of TBA (1% in 50 mM NaOH) was added followed by 0.25 mL of TCA (2.8%). The tubes were incubated for 45 min at 95 ^{0}C. A pink chromogen was extracted after the mixture had cooled to room temperature by centrifugation (at 2000 rpm for 10 min). Thiobarbituric acid-reactive species in the pink chromogen were detected by fluorescence at 515 nm excitation and 553 nm emission. Data were expressed in terms of MDA equivalent, which was estimated by comparison with the standard graph drawn for 1,1,3,3-tetramethoxypropane (which was used as a standard). Which gave the amount ofoxidation.

Experimental procedure for liver homogenate[73]

Male albino rats of Wistar strain, weighing 180-220 g, were used for the studies. The animal were grouped into three groups containing six animals in each group. The first group served as control, the second group was administered CCl_4 (negative control), and the third group was administered the extract. The third group was suspended in 0.5% sodium carboxymethylcellulose and was fed to third group rats via oral route at 50 mg (in terms of catechin equivalents)/kg of body weight for 14 days. The dose was selected on the basis of the LD_{50} value of polyphenols[74]. The animals of first and second group were simultaneously administered saline until the 14th day. The animals of second and third group 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 administration of extract/saline on the 14th day. After 24 h, animal were sacrificed, and the liver was isolated to prepare the liver homogenate.

Five percent liver homogenate was prepared with 0.15 M KCl and centrifuged at 800g for 10 min. The cell-free supernatant was used.

Measurement of lipid peroxidation by thiobarbituric acid[73]

Thiobarbituric acid reacts with malondialdehyde (MDA) to form a diadduct, a pink chromogen, which can be detected spectrophotometrically at 532 nm[74]. The liver homogenate and the different concentration (50, 100 and 200 ppm) of grape pomace extract were taken. One milli Litre of 0.15 M KCl was added. Peroxidation was initiated by adding 100 µL of 0.2 mM ferric chloride. After incubation at 37 ^{0}C for 30 minutes, the reaction wasstopped by adding 2 mL of ice cold HCl (0.25 N) containing 15% trichloro acetic acid (TCA), 0.38% TBAand 0.5% BHT. The reaction mixture were heated at 80 ^{0}C for 60 min. The samples were cooled, centrifuged and absorbance of the supernatants was measured at 532 nm. An identical experiment was performed to determine the amount of lipid peroxidation obtained in the presence of inducing agents without any extract. The percentage inhibition of lipid peroxidation is calculated by the following formula.

Inhibition of lipid peroxidation (%) = 1- (Sample OD / Blank OD) X 100

Estimation of Superoxide Dismutase[73]

The SOD assay was carried out as per the method[75]. 0.5 mL of liver homogenate was taken and 1 mL of 50 mM sodium carbonate, 0.4 mL of 24 µM nitro blue tetrazolium (NBT) and 0.2 mL of 0.1 mM EDTA were added. The reaction was initiated by adding 0.4 mL of 1 mM hydroxylamine hydrochloride. Zero time absorbance was taken at 560 nm followed by recording the absorbance after 5 min at 25 ^{0}C. The control was simultaneously run without liver homogenate. Units of SOD activity were expressed as the amount of enzyme required to inhibit the reduction of NBT by 50%. The specific activity was expressed in terms of units/mg of proteins.

Estimation of Peroxidase[73]

The peroxidase assay was carried out as per the method.[76] 0.5 mL of liver homogenate was taken. 1 mL of 10 mM KI solution and 1 mL of 40 mM sodium acetate solution were added to these tubes. The absorbance was read at 353 nm. 20 µL of hydrogen peroxide (15 mM) was added and change in the absorbancein5 min was recorded. Units of peroxidase activity was expressed as the amount of enzyme required to change the OD by 1 unit per min. The specific activity was expressed in terms of units/mg of proteins.

REFRENCES

1. Vivek Kumar Gupta and Surendra Kumar Sharma, Plants as natural antioxiadant, Natural Product Radience, Vol 5(4), 2006, pp. 324-26.
2. Sasikala. V., Saravana. S. and Parimelazhagan T, Evaluation of antioxidant potential of different parts of wild edible plant *passiflora foetida* L., Journal of Applied Pharmaceutical Science, 01 (04); 2011: 89-96.
3. Rice-Evans C. Flavonoids and isoflavones: absorption, metabolism and bioactivity. Free Rad. Biol. Med. 2004, 36: 827-28.
4. Prior RL, Cao G. Antioxidant phytochemicals in fruits and vegetables. Diet and health implications. Hortic. Sci. 2000, 35: 588-92.
5. Mathew S, Abraham TE. *In vitro* antioxidant activity and scavenging effects of *Cinnamomum verum* leaf extract assayed by different methodologies. Food Chem. Toxicol. 2006, 44: 198-206.
6. Ito N, Fukushima S, Hagiwara A, Shibata M, Ogiso T, Carcinogenicity of butylated hydroxyanisole in F344 rats. J. Natl. Cancer Inst. 1983, 70: 343-347.
7. Gao JJ, Igalashi K, Nukina M. Radical scavenging activity of phenylpropanoid glycosides in *Caryopteris incana*. Biosci. Biotechnol. Biochem. 1999, 63: 983-988.
8. Williams GM, Iatropoulos MJ, Whysner J. Safety assessment of butylated hydroxyanisole and butylated hydroxyltoluene as antioxidant food additives. Food Chem. Toxicol.1999, 37: 1027-1038.
9. Osawa T, Namiki M. A novel type of antioxidant isolated from leaf wax of *Eucalyptus* leaves. Agric. Biol. Chem.1981, 45: 735-39.
10. S. Chanda and R. Dave, In vitro models for antioxidant activity evaluation and some medicinal plants possessing antioxidants properties: An overview, African Journal of Microbiology Research December, 2009, Vol. 3(13) pp. 981-96.
11. Irshad M and Chaudhury PS, oxidant-antioxidant system: Role and significance in human body, *Indian J Exp Biol*, 2002, 40, 1233-39.
12. Naim M, Gestener B, Bondi A, Brick Y. Antioxidant and Antihemolytic. J Agric Food Chem 1976; 24: 1174-77.
13. Ranganathan TS. Liver Textbook of human anatomy, S. Chand and company Ltd 1999.
14. Valiathan MS. Healing plants: *Current Science* 1998; 75: 1122-26.

15. Huang D, Ou B, Prior RL. The chemistry behind antioxidant capacity assays. J. Agric. Food Chem. 2005, 53: 1841-56.
16. Salazar R, Pozos ME, Cordero P, Perez J, Salinas MC. Waksman N. Determination of the antioxidant activity of plants from Northeast Mexico. Pharm. Biol. *2008*, 46: 166-70.
17. Rice-Evans C, Miller N, Paganga G (1997). Antioxidant properties of phenolic compounds. Trends Plant Sci. 1997, 2: 152-59.
18. McDonald S, Prenzler PD, Antolovich M, Robards K. Phenolic content and antioxidant activity of olive extracts. Food Chem.2001, 73: 73-84.
19. Wolfe K, Wu X, Liu RH. Antioxidant activity of apple peels. *J. Agric.* Food Chem.2003, 51: 609-14.
20. Singleton VL, Rossi JA. Colorimetry of total phenolics with phosphomolybdic phosphotungestic acid reagents. Am. J. Enol. Viticult. 1965, 16: 144-58.
21. Singleton VL, Orthofer R, Lamuela-Raventos RM. Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. Methods Enzymol.1999, 299: 152-78.
22. Slinkard K, Singleton VL. Total phenol analysis: automation and comparison with manual methods. Am. J. Enol. Viticult.1977, 28: 49-55.
23. Yildirim A, Oktay M, Bilaoglu V. The antioxidant activity of the leaves of *Cydonia vulgaris*. Turk. J. Med. Sci.2001, 31: 23-27.
24. Benavente-Garcia O, Castillo J, Marin FR, Ortuño A, Del-Rio JA. Uses and properties of *Citrus* flavonoids. J. Agric. Food Chem.1997, 45: 4505-15.
25. Chang C, Yang M, Wen H, Chern J. Estimation of total flavonoid content in propolis by two complementary colorimetric methods. J. Food Drug Anal.2002, 10: 178-82.
26. Ordonez AAL, Gomez JD, Vattuone MA, Isla MI. Antioxidant activities of *Sechium edule* (Jacq.) Swartz extracts. Food Chem.2006, 97: 452-58.
27. Kim DO, Jeong SW, Lee CY. Antioxidant capacity of phenolic phytochemicals from various cultivars of plums. Food Chem.2003, 81: 321-26.
28. Oktay M, Gulcin I, Kufrevioglu OI. Determination of *in vitro* antioxidant activity of fennel (*Foeniculum vulgare*) seed extracts. Leb.-Wissen. Technol.2003, 36: 263-71.
29. Yen GC, Chen HY. Antioxidant activity of various tea extracts in relation to their antimutagenicity. J. Agric. Food Chem.1995, 43: 27-32.

30. Athukorala Y, Kim KN, Jeon YJ. Antiproliferative and antioxidant properties of an enzymatic hydrolysate from brown alga *Ecklonia cava*. Food Chem. Toxicol.2006, 44: 1065-74.
31. Oyaizu M. Studies on products of browning reactions: antioxidative activities of products of browning reaction prepared from glucosamine. Jap. J. Nutr.1986, 44: 307-15.
32. Jayaprakasha GK, Singh RP, Sakariah KK. Antioxidant activity of grape seed (*Vitis vinifera*) extracts on peroxidation models *in vitro*. Food Chem. 2001, 73: 285-90.
33. Blois MS. Antioxidant determinations by the use of a stable free radical. Nature 1958, 181: 1199-50.
34. McCune LM, Johns T. Antioxidant activity in medicinal plants associated with the symptoms of diabetes mellitus used by the indigenous peoples of the North American boreal forest. J. Ethnopharmacol.2002, 82: 197-205.
35. Shimada K, Fujikawa K, Yahara K, Nakamura T. Antioxidative properties of xanthan on the antioxidation of soybean oil in cyclodextrin emulsion. J. Agric. Food Chem. 1992, 40: 945-48.
36. Shon DH, Kim YC, Oh SH, Park EJ, Li X, Lee BH. Hepatoprotective and free radical scavenging effects of *Nelumbo nucifera*. Phytomed.2003, 10: 165-69.
37. Liyana-Pathirana CM, Shahidi F. Antioxidant activity of commercial soft and hard wheat (Triticum aestivum L.) as affected by gastric pH conditions. J. Agric. Food Chem. 2005, 53: 2433-40.
38. Yamasaki K, Hashimoto A, Kokusenya Y, Miyamoto T, Sato T. Electrochemical method for estimating the antioxidative effects of methanol extracts of crude drugs. Chem. Pharm. Bull. 1994, 42: 1663-65.
39. Astudillo L, Schmeda-Hirschmann G, Herrera JP, Cortes M. Proximate composition and biological activity of Chilean Prosopis species. J. Sci. Food Agric.2000, 80: 567-73.
40. Kato K, Terao S, Shimamoto N, Hirata M. Studies on scavengers of active oxygen species. 1. Synthesis and biological activity of 2-*O*alkylascorbic acids. J. Med. Chem. 1988, 31: 793-98.
41. Meyer AS, Isaksen A. Application of enzymes as food antioxidants. Trends Food Sci. Tech. 1995, 6: 300-04.
42. Robak J, Gryglewski RJ. Flavonoids are scavengers of superoxide anions. Biochem. Pharmacol. 1988, 37: 837-41.

43. Nishikimi M, Rao NA, Yagi K. The occurrence of superoxide anion in the reaction of reduced phenazine methosulfate and molecular oxygen. Biochem. Biophy. Res. Commun. 1972, 46: 849-54.
44. Beauchamp C, Fridovich I. Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. Anal. Biochem. 1971, 44: 276-87.
45. Fernandes E, Toste SA, Lima JLFC, Reis S. The metabolism of sulindac enhances its scavenging activity against reactive oxygen and nitrogen species. Free Rad. Biol. Med. 2003, 35: 1008-17.
46. McCord JM, Fridovich I. Superoxide dismutase, an enzymic function for erythrocuprein (Hemocuoprein). J. Biol. Chem. 1969, 244: 6049-55.
47. Zhang HY, Lu CS. A study of the SOD-like activity of some copper (II)-small peptide and amino acid complexes. Acta. Biochim. Biophys. Sin. 1990, 22: 593-94.
48. Noro T, Oda Y, Miyase T, Ueno A, Fukushima S. Inhibitors of xanthine oxidase from the flowers and buds of *Daphne genkwa*. Chem. Pharm. Bull. 1983, 31: 3984-87.
49. Chang WS, Lin CC, Chuang SC, Chiang HC. Superoxide anion scavenging effect of coumarins. Am. J. Chin. Med.1996, 24: 11-17.
50. Schmeda-Hirschmann G, Rodriguez JA, Theoduloz C, Astudillo SL, Feresin GE, Tapia A. Free radical scavengers and antioxidants from *Peumus boldus* Mol. (Boldo). Free Rad. Res. 2003, 37: 447-52.
51. Ruch RJ, Cheng SJ, Klaunig JE (1989). Prevention of cytotoxicity and inhibition of intercellular communication by antioxidant catechins isolated from Chinese green tea. Carcinogen. 1989, 10: 1003-08.
52. Jayaprakasha GK, Jaganmohan Rao L, Sakariah KK. Antioxidant activities of flavidin in different *in vitro* model systems. Bioorg. Med. Chem.2004, 12: 5141-46.
53. Gulcin I, Oktay M, Kirecci E, Kufrevioglu Ol. Screening of antioxidant and antimicrobial activities of anise (Pimpinella anisum L.) seed extracts. Food Chem.2003, 83: 371-82.
54. Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR. Analysis of nitrate, nitrite and 15N nitrate in biological fluids. Anal. Biochem. 1982, 126: 131-38.
55. Sreejayan Rao MN. Nitric oxide scavenging by curcuminoids. J. Pharm. Pharmacol. 1997, 49: 105-07.

56. Klein. S. M.; Cohen, G.; Cederbaum, A. I. Production of formaldehyde during metabolism of dimethyl sulphoxide by hydroxyl radical generating system. *Biochemistry* 1991, *20,* 6006-12.
57. R. P. Singh, K. N. Chidambara Murthy and G. K. Jayaprakasha, Studies on the Antioxidant Activity of Pomegranate (*Punica granatum*) Peel and Seed Extracts Using in vitro Models. J. Agric. Food Chem., 2002, 50, 81-86.
58. Rengasamy ragupathi Raja Kannan, Rajasekaran Arumugam, Perumal Anantharaman, In vitro antioxidant activities of ethanol extract from *Enhalus acoroides* (L.F.) Royle. Asian Pacific journal of Tropical Medicine, 2010, 898-901.
59. Oyaizu M. Studies on product of browning reaction prepared from glucose amine. *Jpn J Nutr* 1986; 44: 307-15.
60. Prieto P, Pineda M, Aguilar M. Spectrophotometric quantification of antioxidant capacity through the formation of phosphomolybdenum complex: specific application to the determination of vitamin E. *Anal Biochem* 1999; 269: 337-41.
61. Prior RL, Wu X, Schaich K. Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. J. Agric. Food Chem.2005, 53: 4290-4302.
62. Ou B, Hampsch-Woodill M, Flanagan J, Deemer EK, Prior RL, Huang D. Novel fluorometric assay for hydroxyl radical prevention capacity using fluorescein as the probe. J. Agric. Food Chem. 2002, 50: 2772-77.
63. Ou B, Huang D, Hampsch-Woodill M, Flanagan JA, Deemer EK. Analysis of antioxidant activities of common vegetables employing oxygen radical absorbance capacity (ORAC) and ferric reducing antioxidant power (FRAP) assays: a comparative study. J. Agric. Food Chem. 2002, 50: 3122-28.
64. Guddadarangavvanahally K. Jayaprakasha et al. Evaluation of Antioxidant activities and Antimutagenicity of turmeric Oil: AByproduct from Curcumin Production. Z. Naturforsch.2002, 57c, 828-35.
65. Jayaprakasha G. K., Jaganmohan Rao L. and Sakariah K. K., An improved HPLC method for the determination of curcumin, demethoxycurcumin and bisdemethoxycurcumin, J. Agric. Food Chem.2002, **50**, 3668-72.
66. Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice- Evans C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radical Bio. Med. 1999; 26: 1231-37.

67. Belsare D. P., Pal S. C. et al. Evaluation of antioxidant activity of chalcones and flavonoids. International journal of chemTech research CODEN (USA): IJCRRG. April-June 2010; .2(2), pp 1080-89.
68. Ajay Sharma, Sudhir Bharadwaj, A.S. Maan, Amit Jain and M.D. Kharya. Screening of Antioxidant activity: An overview. Pharmacognosy review, Jul-Dec, 2007, 1(2), 232.
69. Evren Onay-Ucar, Ali Karagoz, Nazli Arda. Antioxidant activity of *Viscum album* ssp.album.Fitoterapia, 2006, 77, 556- 60.
70. Guillermo Garcia –santos,Federico Herrera,Vanesa Martin et al. Antioxidant activity and neuro protective effects of Zolpidem and several synthesis intermediates. Free radical research, 2004, 38, 1289-99.
71. Naim M, Gestener B, Bondi A, Brick Y. Antioxidant and Antihemolytic. J Agric Food Chem 1976; 24: 1174-77.
72. Zarban Asghar and Ziaee Masood. Evaluation of antioxidant properties of Silymarin and its potential to inhibit peroxyl radicals *in vitro*. Pak. J. Pharm. Sci., July 2008; 21(3), 249-54.
73. Kontush A and Beisiegel U., Measurement of oxidizability of blood plasma. *Methods Enzymol*,1999, **299**: 35-49.
74. Princen, H. M. G.; Van Poppel. G.; Vogelezang, C,; Buytenhek, R.; Kolk, F. J. Supplementation of vitamin E but not β-carotene in vivo protects low-density lipoprotein from lipid peroxidation in vitro. *Arterioscler. Thromb.* 1992, 12, 554-62.
75. R. P. Singh, K. N. Chidambara Murthy and G. K. Jayaprakasha, Studies on the Antioxidant Activity of Pomegranate (*Punica granatum*) Peel and Seed Extracts Using in Vivo Models. J. Agric. Food Chem., 2002, 50, 81-86.
76. Bombardelli,; Morazzom. P.. *Vitis vinifera L. Fitoterapa* 1995. 65. 291-317.
77. Aebi. H. Catalyse in vitro. *Methods Enzymol.* 1984. 105. 121-125.
78. Nicholos, M. A. A spectrophotometric assay for iodide oxidation by thyroid peroxidase. *Anal. Biochem.* 1962. 4. 311-345.