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ANTIOXIDANT ASSAY IN VIVO AND VITRO

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ABSTRACT

Measurement of oxidative stress in animal tissue and human serum /plasma with help of various methods modified time to time are presented and can be carried out in laboratory. Present article highlights some important method of measurement of oxidative stress, it include enzyme estimation , in vitro methods , in vivo methods, some assay related to oxidative damages, screening of antioxidant compounds assay etc .merits and demerits of each method /assay.

Key words: Antioxidant, Catalse, Glutathione peroxidase.

INTRODUCTION

Oxidative damage and detection of such damage in humans and animal is challenge, many methods are available each method has its own merits and demerits. Also it is important to test the antioxidant property of compound *in vitro*. Living tissue has enzymes, certain biomolecules and defence system to fight against free radical injury.

SUPEROXIDE DISMUTASE ASSAY (SOD)

Indirect Method (Flohe and Ötting, 1984; Beyer and Fridovich, 1987).

Among these, the xanthine-xanthine oxidase ferricytochrome *c* (X/XOD/Cyt *c*3+) method described by**i)** McCord and Fridovich (1969) is the first and the most widely used method.

Disadvantage

This method gives error while using in animal tissue

(i) Ascorbic acid present in animal tissues (approximately 10^{-3} M) is a scavenger of superoxide O_2 (Nishikimi, 1975; Nandi and Chatterjee, 1987) and also reduces cytochrome C chemically and interferes with the SOD assay.

(ii) Cytochrome *c* oxidase activity present in the tissues may be mistaken for SOD activity, since reoxidation of reduced cytochrome *c* mimics an inhibition of cytochrome *c* reduction.

(iii) uric acid, the product of the action of xanthine oxidase on xanthine, is a scavenger of oxygen free radicals (Ames *et al*., 1981).

Solution to problem of cytochrome C interference

Interference of cytochrome *c* oxidase may be eliminated by using 10 μM potassium cyanide (KCN) in the assay mixture.

Demerits of using KCN in assay

10 μM KCN often does not fully inhibit cytochrome oxidase in crude tissue homogenates (Crapo *et al*., 1978). Rigo *et al.* (1975) have shown that cyanide, even at a concentration of 1·77 × 10–6 M, may affect SOD. Higher concentrations of KCN significantly inhibit Cu, Zn-SOD. Azzi *et al*., (1975) recommended the use of use of acetylated ferricytochrome c in place of ferricytochrome C.

Importance of using acetylated ferricytochrome C

Acetylated ferricytochrome *c* is not recognized as a substrate by cytochrome *c* oxidase.

Drawbacks of using acetylated ferricytochrome C

i) Not suitable for routine purposes Preparation of acetylated ferricytochrome *c* are time-consuming the yield and stability of the acetylated product are low.

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ii) The rate of change of absorbance using acetylated ferricytochrome *c* is markedly low.
iii) The change of absorbance of 0·02 per min at 550 nm using X/XOD/Cyt *c*3+ became 0·007 when ferricytochrome *c* was replaced by acetylated ferricytochrome *c*.

Nitroblue tetrazolium (NBT) method (Beauchamp and Fridovich, 1971)

Nitroblue tetrazolium (NBT) is also used as a detector of O^{-}_{2}generated by the X/XOD system

Principle

The assay of SOD is based on the inhibition of the formation of NADH-phenazine methosulphate-nitroblue tetrazolium formazon. The colour formed at the end of the reaction can be extracted into butanol and measured at 560nm.

Reagents

1. Sodium pyrophosphate buffer (0.025M, pH 8.3)
2. Phenazine methosulphate (PMS) (186□M)
3. Nitroblue tetrazolium (NBT) (300□M)
4. NADH (780□M)
5. Glacial acetic acid
6. n-butanol
7. Potassium phosphate buffer (50mM, pH 6.4)

Procedure

Preparation of enzyme extract

The different samples tissue (0.5g), were ground with 3.0ml of potassium phosphate buffer, centrifuged at 2000g for 10 minutes and the supernatants were used for the assay.

Assay

The assay mixture contained 1.2ml of sodium pyrophosphate buffer, 0.1ml of PMS, 0.3ml of NBT, 0.2ml of the enzyme preparation and water in a total volume of 2.8ml. The reaction will be initiated by the addition of 0.2ml of NADH. The mixture was incubated at 30□C for 90 seconds and arrested by the addition of 1.0ml of glacial acetic acid. The reaction mixture will be then shaken with 4.0ml of n-butanol, allowed to stand for 10 minutes and centrifuged. The intensity of the chromogen in the butanol layer will be measured at 560nm in a spectrophotometer. One unit of enzyme activity is defined as the amount of enzyme that gave 50% inhibition of NBT reduction in one minute.

Use

X/XOD / NBT assay is better suited for monitoring SOD in Polyacrylamide gels (Beauchamp and Fridovich, 1971). SOD in plant extract are measured.

Drawbacks /Demerits

1. The spectrophotometric assay of SOD using the X/XOD/NBT method is not applicable to crude tissue homogenates.
2. NBT is chemically reduced by low concentrations of ascorbic acid present in tissue homogenates.
3. The adrenal gland where ascorbic acid concentration is comparatively high.
4. Lung and brain tissues where SOD activities are comparatively low.
5. Not usefull to measure SOD in animal tissue.

Autoxidation of epinephrine at alkaline pH (Misra and Fridovich, 1972)

Misra and Fridovich (1972) reported an assay for SOD based on the ability of SOD to inhibit the autoxidation of epinephrine at alkaline pH. An indirect method of inhibiting auto-oxidation of epinephrine to its adrenochrome was used to assay SOD activities in blood plasma (Misra and Fridovich, 1971). Auto-oxidation of epinephrine was initiated by adding 1ml of Fenton reagent prepared as described by Onwurah, (1999) to a mixture of epinephrine (3×10^{-4}M), Na_2CO_3 (10^{-3}M), EDTA (10^{-4}M), and 1.0ml of deionized water at a final volume of 6 ml. The auto-oxidation was read in a spectrophotometer at 480 nm every 30 sec for 5 min. The experiment was repeated with 1.0 ml of the blood plasma from different blood samples collected from different groups of animals. A graph of absorbance against time was plotted for each, and the initial rate of auto-oxidation calculated. One unit of SOD activity was defined as the concentration of the enzyme (mg protein/ml) in the plasma that caused 50 % reduction in the auto-oxidation of epinephrine (Jewett and Rockling, 1993). Superoxide dismutase activity was subsequently calculated for each sample.

Disadvantage

i) Ascorbic acid inhibit the autoxidation of epinephrine
ii) Glutathione (GSH) inhibit the autoxidation of epinephrine
iii)It is observed system containing 3 × l0–4 M epinephrine and 0·05 M sodium carbonate solution, pH 10·2, 1 × 10–5 M ascorbic acid or 5 × 10–5 M GSH completely inhibits the autoxidation of epinephrine.

Modified nitrite method

The activity of superoxide dismutase (SOD) was indicated by the Oyanagui method. Superoxide anion radical, produced in the reaction of xantine with O_2 catalysed by xantine oxydase, reacts with hydroxylamine producing nitric ion. Nitric ion combines with naphthalene diamine and sulfaniline acid producing coloured product; the concentration of this mixture is proportional to the amount of superoxide anion radical produced. Enzymatic activity was expressed in nitric unit (NU) in each ml of blood plasma or g of haemoglobin

[Hb]. 1 nitric unit (NU) means 50% of inhibition by SOD of nitric ion production in method conditions. SOD activity was indicated in blood plasma and erythrocytes. Whole blood was centrifuged. Sediment of erythrocytes were rinsed 3 times using 0.9% NaCl. Then, erythrocytes were haemolysed with deionised water. In 10% haemolysate activity of SOD and concentration of haemoglobin by Drabkin reagent were indicated. In blood plasma, SOD isoenzymes were also indicated – Mn-SOD and ZnCuSOD, using KCN as the inhibitor of the ZnCu-SOD isoenzyme by the Oyanagui method (1984).

Merits

Useful to measure SOD in blood plasma and erythrocytes.

Drawbacks

1. Cannot be used in animal tissue.
2. 10μM KCN often does not fully inhibit cytochrome oxidase in crude tissue homogenates (Crapo *et al*., 1978).
3. Rigo *et al*., (1975) have shown that cyanide, even at a concentration of 1·77 × 10–6 M, may affect SOD. Higher concentrations of KCN significantly inhibit Cu, Zn-SOD.

Pyrogallol autoxidation (Marklund and Marklund, 1974)

Modification

Modifications such as the use of pH 8·5 instead of pH 8·2 in the assay mixture and allowance of a lag period of one and a half min to allow the steady state of oxidation of pyrogallol to be attained before taking the initial reading.

Advantages

i. Reliable.
ii. Reproducible results.
iii. Simple.
iv. Not affected by the concentrations of ascorbic acid and glutathione present in tissue homogenates.
v. Assay of Fe-SOD in Escherichia coli.

Assay

The assay system contained 1 mM DTPA, 40 μg catalase, 50 mM air-equilibrated Triscacodylate buffer, pH 8·5 and tissue homogenate or Tsuchihasi extract of erythrocyte in a final volume of 2 ml. The reaction was initiated by the addition of 100 μl of freshly prepared 2·6 mM pyrogallol solution in 10 mM HCl to attain a final concentration of pyrogallol of 0·13 mM in the assay mixture. The assay mixture was transferred to a 1·5 ml cuvette and the rate of increase in the absorbance at 420 nm was recorded for 2 min from 1 min 30 s to 3 min 30 s in a double beam spectrophotometer with recorder. The lag of1 min 30 s was allowed for steady state of autoxidation of pyrogallol to be attained. The allowance of this lag period was very important for reproducibility of results. The concentration of pyrogallol was so adjusted that the rate of change of absorbance per min was approximately 0·020–0·023. The increase in the absorbance at 420 nm after addition of pyrogallol was inhibited by the presence of SOD.

One unit of SOD is described as the amount of enzyme required to cause 50% inhibition of pyrogallol autoxidation per 3 ml of assay mixture. Results have been expressed in units per g tissue or per mg protein for tissue homogenate and units per g Hb for erythrocyte haemolysate. Protein was estimated according to the method of Lowry *et al.*

Tissue SOD – By Pyrogallol Method

To determine the amounts of Cu, Zn-SOD and Mn-SOD in tissues, 2 mM KCN solution was added to the assay mixture to inhibit Cu, Zn-SOD; Mn-SOD remains unaffected (Fridovich, 1974).

Lung Tissue

Lung tissue SDS was used instead of KCN, because KCN gave an erroneously high value for Mn-SOD when the tissue was contaminated with Hb. For the determination of Mn-SOD in lung tissues, the homogenate was pre-incubated with 2% SDS at 37°C for 30 min before addition to the assay mixture. SDS inhibits Mn-SOD; Cu, Zn-SOD remains unaffected (Geller and Winge, 1984). SDS (2%) did not interfere with the autoxidation of pyrogallol. The decrease in the total activity after addition of SDS represents Mn-SOD.

CATALASE

Principle

Catalase $2\ H_2O_2 \longrightarrow O_2 + 2\ H_2O$

The disappearance of hydrogen peroxide (H_2O_2) is measured spectrophotometrically at 240 nm.

Definition of unit

One unit (U) is defined as the amount of enzyme which decomposes 1 µmol of H_2O_2 per min at 25°C and pH 7.0 under the conditions described below.

Reagents

A. Phosphate buffer, 50 mM; pH 7.0: mix 50 mM Na_2HPO_4 solution and 50 mM KH_2PO_4 solution to make a pH 7.0 solution.

B. Hydrogen peroxide (H_2O_2) solution: add about 0.75 ml of 30% H_2O_2 to 100 ml of phosphate buffer (Reagent A).

(Prepare freshly before measurement and store at 4°C).

Measure the absorbance of the mixture (2.0 ml of phosphate buffer (Reagent A) and 1.0 ml of H_2O_2solution) at 240 nm in 1 cm light path versus phosphate buffer (Reagent A) and check the absorbance of 0.85 (±0.02). Otherwise add more 30% H2 O2 or phosphate buffer (Reagent A) to the H2 O2 solution and repeat the same check.

Sample

Dissolve the lyophilized enzyme to a concentration of 5.0 mg/ml in ice-cold phosphate buffer (Reagent A) and dilute to a volume activity of 0.3–0.6 U/ml with ice-cold phosphate buffer (Reagent A) immediately before measurement.

Procedure

1. Pipette 2.0 ml of sample into a cuvette (light path: 1 cm).
2. Equilibrate at 25°C for about 5 min.
3. Add 1.0 ml of H_2O_2 solution (Reagent B).
4. Record the decrease of absorbance at 240 nm in a spectrophotometer thermostated at 25°C, and calculate the ΔA per min using the linear portion of the curve (ΔAS). The blank solution is prepared by adding phosphate buffer (Reagent A) instead of sample (ΔA0).

Calculation

Activity can be calculated by using the following formula:
Volume activity (U/ml)= ΔA_S - ΔA_O)X3ml
xd*f* ÷ *0.04*36x2ml= Δ Ax34.4x d*f* .
Weight activity (U/mg) =(U/ml) x1/C
0.0436 : Millimolar extinction coefficient of hydrogen peroxide at 240 nm (cm2/µmol)
df : Dilution factor
C : Content of catalase preparation in sample (mg/ml)

Glutathione peroxidase (GPx)

Provides a mechanism for detoxification of peroxides in living cells. This reaction plays a crucial role in protecting cells from damage by free radicals, which are formed by peroxide decomposition. Lipid components of the cell are especially susceptible to reactions with free radicals, resulting in lipid peroxidation. GPx enzymes reduce peroxides to alcohols using glutathione, thus preventing the formation of free radicals.

GPx enzymes will catalyze the reduction of hydrogen peroxide (H2O2) and a wide variety of organic peroxides (R-OOH) to the corresponding stable alcohols (R-OH) and water using cellular glutathione as the reducing reagent.

Most cellular glutathione peroxidases are tetrameric enzymes consisting of four 22 kDa monomers, each of which contains a selenocysteine moiety in the active site. The selenocysteine participates directly in electron donation to the peroxide substrate and becomes oxidized in the process. The enzyme then uses reduced glutathione as a hydrogen donor to regenerate the selenocysteine. GPx enzymes also exist as non-selenium (non-Se) containing enzymes. Cellular GPx is present in all tissues; however, various diseases may influence its level. An increase in the level of glutathione peroxidase has been observed in reticulocytes of diabetic rats. The level returned to normal after administration of insulin. A decrease in the level of the enzyme has been observed in patients suffering from diseases such as Favism (a disease associated with extreme hemolytic crisis) or hairy cell leukemia. In this procedure, the rate of oxidation of glutathione by H202 is used as a measure of peroxidase activity. Glutathione remaining in the solution at a given time is determined by its reaction with DTNB. EDTA is used in the incubation medium to reduce the non-enzymatic reaction rate at a low level.

Reagents

1. Sodium phosphate buffer : 0.3 M, pH 7.0
2. Sodium azide : 1Omhl
3. Reduced glutathione :4mM
4. Hydrogen peroxide :*2.5* mM
5. TCA :lPh
6. Phosphate solution : 0.3 M disodium hydrogen phosphate.
7. DTNB : 40 mg/100 ml of 1% sod~umc ihate
8. EDTA : 0.8 mM
9. Standard : 20 mg of reduced glutathione in 100 rnl distilled water.

This solution contained 20 pg of glutathione/O. 1 ml.

A known volume of the homogenate was added to the incubation medium which contain 0.4 ml of buffer, 0.2 rnl of sodium azide, 0.2'ml of ED%, 0.2 ml of Hydrogen peroxide and 0.2 ml of reduced glutathione. The incubation medium was made upto a final volume of 2.0 ml with water. The tubes were incubated at 37'C for 90 and 180 minutes. The will be terminated by the addition of 1.0 ml of the precipitating agent. The reaction mixture was centrifuged and to the supernatant, 6.0 ml of disodium hydrogen phosphate will be added. One ml of DTNB reagent will be added just prior to the calorimetric analysis. The absorbance will be read at 412 nm against a blank, which contained only 6.0 rnl of disodium phosphate and 1.0 ml of DTNB reagent . The activity will be expressed in terms of *u*gm of glutathione utilizedl/minute/mg protein.

Glutathione reductase (GR, EC 1.6.4.2)

Catalyzes the reduction of oxidized glutathione (GSSG) to reduced glutathione (GSH). This enzyme, which is found in many tissues, enables the cell to sustain adequate levels of cellular GSH. Reduced glutathione is a substrate for the glutathione peroxidases, which provide a mechanism for the detoxification of peroxides and glutathione S-

transferases, which are involved in the conjugation and elimination of xenobiotics from the organism. Reduced glutathione also acts as an antioxidant, reacting with free radicals and organic peroxides.

The Glutathione Reductase Assay Kit enables the spectrophotometric measurement of glutathione reductase activity. The activity can be measured by either the decrease in absorbance caused by the oxidation of NADPH at 340 nm (UV assay) or by the increase in absorbance caused by the reduction of DTNB [5,5 ¢-dithiobis(2-nitrobenzoic acid)] at 412 nm (Colorimetric assay).

Glutathione reductase is a homodimeric enzyme with a molecular mass ranging from 100 kDa for the human erythrocyte enzyme to 118 kDa for the yeast enzyme. It contains one FAD moiety per subunit and shows a K_M of 4-9 mM for NADPH and 55-65 mM for GSSG. The enzyme is inhibited by divalent metal ions (Zn^{2+} or Cd^{2+}), but this can be prevented by the addition of EDTA to the extraction buffer. The activity of this enzyme will be depressed by ammonium sulfate at concentrations greater than 60 mM, but 0.6 M urea, 1% TRITON^{Ò} X-100, or 100 mM KCl have very little effect on the activity.

Reagents

Glutathione Reductase Assay Buffer 100 mM potassium phosphate buffer, pH 7.5, with 1 mM EDTA Glutathione Reductase Dilution Buffer100 mM potassium phosphate buffer, pH 7.5, with 1 mM EDTA and 1mg/ml bovine serum albumin, Glutathione Reductase Positive Control lyophilized powder containing yeast glutathione reductase, potassium phosphate buffer, pH 7.5, with EDTA trehalose as a stabilizer Nicotinamide Adenine Dinucleotide Phosphate, Reduced (NADPH) Glutathione, Oxidized, Disodium Salt 5,5 -Dithiobis(2-nitrobenzoic acid).

Preparation Instructions

Prepare the solutions in the volume required for the number of assays to be performed, according to the detection method to be used.

1. Glutathione Reductase Positive Control Solution - Reconstitute the vial with 1 ml of water to obtain a glutathione reductase activity of >1 unit per ml in a solution containing 100 mM potassium phosphate buffer, pH 7.5, with 1 mM EDTA and38 mg/ml trehalose. For long term storage, divide the solution into aliquots and freeze at -20 °C. The solution will be stable for at least 6 months at -20 °C.
2. 2 mM NADPH Solution - Dissolve a portion of the b-Nicotinamide adenine dinucleotide phosphate, reduced (NADPH) at 1.85 mg/ml in Assay Buffer to prepare a working solution of 2 mM. Store at 4 °C. Prepare the NADPH solution fresh every day.
3. 2 mM Oxidized Glutathione Solution - Dissolve the Glutathione, Oxidized, Disodium salt (GSSG) at 1.42 mg/ml in Assay Buffer to prepare a working solution of 2 mM. Store at 25 °C while performing the test. The solution may be kept up to 7 days at 2-8 °C for temporary storage.
4. 3 mM DNTB Solution - Dissolve the 5,5 ¢-Dithiobis (2-nitrobenzoic acid) (DNTB) at 1.19 mg/ml in Assay Buffer to prepare a working solution of 3 mM. This solution is very unstable; prepare the solution fresh every 4 hours and store at 4 °C.
5. Sample preparation. Dilute the samples to be assayed in Dilution Buffer as needed immediately before assaying. The concentration dependent enzymatic reaction is linear from 0.003-0.03 units per ml of reaction mixture for the colorimetric assay and from 0.003-0.012 units per ml of reaction mixture for the UV assay.

Principle

This assay is based on the reduction of glutathione (GSSG) by NADPH in the presence of glutathione reductase. In addition, 5,5 ¢-dithiobis(2-nitrobenzoic acid) [DTNB] reacts with the reduced glutathione (GSH) formed:

$$NADPH + H^+ + GSSG \xrightarrow{GR} NADP + 2\,GSH$$

The reduced glutathione can then spontaneously react with DTNB:

$$GSH + DTNB \longrightarrow GS\text{-}TNB + TNB$$

TNB = 5-thio(2-nitrobenzoic acid)

The first reaction is measured by the decrease in absorbance at 340 nm using an extinction coefficient (e^{mM}) of 6.22 for NADPH and the second reaction is measured by the increase in absorbance at 412 nm using an extinction coefficient (e^{mM}) of 14.15 for TNB.

Unit definitions

UV assay - One unit will cause the oxidation of 1.0 mmole of NADPH at 25 °C at pH 7.5. Colorimetric assay - One unit will cause the reduction of 1.0 mmole of DTNB to TNB at 25 °C at pH 7.5.

Assay Procedure

1. Equilibrate the Assay Buffer and 2 mM Oxidized Glutathione Solution at 25 °C for at least 10 minutes before starting the assay.
2. Set up the kinetic program in the spectrophotometer. The following programs are recommended.

a. UV assay

Wavelength: 340 nm; Initial delay: 10 seconds; Interval: 10 seconds; Number of readings: 11;

b. Colorimetric assay:

Wavelength: 412 nm; Initial delay: 60 seconds Interval: 10 seconds Number of readings: 11

3. Zero the spectrophotometer with a cuvette filled with water.
4. Place the following solution volumes in a 1 ml quartz cuvette in the order shown below.

Reaction Volumes

Solution	UV Assay	Colorimetric Assay
2 mM Oxidized Glutathione	500ml	500ml
Assay Buffer	350-450 ml	50-150 ml
Sample*	0-100 ml	0-100 ml
3 mM DTNB	-	250 ml
2 mM NADPH	50 ml	50 ml
Total volume	1.00 ml	1.00 ml

* For both (UV or Colorimetric) assays perform a positive control by adding 10 to 20 ml of the Glutathione Reductase Positive Control Solution per control reaction.

Calculations

1. The spectrophotometer should record the DA/minute for the reaction automatically.
2. Ifthe absorbance is measured manually, calculate this value for the blank, positive controls, and all samples.
3. Samples that give a large deviance from linearity should not be taken into account. This may happen with very dilute or very concentrated samples. The range of enzymatic activity that can be measured varies with the assay used. The concentration dependent enzymatic reaction is linear from 0.003-0.03 units per ml of reaction mixture for the colorimetric assay and from 0.003-0.012 units per ml of reaction mixture for the UV assay.
4. The concentration of enzyme can be calculated using the formula:

units/ml =(DA_{sample} - DA_{blank}) x (dilution factor)/emM X (volume of sample in ml)

For NADPH e^{mM} = 6.22 $mM^{-1}cm^{-1}$

For TNB[6] e^{mM} = 14.15 $mM^{-1}cm^{-1}$

Estimation of GSH in liver tissue

GSH will be determined by the method of Ellman (1959) [18]. 0.5mL of tissue homogenate will be precipitated with 2mL of 5% TCA and centrifuged at 3200Xg for 20 minutes. After centrifugation 1mL of the supernatant will be taken and added to 0.5mL of Ellman's reagent (2,2´-dinitro-5,5´-dithiobenzoic acid) and 3mL of phosphate buffer (pH 8.0). Then the absorbance will be measured at 412nm.The values will be expressed as mg/100 g tissue.

***In Vitro* testing antioxidant property**

To test the anti-oxidant activity of compound in vitro following test can be used

ABTS scavenging activity

The reaction was initiated by the addition of 1.0 ml of diluted ABTS to 10 µl of different concentrations of plant /herbal extract of the sample or 10 µl methanol as control. The absorbance will be read at 734 nm and the percentage inhibition will be calculated.

The inhibition will be calculated according to the equation

$I = A_1/A_0 \times 100$

where A_0 is the absorbance of control reaction, $A_1$is the absorbance of test compound.

Hydroxy radical activity

The reaction mixture 3.0 ml contained 1.0 ml of 1.5 mM $FeSO_4$, 0.7 ml of 6 mM hydrogen peroxide, 0.3 ml of 20 mM sodium salicylate, and different dilution of the plant /herbal extract will be added. After incubation for 1 hour at 37°C, the absence of the hydroxylated salicylate complex will be measured at 562 nm. The percentage scavenging effect will be calculated as

Scavenging activity = $[1-(A_1-A_2)/A_0] \times 100\%$,

Where A_0 is absorbance of the control (without extract), A_1 is the absorbance in the presence of the extract, and A_2 is the absorbance without sodium salicylate.

Reducing power

The reaction mixture contained 2.5 ml various concentrations of spagyric essence of the sample, 2.5 ml of 1% potassium ferric cyanide and 2.5 ml of 0.2 M sodium phosphate buffer. The control contained all the reagents except the sample. The mixture will be incubated at 50°C for 20 minutes, and will be terminated by the addition of 2.5 ml of 10% (W/V) trichloroacetic acid, followed by centrifugation at 3000 rpm for 10 minutes. 2.5 ml of the supernatant upper layer will be mixed with 2.5 ml distilled water and 0.5 ml of 0.1% ferric chloride, and absorbance will be measured at 700 nm against blanks that contained distilled water and phosphate buffer. Increased absorbance indicated increased reducing power of the sample. Ascorbic acid will be used for comparison.

Superoxide anion radical scavenging activity

This assay was based on the reduction of nitro blue tetrazolium (NBT) in the presence of NADH and phenazine methosulphate (PMS) under aerobic condition .The 3.00 ml reaction mixture contained 50 µl of lM NBT, 150 µl of lM nicotinamide adenine dinucleotide (NADH) with or without sample and Trisbuffer (0.02 M, pH 8.0). The reaction will be started by adding 15µl of lM phenazine methosulfate (PMS) to the mixture and the absorbance change will be recorded to 560 nm after 2 minutes. Percent inhibition will be calculated against a control without the extract.

Chelating activity

The reaction mixture contained 1.0ml of various diluted plant /herbal extract , 0.1 ml of 2 mM $FeCl_2$ and 3.7 ml methanol. The control contained all the reaction reagents except the sample. The reaction will be

initiated by the addition of 2.0 ml of 5 mM ferrozine. After 10 minutes at room temperature, the absorbance of the mixture will be determined at 562 nm against a blank. A lower absorbance of the reaction mixture indicated a higher iron chelating ability. The capacity to chelate the ferrous ion will be calculated by

% chelation = [1-(ABS $_{sample}$/ABS $_{control}$] x 100.

Ferric-reducing antioxidant power (FRAP) assay

The stock solution of 10 mM 2,4,6-tripyridyl-s- triazine (TPTZ) in 40mM HCL, 20 mM $FeCl_3$. $6H_2O$ and 0.3Macetate buffer (pH 3.6) will be prepared. The FRAP reagent contained 2.5 ml TPTZ solution, 2.5 ml ferric chloridesolution and 25 ml acetate buffer. It will be freshly prepared and warmed to 37°C. FRAP reagent (900 µl) will be mixed with 90 µl water and 30 µl test ethanolic extract of the sample and standard antioxidant solution. The reaction mixture will be then incubated at 37°C for 30 minutes and the absorbance will be recorded at 595 nm. An intense blue color complex is formed when ferric tripyridyltriazine (Fe^{3+}-TPTZ) complex will reduced to ferrous (Fe^{2+}) form. The absorption at 540 nm will be recorded.

Scavenging of hydrogen peroxide

It can be generated through a dismutation reaction from superoxide anion by superoxide dismutase. It can generate the hydroxyl radical in the presence of metal ions and superoxide anion

$$O_2 + H_2O_2 \longrightarrow OH^- + OH^+ + O_2$$

A solution of hydrogen peroxide (20mM) will be prepared in phosphate buffered saline (PBS, pH 7.4). Various concentrations of 1ml of the plant /herbal extract or standards in methanol were added to 2 ml of hydrogen peroxide solutions in PBS. The absorbance was measured at 230 nm, after 10 min against a blank solution that contained extracts in PBS without hydrogen peroxide.

TBARS Assay

A modification of thiobarbituric acid reactive substances (TBARS) assay was used to determine the level of lipid peroxide formed using egg yolk homogenate as lipid-rich media. Egg homogenate (0.5 ml, 10% v/v) will be added to 0.1 ml of extract (1mg/ml) and the volume made up to 1 ml with distilled water. Then, 0.05 ml of FeSO4 will be added and the mixture incubated for 30 minutes. Acetic acid (1.5 ml) and thiobarbituric acid (1.5 ml) in SDS will be sequentially added. The resulting mixture will be vortexed and heated at 95ºC for 60 minutes. After cooling, 5 ml of butanol will be added and the mixture centrifuged at 3000 rpm for 10 minutes. The absorbance of the organic upper layer will be measured at 532 nm and converted to percentage inhibition using the formula:

Inhibition of lipid peroxidation (%) = (1 – E/C) x 100

Where C = absorbance of fully oxidized control and E = absorbance in the presence of extract

DPPH free radical scavenging assay

DPPH (1, 1-diphenyl-2-picrylhydrazyl) free radical scavenging assay 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 non radical form DPPH. This transformation results in a colour change from purple to yellow, which is measured spectrophotometrically. The disappearance of the purple colour is monitored at 517 nm. The reaction mixture (3.0 ml) consists of 1.0 ml of DPPH in methanol (0.3 mM), 1.0 ml of the extract (different concentrations of 1:1, 1:10, 1:100, 1:1000) and 1.0 ml of methanol. It is incubated for 10 min in dark and then the absorbance is measured at 517 nm. Ascorbic acid has taken as positive control.

$$\% \text{ DPPH radical scavenging activity} = \frac{(\text{Absorbance of Control} - \text{Absorbance of test Sample}) \times 100}{(\text{Absorbance of Control})}$$

Nitric oxide radical scavenging (NO) assay

Nitric oxide was generated from sodium nitroprusside and measured by the Griess reaction13. Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide, which interacts with oxygen to produce nitric ions that can be estimated by use of Griess reagent. Scavenger of nitric oxide competes with oxygen leading to reduced production of nitric oxide. Sodium nitroprusside (5 M) in phosphate–buffered saline (1xPBS PH 7.4) was mixed with 3ml of different plant extracts (different concentrations i.e. 1:1 ,1:10,1:100,1:1000) and incubated at 25^0C for 150 min. The samples from the above were reacted with Griess reagent. The absorbance of the chromophore formed during the diazotization of nitrite with sulphanilamide and subsequent coupling with napthylethylene diamine was read at 546 nm. Ascorbic acid has taken as positive control. The percentage scavenging of nitric oxide of plant extracts was calculated using the following.

$$\text{NO Scavenging (\%)} = \frac{(\text{Absorbance of Control} - \text{Absorbance of test Sample}) \times 100}{(\text{Absorbance of Control})]}$$

REFERENCES

Azzi A, Montecucco C and Richter C. The use of acetylated ferricytochrome c for the detection of superoxide radicals produced in biological membranes. *Biochem. Biophys. Res. Commun*., 65, 1975; 597.

Beauchamp C and Fridovich I. Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. *Anal. Biochem*., 44, 1971, 276.

Beyer WF and Fridovich I. Assaying for superoxide dismutase activity: some large consequences of minor changes in conditions. *Anal. Biochem*., 161, 1987, 559.

Bridges SM and Salin ML. Distribution of iron-containing superoxide dismutase in vascular plants. *Plant Physiol*., 68, 1981, 275.

Crapo JD, McCord JM and Fridovich I. Preparation and assay of superoxide dismutases. *Methods Enzymol*., 53, 1978, 382.

Davis BJ. Disc electrophoresis. II. Method and application to human serum proteins. *Ann. N. Y. Acad. Sci*., 121, 1964, 404.

Flohé L and Ötting F. Superoxide dismutase assays. *Methods Enzymol*., 105, 1984, 93.

Fridovich I. Superoxide dismutases. *Adv. Enzymol*., 41, 1974, 35.

Fridovich I. Superoxide radical: an endogenous toxicant. *Annu. Rev. Pharmacol. Toxicol*., 23, 1983, 239.

Geller BL and Winge DR. *Methods Enzymol*., 105, 1984, 105.

Gram TE, Okine LK and Gram RA. *Annu. Rev. Pharmacol. Toxicol*., 26, 1986, 259.

Hartman D. The aging process. *Proc. Natl. Acad. Sci.* USA, 78, 1981, 7124-7128.

Leibovitz BE and Siegel BV. Aspects of free radical reactions in biological systems: aging. *J. Gerontol*., 35, 1980, 45-56.

Lowry OH, Rosebrough NJ, Farr AL and Randall RJ. Protein measurement with the folin phenol reagent. *J. Biol. Chem*., 193, 1951, 265-275.

Marklund SL and Marklund G. Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. *Eur. J. Biochem*., 47, 1974, 469.

McCord JM and Fridovich I. Superoxide Dismutase *an Enzymic Function for Erythrocuprein (Hemocuprein). J. Biol. Chem*., 244, 1969, 6049.

Misra HP and Fridovich I. The Role of Superoxide Anion in the Autoxidation of Epinephrine and a Simple Assay for Superoxide Dismutase. *J. Biol. Chem*., 247, 1972, 3170.

Nandi A and Chatterjee IB. Scavenging of superoxide radical by ascorbic acid. *J. Biosci*., 11, 1987, 435-441.

Nishikimi M. Oxidation of ascorbic acid with superoxide anion generated by the xanthine-xanthine oxidase system. *Biochem. Biophys. Res. Commun*., 63, 1975, 463-468.

Oberly LW and Buettner GR. Role of superoxide dismutase in cancer: a review. *Cancer Res*., 39, 1979, 1141-1149.

Oberly LW and Spitz DR. Assay of superoxide dismutase activity in tumor tissue. *Methods Enzymol*., 105, 1984, 457-464.

Peeters-Jorris C, Vandevoorde AM and Bandhuin P. Subcellular localization of superoxide dismutase in rat liver. *Biochem. J*., 150, 1975, 31-39.

Richterich R. Clinical chemistry, Basel, Switzerland, New York, 1969.

Rigo A, Viglino P and Rotilio G. Oxidation of reduced Cu, Zn superoxide dismutase by molecular oxygen. A kinetic study. *Anal Biochem*, 68, 1975, 1.

Steinhagen-Thiessen E, Reznick A, Ringe JD and Gershon D. in Superoxide and superoxide dismutase in chemistry, biology and medicine (ed. G. Rotilio) (Amsterdam: Elsevier). 1986, 464.

Sykes JA, McCormack FX and O'Brien JJ. *Cancer Res*., 1978, 38, 2759.

Takada Y, Noguchi T, Okabe T and Kajiyama M. Superoxide dismutase in various tissues from rabbits bearing the Vx-2 carcinoma in the maxillary sinus. *Cancer Res*., 42, 1982, 4233-4235.

Troll W and Weisner R. The role of oxygen radicals as a possible mechanism of tumor promotion. *Annu. Rev. Pharmacol Toxicol*., 25, 1985, 509-528.

Westman NG and Marklund SL. *Cancer Res*., 41, 1981, 2962.