List of appendices

APPENDIX – I

ESTIMATION OF BLOOD GLUCOSE

Aim : To estimate the amount of Blood glucose in the given sample

Principle:

Glucose is determined after enzymic oxidation in the presence of glucose oxidase. The hydrogen peroxide formed reacts, under catalysis of peroxidase, with 4 hydroxy benzoic acid and 4-aminophenazole to form a red-violet quinomine as indicator.

Reagents

Reagent I	:	Enzyme reagent
Reagent II	:	Diluent
Glucose standard	:	100mg/dl

Procedure:

Pipette into test tubes

	Blank	**Standard**	**Sample**
Sample	-	-	10µl
Standard	-	10µl	-
Reagent	1000µl	1000µl	1000µl

Mix and incubate for 30 minutes at room temperature. Measure absorbance of sample (AT) and standard (AS) against reagent blank at 505nm. The colour is stable for 30 minutes at room temperature.

Calculation and linearity

Total glucose (mg/dl) = AT/AS x Concentration of standard

The method is linear upto a concentration of 1000mg/dl. Dilute samples above this concentration 1:1 with 0.9 percent saline solution and repeat the assay.

APPENDIX- II

ESTIMATION OF CATALASE ACTIVITY

(Luck, 1974)

The enzyme catalase has a double function and it catalyses the following

a. It decomposes hydrogen peroxide to give water and oxygen.

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

B.It oxidizes H^+ donors, for example methanol, formic acid, phenol with the consumption of one mole of peroxide.

$$ROOH + AH_2 \xrightarrow{\text{Catalase}} H_2O + ROH + A$$

Principle

The UV light absorption of hydrogen peroxide solution can be easily measured between 230 and 250nm. On decomposition of hydrogen peroxide by catalase, the absorption decreases with time. The enzyme activity could be arrived at from this decrease. But this method is applicable only to enzyme solution, which do not absorb strongly at 230-250nm.

Reagents

1. Phosphate buffer 0.067M (pH 7.0)

Dissolved 3.522g of KH_2PO_4 and 7.268g of $KHPO_4$. 2 H_2O in distilled water and made up the volume to one litre.

2. Hydrogen peroxide – Phosphate buffer

Dissolved 0.16 ml of H_2O_2 (10% W/V) to 100 ml phosphate buffer, prepared fresh. The absorbance of the solution should be about 0.5 at 240nm with 1cm light path.

Procedure

Enzyme extract:

The sample is homogenized in a prechilled mortar and pestle with M/150 phosphate buffer (assay buffer diluted 10 times) at 1 - 4°C and centrifuged. Stirred the sediment with cold phosphate buffer, allowed to stand in the cold with occasional shaking and then repeated the extraction once or twice. The extraction should not take more than 24 hr. The combined supernatants were used for the assay. Used fresh extract for assay.

Assay

Read against a control cuvette 3ml of H_2O_2 containing the enzyme solution as in the phosphate buffer (M/15). Pipetted into the experimental cuvette 3ml of H_2O_2 phosphate buffer. Mixed in 0.01 – 0.04ml sample with the glass or plastic rod flattened at one end. Noted the time it required for a decrease in absorbance from 0.45 to 0.4. This value was used for calculations. If 't' was more than 60 seconds, repeated the measurement with more concentrated solution of the sample.

Calculation

Calculated the concentration of H_2O_2 using the extinction coefficient 0.036μ mole/ml.

APPENDIX – III

ESTIMATION OF PEROXIDASE ACTIVITY

(Reddy *et al.*, 1995)

Principle

Peroxidase converts H_2O_2 to water and oxygen in the presence of hydrogen donor (pyrogallol or dianisidine) the oxidation of pyrogallol or dianisidine to coloured product called purpurogalli is measured colorimetically.

Pyrogallol + H_2O_2 ⟶ Oxidized pyrogallol +H_2O

Reagents

1. Pyrogallol-0.05 M phosphate buffer (pH 6.5)
2. 1% H_2O_2

3. Enzyme extract: Macerated one gram of the sample with 5ml (w/v) of 0.1M phosphate buffer (pH 6.5) in a homogenizer. Centrifuged the homogenate at 300g for 15 minutes. Used the supernatant as the enzyme source. All procedures were carried out at 0-5°C.

Procedure

Pipetted out 3ml of 0.05 M-pyrogallol solution and 0.5 to 0.1ml of enzyme extract in a test tube. Adjusted the spectrophotometer to read '0' at 400nm. Added 0.5ml of 1% H_2O_2 in the test cuvette. Recorded the change in the absorbance every 30 seconds up to 3 minutes.

APPENDIX - IV

ESTIMATION OF SUPEROXIDE DISMUTASE ACTIVITY

(Misra and Fridovich, 1972)

Principle

Superoxide dismutase uses the photochemical reduction of riboflavin as oxygen generating system and catalyses the inhibition of Nitro Blue Tetrozolium (NBT) reduction, the extent of which can be assayed spectrophotometrically.

Reagents

1. 50mM potassium phosphate buffer, pH 7.8
2. 45µM Methionine
3. 5.3µM riboflavin
4. 84µM Nitro Blue Tetrozolium (NBT)
5. 20mM potassium cyanide

Procedure

The incubation medium contained a final volume of 3ml, 50mM potassium phosphate buffer (pH 7.8), 45μM methionine, 5.3μM riboflavin, 84μM NBT and 20mM potassium cyanide. The tubes were placed in an aluminum Foli – lined box maintained at 25°C and equipped with 15W fluorescent lamps. Reduced NBT was measured spectrophotomerically at 600nm after exposure to light for 10 minutes. The maximum reduction was evaluated in the absence of the amount of enzyme giving 50% inhibition of the reduction of NBT.

APPENDIX – V

ASSAY OF GLUTATHIONE-S-TRANSFERASE

(Habig *et al.*, 1974)

The enzyme was assayed by the conjugate GSH and CDNB, the extent of conjugation causing a proportionate change in the absorbance at 340nm.

Reagents

1. 1mM –Chloro 2,4-dinitrobenzene (CDNB) in ethanol
2. 1mM – Glutathione
3. 0.1 M phosphate buffer

Procedure

The assay was done at 5°C under condition giving activities linear with respects to incubation time and protein concentration for at least 3 minutes. The enzyme activity was determined by monitoring the change in absorbance at 340nm in a spectrophotometer. 0.1 ml of both substrates (GSH and CDNB was taken in 0.1M phosphate buffer (pH 6.5) at room temperature to make a volume of 2.9 ml.

The reaction was started by the addition of 0.1 ml of sample to this mixture; the readings were recorded against distilled water blank for a minimum of three minutes. The complete assay mixture without the sample served as the control to monitor non-specific binding of the substrate. Care was taken to ensure that final concentration of ethanol in the mixture was always less than 4%.

Calculation

GST activity was calculated using the extinction coefficient of the product formed and the values have been expressed as nmoles and CDNB conjugated/minutes/ g sample.

APPENDIX – VI

ESTIMATION OF ASCORBIC ACID

(Roe and Kuether, 1953)

Principle

Ascorbate is converted to dehydroascorbate by treatment with activated charcoal and bromine. Dehydroascorbic acid then reacts with 2, 4- dinitrophenyl hydrazine to form osazones, which dissolves in sulpuric acid to give an orange coloured solution whose absorbance can be measured spectrophotomerically at 540nm.

Reagents

1. 4%TCA
2. 9 N H_2SO_4
3. 2% 2, 4- dinitrophenyl hydrazine: dissolved 2g of DNPH in 100ml of 9N H_2SO_4
4. 10% thiourea
5. 80% sulpuric acid
6. Stock standard solution: Dissolved 100mg of ascorbic acid in 100ml 4%TCA
7. Working standard: diluted 10ml of the stock solution to 100ml with 4%TCA

Procedure

About 1g of the sample was homogenized in 4% TCA up to 10ml. Centrifuged at 2000rpm for 10 minutes. To the supernatant obtained, a pinch of activated charcoal was added, shaken well and kept for 10minutes. Centrifuged once again and removed the charcoal residue. The volume of the clear supernatants was noted. 0.5 and 1.0 ml aliquots of this supernatant were taken for the assay. The assay volumes were made up 2.0 ml with 4%TCA. 0.2 to 1.0 ml of the working standard solution containing 20-100µg of ascorbate respectively were pipetted out into clean dry test tube, the volume of which were also made up to 2.0 ml with 4% TCA. Added 0.5 ml of DNPH reagent to all the test tubes, followed by 2drops of 10% thiourea solution. Incubated at 37°C for 3 hours.

The osazones formed were dissolved in 2.5 ml of 85% sulpuric acid, in cold, drop by drop, with no appreciable rise in temperature. To the blank alone , DNPH reagent and thiourea were added after the addition of H_2SO_4. The tubes were incubated for 30 minutes at room temperature, and the absorbance was read spectrophotometrically at 540nm. Calculated the content of ascorbic acid in the sample using the standard graph.

APPENDIX – VII

ESTIMATION OF α - TOCOPHEROL

(Emmerie-Engel method, 1938 as described by Rosenberg, 1992)

Principle

Tocopherol can be estimated using Emmerie-Engle reaction which based on the reduction of ferric to ferrous ions by tocopherols, which then forms a red colour with 2, 2'-dipyridyl. Tocopherol and carotenes are first extracted with xylene and the extraction read at 460nm to measure carotenes. A correlation is made for these after adding ferric chloride and reading at 520nm.

Reagents

1. Absolute alcohol
2. Xylene
3. 2, 2'-dipyridyl
4. Standard solution: Dissolved 10mg/ 10 ml of α-tocopherol in absolute alcohol. 91mg of α-tocopherol is equivalent to 100mg of tocopherol acetate.

Extraction

The sample was homogenized with water in a blender. Weighed accurately, 2.5g of the homogenized sample into a conical flask. Added 50 ml of 0.1N H_2SO_4 slowly without shaking. Stoppered and allowed to stand overnight. The next day contents of the flask were shaken vigorously and filtered through whatman No.1 filter paper, discarding the initial 10-15ml of filtrate. Aliquots of the filtrate were used for the estimation.

Procedure

Into 3 stoppered centrifuge tubes (test, standard and blank), pipetted out 1.5 ml of extract, 1.5 ml of standard, 1.5 ml of water respectively. To the test and blank added 1.5 ml of ethanol and to the standard, added 1.5 ml of water. Added 1.5 ml xylene to all the test tubes, stoppered, mixed well and centrifuged. Transferred 1.0 ml of xylene layer into another stoppered tube, taking care not to include any other ethanol or protein. Added 1.0 ml 0f 2, 2'-dipyridyl reagent to each tube, stoppered and mixed. Pipetted out 1.5 ml of the mixture into colorimeter cuvettes and read the extinction of the test and standard against the blank at 460nm. Then in turn beginning with the blank, added 0.33 ml of ferric chloride solution. The amount of vitamin E can be calculated using the formula,

$$\text{Amount of tocopherols} = \frac{\text{Reading at 520nm - Reading at 460nm}}{\text{Reading of standard at 520nm x 0.29 x 15}}$$

APPENDIX – VIII

ESTIMATION OF REDUCED GLUTATHIONE

(Moron *et al.*, 1979)

Principle

Reduced glutathione (GSH) is measured by its reaction with DTNB (5, 5-dithio-2-nitrobenzoic acid) (Ellman's reaction) to give a compound that absorbs at 412nm.

Reagents

1. DTNB
2. 5% TCA
3. 0.2M Sodium phosphate buffer

Procedure

1g of the sample was homogenized in 5% TCA to give a 20% homogenate. The precipitated protein was centrifuged at 1000rpm for 10 minutes. The homogenate was cooled on

ice and 0.1 ml of supernatant was taken for the estimation. The volume of the aliquot was made up to 1.0 ml with 0.2M Sodium phosphate buffer (pH 8.0), 2 ml of freshly prepared DTNB solution (0.6mM) in 0.2M phosphate buffer (pH 8.0), was added to the tubes and intensity of the yellow colour formed was read at 412nm in a spectrophotometer after 10minutes. A standard curve of GSH was prepared using concentration ranging from 2 to 10nmoles of GSH IN 5% TCA.

APPENDIX – IX

ESTIMATION OF PROTEIN
(Lowry *et al.*, 1959)

PRINCIPLE

This method is based on the principle that different proteins contains different amounts of amino acid residues, which reacts with Folin-Ciocalteau reagent giving a blue color, which is read in a spectrophotometer at 750 nm.

REAGENTS

1. Alkaline copper sulphate

   2% Na_2CO_3 in 0.1 N NaOH

   2% Sodium potassium tartarate-1 ml

   2% Copper sulphate-1 ml

2. Folin-Ciocalteau reagent stock

   2N Folin-Ciocalteau reagent was diluted with water (1:1 v/v)

3. Standard protein solution

   Standard BSA containing 20 mg/100 ml of 0.9% NaCl

PROCEDURE

Into a series of clean dry test tubes pipetted out 0.2 to 10 ml of standard BSA solution corresponding to 40 to 200 µg of protein. Made up the volume to 1.9 ml with 0.1N NaOH. Added 1.0 ml of alkaline copper sulphate solution mixed well and incubated for 3 minutes at 37°C. Then added 0.5 ml of alkaline copper sulphate solution mixed well and incubated for 3 minutes at 37°C. The optical density was read at 750 nm in a spectrophotometer.

APPENDIX X

ASSAY OF LIPID PEROXIDATION
(Okhawa *et al.*, 1979)

Principle

Malondialdehyde formed (MDA) from the break down of polyunsaturated fatty acid serves as a convenient index for the extent of per oxidation reaction. MDA a product of lipid peroxidation that reacts with TBA give a pink colour product having absorbance maximum at 535nm.

Reagents

1. 10%TCA
2. 0.1M Thiobarbituric acid

Procedure

0.1ml of homogenate was treated with 1ml of 10% TCA and 1ml of 0.1M TBA and mixed thoroughly. The mixture was heated in a boiling water bath for 20minutes. Centrifuged at 1000g for 10mintes and the absorbance were read at 535nm against blank that contains all reagents minus homogenate. The MDA equivalent of the sample calculated using the extinction coefficient 1.56×10^5 μM/cm.

Calculation

$$\text{Concentration of MDA} = \frac{A}{E \times L}$$

Where A = O.D at 535nm

E = Extinction coefficient of MDA

L = Length of cuvette (1cm)

APPENDIX – XI
DETERMINATION OF INHIBITION OF SUPEROXIDE GENERATION
(Mc Cord and Fridovich,1995)

The extent of superoxide generation was studied on the basis of inhibition in the production of Nitro Blue Tetrazolium (NBT) formazon of the superoxide ion on the plant sample measured colorimetrically at 560nm.

Reagents

1. EDTA (0.1 M containing 1.5mg NaCN/100ml)
2. NBT (1.5mM)
3. 0.12mM riboflavin
4. 0.067M phosphate buffer, pH 7.8
5. Dimethylsulfoxide

Procedure

The assay tubes contained test sample (20mg concentration) with 0.2 ml of EDTA , 0.1 ml NBT, 0.05 ml riboflavin and 2.55 ml of phosphate buffer. The control tubes were also set up in DMSO and were added instead of sample.

All the tubes were vortexed and measured the initial optical density at 560nm. After that, these tubes were placed in an area where they received uniform illumination for 30minutes. Again the optical density was measured at 560nm. The difference in optical density before and after illumination is the quantum of superoxide production and the percentage of inhibition by the test sample was calculated by comparing with the optical density of control.

APPENDIX – XII

DETERMINATION OF INHIBITION OF NITRIC OXIDE GENERATION (Green and Hill, 1984)

Aqueous solution of sodium nitroprusside spontaneously generates nitric oxide (NO) at physiological pH, which interacts with oxygen to produce nitrite ion, which is measured colorimetrically.

Reagents

1. Phosphate buffered saline
2. Sodium nitroprusside (100mM)
3. Griess reagent (1% sulfanilamide, 2% H_3PO_4, 0.01% Naphthalene diamine dihydrochloride)

Procedure

3ml of reaction mixture containing sodium nitroprusside in PBS and extract was incubated at 25°C for 150 minutes. Controls were kept without test compound in an identical manner. After incubation, 0.5ml of Griess reagent was added. The absorbance of the chromopore formed was read at 546nm.

The percentage inhibition of nitric oxide generation was measured by comparing the absorbance values of control and those of test compounds.

APPENDIX – XIII

DPPH SCAVENGING ACTIVITY

(Mensor *et al.*, 2001)

Principle

DPPH radical reacts with an antioxidant compound, which can donate hydrogen and gets reduced. The change in colour from deep violet to yellow can be measured at 515nm.

Reagents

1. DPPH – 2,2'-diphenyl-2-picryl hydrazyl hydrate (0.3mM)
2. Methanol

Procedure

A methanolic solution of 0.3mM DPPH (0.5ml) was added to equal volumes of sample homogenate (20% homogenate was prepared in Tris EDTA buffer, pH 7.2) and allowed to react at room temperature. DPPH in methanol without plant extracts served as positive control. After 30minutes, the mixture was centrifuged and the absorbance of the supernatant was measured at 515nm and converted into percentage radical scavenging activity as follows.

$$\text{Scavenging activity (\%)} = 100 \quad \frac{A_{518}\,(\text{sample}) - A_{518}\,(\text{Blank})}{A_{518}\,(\text{Blank})} \times 100$$

APPENDIX - XIV

ABTS RADICAL SCAVENGING EFFECTS

(Shirwaikar *et al.*, 2006)

Shirwaikar *et al.*, (2006) have proposed an assay for the determination of antioxidant activity of compounds by their ability to scavenge the ABTS cation.

Reagents:

ABTS solution – 7mM ABTS in 2.45mM Ammonium Persulphate

100% Ethanol

Procedure:

ABTS radical cations ($ABTS^{+}$) were produced by reacting 7mM ABTS solution with 2.45mM Ammonium Persulphate. The mixture was allowed to stand in the dark at room temperature for 12 to 16 hours before use. 100µl of 7mM ABTS-APS solution was added to 100µl of the plant sample and the total volume was made up to 700µl with Ethanol. A mixture of 100µl of ABTS-APS solution and 600µl of Ethanol was taken as positive control while 1ml of

Ethanol serves as blank. The absorbance was read at 745 nm. The percent inhibition was calculated using the formula,

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

where,

A_0 - Absorbance of positive control

A_1 - Absorbance of the sample

APPENDIX-XV

HISTOPATHOLOGICAL TECHNIQUE

(Culling, 1979)

At the end of the treatment period, the liver of normal control, Streptozotocin induced diabetic control and extract treated diabetic rats, were removed and histopathological studies were carried out to reveal the Free radical scavenging effect of the the selected medicinal plant *Aristolochia bracteolata*. The following steps were followed in the histopathological techniques:

Autopsy bits were

1) Preserved in 10 per cent formalin solution for minimum one hour.
2) Dehydrated by 3 changes of acetone (each 500ml).
3) Cleaned from acetone by 3 changes of xylene (each 500ml) for about 3 hours.
4) Incubated in paraffin wax-2 changes for 3-4 hours in an incubator at 58 - 60°C.
5) After incubation in paraffin, the tissues were embedded in paraffin wax.
6) Cutting of the embedded bits to get sections (sections were 1 - 3µm thick)
7) Sections were taken on the glass slide.
8) Sections on glass slide were cleaned from wax by immersing in xylene.
9) Sections were histochemically reacted with haematoxylin and eosin staining to evaluate the morphology and cellular composition.