APPENDICES

APPENDIX - I

QUALITATIVE PHYTOCHEMICAL ANALYSIS

ALKALOIDS (Raaman, 2006)

Solvent free extract, 50mg of the plant sample was stirred with one ml of dilute hydrochloric acid and filtered. The filtrate was tested for alkaloids.

Mayer's Test: To the filtrate, a drop of Mayer's reagent was added along the sides of the test tube. A white precipitate indicates the test as positive.

FLAVONOIDS (Raaman, 2006)

Alkaline reagent test: Two ml of aqueous solution of the extract was treated with 1 ml of 10% ammonium hydroxide solution. Yellow fluorescence indicates the presence of flavonoids.

SAPONINS (Raaman, 2006)

50 mg of the plant sample was ground with 3 ml of distilled water and diluted with the same, madeup to 20ml. The suspension was shaken in a graduated cylinder. After 15 min, a two cm layer of foam indicates the presence of saponins.

PHENOLS (Raaman, 2006)

Ferric chloride test: 50mg of the sample was dissolved in 5ml of distilled water. To this, few drops of neutral 5% ferric chloride solution was added. A dark green colour indicates the presence of phenolic compounds.

GLYCOSIDES (Raaman, 2006)

50mg of the plant sample was hydrolysed with concentrated hydrochloric acid for 2 hours on a water bath, filtered and the hydrolysate was subjected to the following test.

Borntrager's test: From the filtered hydrolysate, 3ml of chloroform layer was separated and 2ml of 10% ammonia solution was added to it. Pink colour indicates the presence of glycosides.

REDUCING SUGAR (Iyengar, 1995)

To 0.5ml of the aqueous extract of the plant sample, 1ml of water and 5-8 drops of Fehling's solution was added at hot and observed for brick red precipitate.

TANNINS (Iyengar, 1995)

One ml of water and 1-2 drops of ferric chloride solution was added to 1 ml of aqueous extract of the plant sample. Blue colour was observed for gallic tannins and green black for catecholic tannins.

PHYTOSTEROLS (Siddiqui and Ali, 1997)

Libermann-Burchard reaction: 4mg of the plant sample was treated with 0.5 ml of acetic anhydride and 0.5ml of chloroform. Then concentrated sulphuric acid was added slowly and green bluish colour for steroids was observed.

TERPENOIDS (Siddiqui and Ali, 1997)

4 mg of the sample was treated with 0.5 ml of acetic anhydride and 0.5ml of chloroform. Concentrated sulphuric acid was added slowly along the sides of the test tube. Red violet colour was observed for terpenoids.

ANTHROQUINONE (Ayoola *et al.*, 2008)

0.5 g of the sample was boiled with 10ml of dilute sulphuric acid and filtered while hot. The filtrate was shaken with 5 ml of chloroform. The chlorofom layer was pipetted out into another test tube and one ml of dilute ammonia was added. The resulting solution was observed for colour changes.

APPENDIX-II

DETERMINATION of ALKALOIDS
(Harbone, 2005)

Two hundred ml of 10% acetic acid in ethanol was added to 5g of powdered sample, covered and allowed to stand for 4 hours. The filtrate was then concentrated on a water bath to $1/4^{th}$ of its original volume. Concentrated ammonium hydroxide was added dropwise to the extract until the precipitation was complete. The whole solution was allowed to settle; collected precipitate was washed with dilute ammonium hydroxide and then filtered. The residue was dried, weighed and expressed as alkaloids.

APPENDIX-III

ESTIMATION OF PHENOLS
(Malick and Singh, 1980)

Principle

Phenols react with phosphomolybdic acid in Folin-ciocalteau's reagent in alkaline medium and produce blue coloured complex (molybdenum blue).

Reagents

1. 80% ethanol
2. Folin – ciocalteau's reagent
3. 20% Sodium carbonate
4. Standard solution – 100mg catechol in 100ml water. Dilute 10 times for a working standard.

Procedure

Weighed exactly 0.5 to 1.0g of the sample and ground it with a pestle and mortar in 10-times volume of 80% ethanol. Centrifuged the homogenate at 10,000 rpm for 20 min. Saved the supernatant. Re-extracted the residue with five times the volume of 80% ethanol, centrifuged and pooled the supernatants. Evaporated the supernatant to drynesss. Dissolved the residue in a known volume of distilled water (5ml). Pipetted out different aliquots (0.2 to 2.0 ml) into test tubes. Madeup the volume in each tube to 3 ml with distilled water. 0.5 ml of Folin-Ciocalteau's reagent was added. After 3 min, 2 ml of 20% sodium carbonate solution was added to each tube. The tubes were mixed and kept in a boiling water bath for exactly one min, cooled and measured the absorbance at 650 nm against a reagent blank. A standard curve was prepared using different concentration of catechol (20-100µg).

Calculation

From the standard curve the concentration of phenols in the test sample was found and expressed as mg phenols/100g material.

APPENDIX-IV

ESTIMATION OF REDUCING SUGAR
Nelson-Somogyi method(Somogyi, 1952)

Principle

The reducing sugars when heated with alkaline copper tartarate reduce the copper from the cupric to cuprous state and thus cuprous oxide is formed. When cuprous oxide is treated with arsenomolybdic acid, the reduction of molybdic acid to molybdenum blue takes place. The blue colour developed is compared with a set of standards in a colorimeter at 620 nm.

Reagents

1. Alkaline copper tartarate: (A) Dissolve 2.5g anhydrous sodium carbonate, 2g sodium bicarbonate, 2.5g potassium sodium tartarate and 20g anhydrous sodium sulphate in 80 ml water and make up to 100ml.

(B) Dissolve 15g copper sulphate in a small volume of distilled water. Add one drop of sulphuric acid and make up to 100ml.

Mix 4ml of 'B' and 96 ml of 'A' solution before use.

2. Arsenomolybdate reagent: Dissolve 2.5 g ammonium molybdate in 45 ml water. Add 2.5ml sulphuric acid and mix well. Then add 0.3 g disodium hydrogen arsenate dissolved in 25ml water. Mix well and incubate at 37°C for 24 to 48 hours.
3. Standard Glucose solution:

   Stock solution: 100mg in 100 ml of distilled water (1mg/ml)

   Working solution: 10 ml of stock solution diluted to 100ml with distilled water (100µg/ml)

Procedure

Weighed 100mg of the sample and extract the sugars with hot 80% ethanol twice (5ml each time). The supernatant was collected and evaporated by keeping it on a water bath at 80°C. 10ml of water was added to it and the sugars are dissolved. Pipetted out aliquots of 0.1 and 0.2 ml into separate test tubes. Working standard solution 0.2-1 ml was pipette out into a series of test tubes. The volume in all the tubes was made up to 2ml with distilled water. 2ml of distilled water alone served as blank. Added 1ml of alkaline copper tartarate reagent to all the tubes and kept in a boiling water bath for 10 min. The tubes were cooled and 1ml of arsenomolybdic acid reagent was added to all the tubes. The volume in each tube was made up to 10ml with distilled water. Read the absorbance of blue color at 620 nm after 10 min. From the graph drawn, calculate the amount of reducing sugars present in the sample.

Calculation

Absorbance corresponds to 0.1ml of test = X mg of glucose

10 ml contains X / 0.1 x 10mg of glucose = % of reducing sugar.

APPENDIX-V
ESTIMATION OF TANNINS
Vanillin-hydrochloride method
(Robert, 1971)

Principle

The vanillin reagent will react with any phenol that has an unsubstituted resorcinol or phloroglucinol nucleus and forms a coloured substituted product which is measured at 500 nm.

Reagents

1. Vanillin hydrochloride reagent: Mix equal volumes of 8% hydrochloric acid in methanol and 4% vanillin in methanol. The solutions must be mixed just before use and avoid using even if it is slightly coloured.
2. Standard solution: Prepare standard catechin solution containing 1mg/ml methanol. Dilute this stock solution ten times, 10ml to 100ml (100µg/ml) concentration used as working standard solution.
3. Preparation of extract: 1 g of the ground sample in 50 ml methanol. Mix occasionally by swirling. After 20-28 h, centrifuge and collect the supernatant.

Procedure

Pipetted out 1ml of the supernatant. To this 5 ml of vanillin hydrochloride reagent was added and read in a spectrophotometer at 500nm after 20 min. Blank contained vanillin hydrochloride reagent alone. Standard graph was prepared with 20-100µg catechin from the working standard.

Calculation

From the standard graph, calculated the amount of catechin, i.e., tannins, in the sample as per the absorbance values and express the results as catechin equivalents.

APPENDIX-VI
ESTIMATION OF CHLOROPHYLL CONTENT
(Aron, 1949)

Principle

Chlorophyll was extracted in 80% acetone and the absorption at 663nm and 645nm were read in spectrophotometer using the absorption coefficients, the amount of chlorophyll was calculated.

Reagents

Analytical grade acetone was diluted to 80% (prechilled).

Procedure

Accurately weighed 1g of plant sample, was ground to a fine pulp with the addition of 20 ml of 80% acetone with a mortar and pestle. It was centrifuged (5000 rpm) for 5 min and the supernatant was transferred to 100ml volumetric flask. The residue was ground with 20 ml of 80% acetone, centrifuged and the supernatant was transferred to the same volumetric flask. This procedure was repeated until the residue becomes colourless. The mortar and pestle was washed thoroughly with 80% acetone and clean washing was collected in the volumetric flask. The volume was madeup to 100ml with 80% acetone. The absorbance of the solution was read at 645, 663 and 652 nm against the solvent (80% acetone) flask.

Calculation

The amount of chlorophyll present in the extract was calculated and expressed in mg chlorophyll/g tissue using the following equation:

1. mg chlorophyll 'a' /g tissue
   $= 12.7 \times (Absorbance_{663}) - 2.69 \times (Absorbance_{645}) \times V/ 1000 \times W$
2. mg chlorophyll 'b' /g tissue
   $= 22.9 \times (Absorbance_{645}) - 4.68 \times (Absorbance_{663}) \times V/ 1000 \times W$
3. mg total chlorophyll /g tissue
   $= 20.2 \times (Absorbance_{645}) + 8.02 \times (Absorbance_{663}) \times V/ 1000 \times W$

Where, A= Absorbance at specific wavelength

V= Final volume of chlorophyll extract in 80% acetone

W= Fresh weight of tissue extracted.

APPENDIX - VII
ESTIMATION OF CARBOHYDRATES
(Hedge and Hofreiter, 1962)

Principle

Carbohydrates are first hydrolyzed into simple sugar using dilute hydrochloric acid. In hot acidic medium glucose is dehydrated to hydroxyl furfural. This compound forms a green coloured product with anthrone, which has an absorption maximum at 630 nm.

Reagents

1. 2.5 N HCl
2. Anthrone reagent: Dissolved 200 mg anthrone in 100 ml of ice cold 95 % H_2SO_4 (prepared fresh before use)
3. Stock standard: Dissolved 100 mg of glucose in 100ml distilled water.
4. Working standard: 10 ml of stock solution is made up to 100 ml of distilled water.

Procedure

Weighed 100 mg of the sample in a boiling tube. Hydrolyzed by keeping it in water bath for 3 hours with 5ml of 2.5 N HCl and cooled to room temperature. Neutralized it with solid sodium carbonate until the effervescence ceases. Made up the aliquots for analysis. Prepared the standards by making 0, 0.2, 0.4, 0.6, 0.8 and 1.0 ml of the working standards. '0' served as the blank. Made up the volume to 1 ml in all test tubes including the sample tubes by adding distilled water. Then added 4 ml of anthrone reagent. Heated for eight minutes in boiling water bath. Cooled rapidly and read the green to dark green colour at 630 nm.

APPENDIX - VIII
ESTIMATION OF PROTEIN
(Lowry *et al.*, 1951)

Principle

The aminoacid tyrosine and tryptophan present in the protein will react with the Folin Ciocaltaeu reagent. By the reduction of phosphomolybdic acid phosphotungstic components it will produce blue colour. Also the colur developed by the biuret reaction of the protein with the alkaline cupric tartarate is measured in micro Lowry's method.

Reagents:

1. Solution A: 1 % copper sulphate
2. Solution B: 2% sodium potassium tartarate.
3. Solution C: 2% sodium carbonate in 0.1 N NaOH.
4. Solution D: mixed just before use, 1 ml of solution A, 1 ml of solution B and 100 ml of solution C.
5. Solution E: 1 N Folin Ciocalteau reagent (stored protected from light).

Procedure

Pipetted out 0.2, 0.4, 0.6, 0.8 and 1.0 ml of the working standard and known volume of the sample in duplicates to different tubes. Made up the volume to 1 ml with 0.1 N NaOH. Added 3 .0 ml of solution D, followed by 0.3 ml of solution E to each tube, mixed well and incubated for 3 minutes at 37°C. Read the colour developed at 750 nm against a reagent blank.

APPENDIX- IX
ESTIMATION OF CATALASE ACTIVITY
(Luck, 1974)

The enzyme catalase has a double function as it catalyzes the following reactions:

a) Decomposition of hydrogen peroxide (H_2O_2) to give water and oxygen.

$$2H_2O_2 \xrightarrow{\text{Catalase}} 2H_2O + O_2$$

b) Oxidation of 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.

Reagents

1. Phosphate buffer (0.067M PH 7.0): Dissolve 3.522g of KH_2PO_4 and 7.268g of $KHPO_4.2H_2O$ 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 1 cm 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 0.45-0.4. 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 –X
ESTIMATION OF GLUTATHIONE PEROXIDASE ACTIVITY
(Rotruck *et al.*, 1973)

Principle

A known amount of enzyme preparation was used to react with hydrogen peroxide in the presence of GSH for a specified time period when the screening was measured by the method of Ellman.

$$2GSH + H_2O_2 \xrightarrow{Se - GPx} GSSH + 2H_2O$$

Reagents

1. 0.4M Tris Buffer
2. 10 µM Sodium azide
3. 10 % TCA
4. 0.4 µM EDTA
5. 19 µM Hydrogen peroxide
6. 2µM Glutathione

Procedure

To 2ml of Tris buffer, 0.2 ml of EDTA ,0.1ml of sodium azide and 0.5 ml of plant extract were added followed by 0.1 ml hydrogen peroxide were added to the mixture , mixed well and incubated at 37° C for 10 minutes along with the tube containing all the reagent except sample. After 10 min the reaction was arrested by the addition of 0.5ml of 10% TCA centrifuged and supernatant was assayed for glutathione by the method of Ellman.

The activities are expressed as µg GSH consumed / min / mg protein.

APPENDIX – XI

ESTIMATION OF GLUTATHIONE -S-TRANSFERASE ACTIVITY

(Beutler, 1984)

Principle

Glutathione-s-transferase catalyses the reaction of 1-chloro-2,4 dinitrobenzene (CDNB) with the SH group of glutathione.

CDNB + GSH ⟶ CDNB-S-glutathione

Reagents

1. 25mM - Chloro 2,4-dinitrobenzene (CDNB) in 95% ethanol
2. 20mM - Glutathione
3. 0.5M Phosphate buffer, pH 6.5
4. 50mM Tris-HCl buffer, pH 7.2
5. 0.4M Sorbitol
6. 10mM Sodium chloride

Procedure

Enzyme extract

5g of the sample was homogenized with 50mM Tris–HCL buffer (pH7.2), 0.4M sorbitol and 10mM sodium chloride and made up to 20 ml. The homogenate was centrifuged at 2000g for 10 minutes and used the supernatant for the assay.

Assay

0.5 ml of phosphate buffer was taken in a test tube and 0.1 ml of CDNB was added. Added 8.8 ml of distilled water, incubated tubes at 37°C for 10 min. Then 0.5 ml of glutathione was added to 0.2 ml of enzyme extract to the reaction mixture. Run a blank like test except adding enzyme. Measured the absorbance at 340 nm for every 30 sec for 3 min.

Calculation

Glutathione-s-transferase activity in the extract is expressed as µmoles of CDNB-GSH conjugate/ min/ mg protein.

APPENDIX- XII

ESTIMATION OF PEROXIDASE ACTIVITY

(Reddy *et al.*, 1995)

Principle

Peroxidase converts hydrogen peroxide 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 colorimetrically.

Pyrogallol + H_2O_2 ⟶ Oxidized pyrogallol + H_2O

Reagents

1. Pyrogallol(0.05M); Phoshphate buffer (pH 6.5): 630 mg of pyrogallol in 100ml of 0.1M Phosphate buffer.
2. Hydrogen peroxide (1%)

Enzyme extract

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

Procedure

Pipetted out 3ml of 0.05 M pyrogallol solution and 0.5 to 1.0 ml of enzyme extract in a test tube. Adjusted the spectrophotometer to read '0' at 400 nm. Added 0.5 ml of 1% hydrogen peroxide in the test cuvette. Recorded the change in the absorbance every 30 seconds upto 3 minutes.

Calculation

Change in absorbance / min	= X
Weight of the plant material taken	= 300 mg
Volume of the extract taken for the assay	= 0.02 ml
Change in absorbance for 1.5 ml extract	= (X / 0.02) x 1.5 – Y
(i.e) Peroxidase activity in 300 mg plant tissue	= Y
Peroxidase activity / g plant tissue	= Yx (1000/300) Units

APPENDIX – XIII
ESTIMATION OF POLYPHENOL OXIDASE ACTIVITY
(Esterbauer *et al.*, 1977)

Principle

Polyphenol oxidases are copper proteins of wide occurrence in nature, which catalases the aerobic oxidation of certain phenolic substrate to quinines, which are auto oxidized to dark brown pigments generally known as melanins. The polyphenol oxidases (PPO) comprise catechol oxidase and laccase. One unit of either catechol oxidase or laccase is defined as the amount of enzymes that transforms 1µ mole of quinine per minute under the assay conditions.

Reagents

1. 50mM Tris- HCl (pH 7.2)
2. 0.4 M Sorbitol
3. 10mM sodium chloride
4. 0.1 M Phosphate buffer (pH 6.5)
5. 0.01M Catechol solution

Procedure

Enzyme extract

Ground about 5g of the plant tissue and made up to 20ml with the medium containing 50mM Tris-HCl (pH 7.2), 0.4 M sorbitol and 10mM sodium chloride. Centrifuged the homogenate at 2000 rpm for 10 min and used the supernatant for the assay.

Assay

Added 2.5 ml of 0.1 M phosphate buffer (pH 6.5), 0.3 ml of catechol solution (0.01M) into the cuvette and set the spectrophotometer at 495nm. Now added the enzyme extract (0.2 ml) and started recording the change in absorbance for every 30 seconds upto 5 minutes.

Calculation

Enzyme units in the test = K * (Δ / min)

K for catechol oxidase = 0.272 ; K for laccase = 0.242.

APPENDIX -XIV
ESTIMATION OF SUPEROXIDE DISMUTASE ACTIVITY
(Misra and Fridovich, 1972)

Principle

Superoxide dismutase uses the photochemical reduction of riboflavin as oxygen generating systems and catalyses the inhibition of Nitro Blue Tetrozolium (NBT) reduction, the extent of which can be assayed spectrophotometrically at 600nm.

Reagents

1. 50mM Potassium phosphate buffer, (pH 7.4)
2. 45µM Methionine
3. 5.3µM Riboflavin

4. 84µM Nitro Blue Tetrozolium (NBT)
5. 20mM Potassium cyanide

Procedure

The incubation medium contained a 300µl of each reagent (50mM Potassium phosphater buffer (pH 7.8), 45mM Methionine, 5.3mM Riboflavin, 84mM Nitro Blue Tetrozolium (NBT), and 20mM Potassium cyanide. To the test 300µl of sample was added. The final volume was made upto 3ml with water. The tubes were placed in an aluminum Foil lined box maintained at 25°C and equipped with 15W fluorescent lamps. Reduced NBT was measured spectrophtomerically at 600nm after exposure to light for 10 minutes. The maximum reduction was evaluated in the absence of enzyme giving 50% inhibition of the reduction of NBT.

APPENDIX – XV

EXTRACTION AND ESTIMATION OF CAROTENOIDS

(Zakaria *et al.*, 1979)

Principle

The total carotenoids in the sample were extracted in petroleum ether. The total carotenoids were estimated in a UV/Visible spectrophotometer at 450nm.

Reagents

1. Petroleum ether
2. Anhydrous sodium sulphate
3. Calcium carbonate
4. 12% alcoholic potassium hydroxide

Procedure

Weighed 5-10g of the sample. Saponified for about 30 minutes in a shaking water bath at 37°C after extracting the sample in 12% alcoholic KOH. Transferred the saponified extract into a separating funnel packed with glass wool and $CaCO_3$ containing 10 to 15ml of petroleum ether layer. Transferred the lower aqueous phase to another separating funnel and the petroleum ether extract containing the carotenoid pigments to amber coloured bottle. Repeated the extraction of the aqueous phase. To the petroleum ether extract added a small quantity of anhydrous Na_2SO_4 to remove the turbidity. Noted the final volume of the petroleum ether extract and diluted if needed by a known dilution factor.

The absorbance of the extract at 450nm and 503nm was noted in a spectrophotometer.

$$\text{Amount of total carotenoids (µg/g)} = \frac{\text{Absorbance}_{450} \times 4 \times \text{Volume of extract}}{\text{Weight of leaves (g)}}$$

APPENDIX – XVI

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 sulphuric acid to give an orange coloured solution whose absorbance can be measured spectrophotomerically at 540nm.

Reagents

1. 4%TCA
2. 9N 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% sulphuric acid
6. Stock standard solution: Dissolved 100mg of ascorbic acid in 100ml of 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 10 minutes. 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 2.0ml with 4%TCA. 0.2 to 1.0ml of the working standard solution containing 20-100 µg of ascorbate respectively were pipetted out into clean dry test tube, the volume of which were also made up to 2.0ml with 4%TCA. Added 0.5ml of DNPH reagent to all the test tubes, followed by 2 drops of 10% thiourea solution. Incubated at 37°C for 3 hours.

The osazones formed were dissolved in 2.5ml 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 – XVII
ESTIMATION OF α-TOCOPHEROL
(Emmerie-Engel method, 1938 as described by Rosenberg, 1992)

Principle

Tocopherol can be estimated using Emmerie – Engel 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/ 10ml of α-tocopherol in absolute alcohol 91mg of œ- tocopherol is equivalent to 100mg of tocopherol acetate.

Procedure

The sample was homogenized with water in a blender. Weighed accurately 2.5g of the homogenized sample into a conical flask. Added 50ml 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.

Into 3 stoppered centrifuge tubes (test, standard and blank), pipetteed out 1.5ml of extract, 1.5ml of standard, 1.5ml of water respectively. To the test and blank added 1.5ml of ethanol and to the standard added 1.5ml of water. Added 1.5ml xylene to all the test tubes, stoppered, mixed well and centrifuged. Transferred 1.0ml of xylene layer into another stoppered tube, taking care not to include any other ethanol or protein. Added 1.0ml of 2, 2'- dipyridyl reagent to each tube, stoppered and mixed. Pipetted out 1.5ml 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 0.33ml of ferric chloride solution. The amount of vitamin E can be calculated using the formula,

$$\text{Amount of tocopherols } (\mu g) = \frac{\text{Reading at 520nm} - \text{Reading at 460nm}}{\text{Reading of standard at 520nm}} \times 0.24 \times 15$$

APPENDIX – XVIII
ESTIMATION OF FLAVONOIDS
(Zhishen *et al.*, 1999)

Reagents

1. 5% Sodium nitrite
2. 10% Aluminium chloride
3. 1 mM Sodium hydroxide
4. Standard solution: 0.011g of catechin dissolved in 100 ml water (110µg/ml)

Procedure

0.1 ml of methanolic extract of plant sample was added to 0.3 ml of distilled water. To this 0.03 ml of 5% sodium nitrate was added to the tubes and incubated for 5 min at 25°C. After incubation, 0.03 ml of 10% aluminium chloride was added and again incubated for 5 min. To this 1mM sodium hydroxide (0.2ml) was added and made up to 1 ml with distilled water. The absorbance readings at 510 nm was noted. The final absorbance of each sample was compared with a standard curve made from catechin. From the standard graph, the amount of flavonoids present in the sample was calculated.

APPENDIX –XIX
ESTIMATION OF POLYPHENOLS
(Malick and Singh, 1980)

Principle

Phenols react with phosphomolybdic acid in Folin - ciocalteau reagent in alkaline medium and produce blue coloured complex (molybdenum blue), which is read in a spectrometer at 650nm.

Reagents

1. 80% ethanol
2. Diluted Folin - Ciocalteau reagent
3. 20% sodium carbonate
4. Stock solution: Dissolved 100mg of catechol is made up with 100ml distilled water
5. Working standard: 10ml of stock standard was diluted to 100ml. 1.0ml of this contains 100µg of catechol.

Procedure

1g of sample was homogenized using 20ml of 80% ethanol. The homogenate was centrifuged at 10,000rpm for 20 minutes. The supernatant was saved. The residue was reextracted with 10ml of 80% ethanol, centrifuged and collected the supernatant and evaporated to dryness. The residue was dissolved in a known volume of distilled water (50ml) and 2.0ml was taken for the experiment. A working standard of 0.5 – 2.5ml catechol solution corresponding to 50 - 250µg of catechol were pipetted out into a series of test tubes. The volume was made upto 2.5ml with water. To all the tubes added 0.5ml of diluted Folin – ciocalteau reagent. After 3 minutes, added 2.0ml of 20% Na_2CO_3 solution to each tube and mixed thoroughly.

The tubes were placed in a boiling water bath for exactly one minute. Cooled and measured at 650nm against a reagent blank. Constructed a standard graph by plotting the concentration of catechol on X-axis and absorbance on Y-axis. From the graph, the amount of polyphenols present in the sample was estimated and expressed as mg of polyphenols per g of the sample.

APPENDIX – XX
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 solution (Ellman's reagent)-0.6 mM DTNB in 0.2M Posphate buffer
2. 5%TCA
3. 0.2M Sodium phosphate buffer, pH 8.0
4. Standard solution: Dissolved 20 mg of reduced glutathione in 100ml of water

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 0.1ml of supernatant was taken for the estimation. The volume of the aliquot was made up to 1.0ml with 0.2M sodium phosphate buffer (pH 8.0), 2ml 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 10 minutes.

A standard curve of GSH was prepared using concentration ranging from 2 to 10 nmoles of GSH in 5%TCA.

APPENDIX-XXI
ABTS RADICAL SCAVENGING ACTIVITY
(Shirwaiker *et al.*, 2006)

Principle

This assay is used for the determination of antioxidant activity of compounds by their ability to scavenge ABTS cation. In this decolourisation assay, ABTS, the oxidant is generated by persulphate oxidation of 2,2-azinobis (3-ethyl benzoline-6-sulphonic acid) ($ABTS^{\cdot}$), based on the inhibition of the absorbance of the radical cation $ABTS^{\cdot+}$ which has a characteristic long wavelength absorption spectrum.

Reagents

ABTS solution: 7mM with 2.4 mM ammonium persulphate

Procedure

ABTS radical cations ($ABTS^{+}$) were produced by reacting ABTS solution with 2.45 mM ammonium persulphate. The mixture was allowed to stand in the dark at room temperature for 12-16 hours before use. 0.5 ml of the extracts of the plant was added to 0.3 ml of ABTS solution and the final volume was made up to 1ml with ethanol. The absorbance was read at 745nm and the percent inhibition was calculated using the formula,

$$\text{Inhibition (\%)} = \frac{\text{Control - Test}}{\text{Control}} \times 100$$

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

Procedure

A methanolic solution of 0.3 mM DPPH (0.5ml) was added to equal volume 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 extract served as positive control. After 30 minutes, the mixture was centrifuged and the absorbance of the supernatant was measured at 515 nm and converted into percentage radical scavenging activity as follows:

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

APPENDIX – XXIII
HYDROGEN PEROXIDE SCAVENGING ACTIVITY
(Ruch *et al.*, 1989)

The ability of the plant extract to scavenge H_2O_2 was determined according to the method of Ruch *et al.*(1989). A solution of H_2O_2 (4mM) was prepared in phosphate buffer (pH 7.4) concentration was determined spectrophotometrically from its absorption at 230 nm was determined after 10 minutes against a blank solution containing phosphate buffer without H_2O_2.

The scavenging activity of H_2O_2 by plant extract and the standard compounds was calculated using the formula,

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

Where, A_0 = Absorbance of control

A_1 = Absorbance in presence of sample and standards.

APPENDIX – XXIV
HYDROXYL ION SCAVENING ACTIVITY
(Elizabethbeth and Rao, 1990)

Principle

The hydroxyl radical scavenging activity was measured by studying the inhibition between deoxyribose and the extract for OH radical generation with Fe^{3+} / ascorbate , EDTA / H_2O_2 system. The OH radical attack deoxyribose which eventually result in TBARS formation. TBARS is formed when the hydroxyl radicals attack deoxyribose, which can be quantified spectrophotometrically.

Reagents

1. Deoxyribose (2.8mM)
2. Ferric chloride (0.1mM)
3. EDTA (0.1 mM)

4. Hydrogen peroxide (1mM)
5. Ascorbate (0.1 mM)
6. Potassium dihydrogen phosphate - Potassium hydroxide buffer (20mM, pH 7.4)
7. TBA (1%)
8. HCl (25%)

Procedure

The reaction mixture contained deoxyribose (2.8mM), ferric chloride (0.1mM), EDTA (0.1 mM) and Potassium dihydrogen phosphate-potassium hydroxide buffer (20 mM, pH 7.4). 20 µl of the sample was added such that the final volume was 1 ml. The reaction mixture was incubated for 1 hr at 37°C. Deoxyribose degradation was measured as TBARS by adding 0.5ml of TBA and 0.5 ml of HCl, boiled in a water bath for 20 min, cooled and measured the absorbance at 532 nm.

APPENDIX – XXV
FERROUS ION CHELATING ACTIVIY
(Carter, 1971)

The ferrous ion chelating (FIC) activity was measured by the decrease in absorbance at 562 nm of the iron (II)-ferrozine complex. One ml of 0.125mM ferrous sulphate, was added to 1.0 ml of sample with different dilutions, followed by 1.0 ml of 0.3125mM ferrozine. The mixture was allowed to equilibrate for 10 min before measuring the absorbance. Sample solutions with appropriate dilutions were used as blanks as the extract may also absorb at this wavelength. The ability of the sample to chelate ferrous ion was calculated relative to the control (consisting of iron and ferrozine only), using the formula,

$$\text{Chelating activity (\%)} = \frac{\text{Control - Test}}{\text{Control}} \times 100$$

APPENDIX – XXVI
DETERMINATION OF INHIBITION OF LIPID PEROXIDATION IN GOAT LIVER HOMOGENATE
(Okhawa *et al.*, 1979)

Goat liver was procured fresh from a slaughter house and washed free of blood using Tris-HCl buffer (40mM, pH 7). A 20% homogenate of the liver was prepared in the same buffer using a mortar and pestle. The homogenate was clarified to remove debris and used as the membrane source for the induction of lipid peroxidation, mediated by $FeSO_4$, as a prooxidant, application of the relevant plant tissue extract in the medium was tried with an object of assessing the extent of inhibition of lipid peroxidation.

Reagents

1. Ferrous sulphate (10mM)
2. Thiobarbituric acid (1%)
3. TBS-10mM Tris, 0.15 M NaCl, pH 7.4
4. Ethanol (70%)
5. Acetone

Procedure

The reaction mixture 0.5 ml containing 0.1 ml each of liver homogenate, plant extract (50µl) and TBS to make a final volume to 500µl. A blank containing no lipid source but only ferrous sulphate, no plant extrat and TBS to final volume of 0.5ml was prepared.

As assay medium corresponding to 100% oxidation was prepared by adding all the other constituents except the plant extract. The experimental medium corresponds to autooxidation contained only of liver homogenate. Tubes are incubated at 37° C for 1 hr. After incubation, 0.5 ml of 70% ethanol was added to all tubes to arrest the reaction. 1ml of 1% TBA was added to all the tubes. The tubes were then incubated in a boiling water bath for 20 minutes. After cooling to room temperature, added with 0.5 ml of acetone. The intensity of pink colour produced is measured at 535 nm in a spectrophotometer.

APPENDIX – XXVII
DETREMINATION OF SUPEROXIDE PRODUCTION *INVITRO*
(Mc Cord and Fridovich, 1968)

Principle

The extent of superoxide generation was studied on the basis of inhibition in the production of superoxide ion by the plant sample,which was measured colorimetrically at 560nm.

Reagents

1. ETDA (0.1M containing 1.5mgNaCN/100ml)
2. NBT (1.5mM)
3. 0.12mM riboflavin
4. 0.067M phosphate buffer,(pH7.8)

Procedure

The assay tubes contained test sample (20mg concentration) with 0.2ml of EDTA, 0.1ml NBT, 0.05ml riboflavin and 2.55ml of phosphate buffer. The control tubes were also set up in DMSO and were added instead of sample. The initial optical density of the solution were recorded at 560nm. After that, these tubes were placed in an area where they received uniform illumination for 30 minutes. 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 – XXVIII
DETERMINATION OF NITRICOXIDE PRODUCTION *INVITRO*
(Green and Hill, 1984)

Principle

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 buffer 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 – XXIX

BRINE SHRIMP LETHALITY ASSAY

(Zakaria *et al.*, 2007)

Brine shrimp nauplii (*Artemia salina*) were obtained by hatching brine shrimp eggs in artificial sea water (3.8% noniodized sodium chloride solution) for 48 hours. 500µl of the plant extracts of different concentration were added to 4.5ml of brine solution with ten nauplii for each extracts in vials. These vials were maintained at room temperature for 24 hours under the light and surving larvae were counted using a magnifying lens. Experiments were conducted along with potassium dichromate as positive control. The mortality concentration data was calculated by the formula,

$$\text{Percentage Mortality} = \frac{\text{No. dead nauplii}}{\text{Total no. of subjects}} \times 100$$

LC_{50} values were obtained by best-fit line plotted concentration versus percentage lethality.

APPENDIX – XXX

ANTIBACTERIAL ACTIVITY

AGAR WELL DIFFUSION METHOD

(NCCLS, 1997)

Agar plates were prepared using sterile Muller-Hinton (MH) agar medium. Selected bacterial strains of 24 hour culture were evenly spread onto the surface of the agar plates using sterile swab sticks. Wells were cut into agar with sterile gel puncture. Twenty microlitres of different plant extracts in the concentration (20mg/ml) were added in the wells. DMSO per well served as negative control and chloramphenicol disc was served as positive control. The plates were incubated at 37°C for 24 hours and observed for zones of inhibition was measured and the antibacterial acitivity expressed in terms of the mean diameter of the zone inhibition in millimeters. The absence of a zone inhibition was inferred as the absence of activity. Each extract was tested in triplicates.