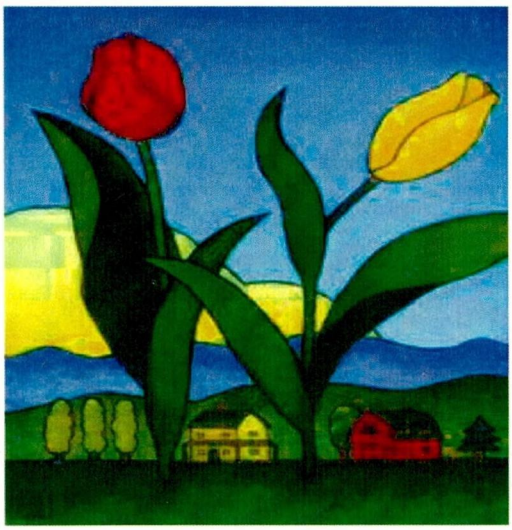

APPENDICES

APPENDIX - I

ANALYSIS OF PHYTOCHEMICALS

ALKALOIDS (Raaman, 2006)

50 mg of solvent free extract 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 indicated the presence of flavonoids.

SAPONINS (Raaman, 2006)

50 mg of the plant extract 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 indicated 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 extract 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.

CARBOHYDRATES (Iyengar, 1995)

To 0.5ml of the 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 extract of the plant sample. Blue colour was observed for gallic tannins and green black for catecholic tannins.

STEROIDS (Siddiqui and Ali, 1997)

Libermann- Burchard reaction: 4mg of the plant extract 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 extract 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.

ANTHRAQUINONES (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 5mo of chloroform. The chloroform layer was pipette out into another test tube and 1 ml of dilute ammonia was added. The resulting solution was observed for colour changes.

APPENDIX – II

TOTAL ANTIOXIDANT ACTIVITY

(Prieto *et al.*, 1999)

PRINCIPLE

The phosphomolybdenum method was used to evaluate the total antioxidant activity of the plant extract. Antioxidants can reduce Mo (IV) to Mo (V) and the green phosphate Mo (V) compounds at acidic pH, which have an absorption peak at 695 nm, were generated subsequently.

REAGENTS

- ❖ 0.6 M Sulphuric acid
- ❖ 28 mM sodium phosphate
- ❖ 4 mM ammonium molybdate

PROCEDURE

0.3 ml of the sample was mixed with 3.0 ml of the reagent solution. Reaction mixture was incubated at 95^0 C for 90 min under water bath. Absorbance of all the mixtures was measured at 695 nm. Total antioxidant activity is expressed as the number of equivalents of ascorbic acid in milligrams per gram of extract.

Total antioxidant activity = $100 [1- (A_o-A_t) / (A_o^o - A_t^o)]$

Where A_o is the OD of the sample at time t_o minutes and A_t is the time of the sample at time t = 90minutes. A_0^o and A_t^o represent the OD of the control at time t = 0 minutes and t = 90 minutes respectively.

APPENDIX - III

ESTIMATION OF TOTAL PHENOLS

(Malick and Singh, 1980)

PRINCIPLE

Phenols react with phosphomolybdic acid in Folin-Ciocalteau reagent in alkaline medium and produce a blue colored complex (molybdenum blue) that can be estimated calorimetrically.

REAGENTS

- 80% ethanol
- Folin- Ciocalteau reagent
- 20% sodium carbonate
- Stock standard solution – Dissolved 100mg of catechol in 100ml of distilled water in a standard flask.
- Working standard solution- Diluted 10ml of the stock solution to 100ml of distilled water. 1ml of this solution contains 100µg of catechol.

PROCEDURE

Weighed exactly 0.5 to 1.0 g of the plant sample and ground it with a mortar and pestle and in 10X volume of 80% ethanol. Centrifuged the homogenate at 10,000 rpm for 20 minutes. Saved the supernatant. Re- extracted the residue with five times the volume of 80% ethanol, centrifuged and pooled the supernatants. Evaporated the supernatants to dryness. Dissolved the residue in a known volume of distilled water. Pippeted out different aliquots (0.2 to 2.0ml) into test tubes. Made up the volume in each tube to 3.0ml with water. Added 0.5ml of Folin Ciocalteau reagent. After 3 minutes added 2.0ml of 20% sodium carbonate solution to each tube. Mixed thoroughly, placed the tubes in boiling water bath for exactly 1 minute, cooled and measured the absorbance at 650nm against reagent blank.

APPENDIX-IV

TOTAL FLAVONOIDS

(Zhiensen *et al*, 1999)

PRINCIPLE

Total flavonoid react with phosphomolybdic acid in Folin-Ciocalteau reagent in alkaline medium and produce a blue colored complex (molybdenum blue) that can be estimated calorimetrically.

REAGENTS

- Sodium nitrate (15%)
- Aluminium chloride (10%)
- Sodium hydroxide (4%)
- Stock standard solution – Dissolved 100mg of catechin in 100ml of distilled water in a standard flask
- Working standard solution- Diluted 10ml of the stock solution to 100ml of distilled water. 1ml of this solution contains 100 µg of catechin.

PROCEDURE

Sample (0.5ml) mixed with 2ml of distilled water and subsequently with 0.15ml $NaNO_2$ solution (15%). After 6 min, 0.15ml of $AlCl_3$ solution (10%) was added and allowed to stand for 6 min, then 2ml NaOH solution (4%) was added to the mixture. Immediately water was added to bring the final volume to 5ml and the mixture was thoroughly mixed and was allowed to stand for another 15 min. Absorbance was then determined at 510nm against the prepared water blank. Results were expressed as catechin equavlents (mg catechin/ g extract).

APPENDIX-V

ESTIMATION OF PROTEIN

(Lowry *et al.*, 1951)

PRINCIPLE

The blue color developed by the reduction of the phosphomolybdic – phosphotungstic components in the Folin-Ciocalteau reagent by the amino acids tyrosine and tryptophan present in the protein, the colour developed by the biuret of the protein with the Alkaline cupric tartarate are measured in the Lowry's method.

REAGENTS

- Solution A : 1% Copper sulphate
- Solution B : 2% Sodium potassium tartarate
- Solution C : 2% Sodium carbonate in 0.1 NaOH
- Solution D : Mixed just before use.1ml of solution A, 1ml of solution B, 100ml of solution C
- Solution E : 1N Folin-Ciocalteau reagent (mixed equal volumes of commercially available reagent and distilled water just before use)
- Standard BSA: 50mg of Bovine serum albumin in 50ml (stock). Diluted 1:10 for working standard.

PROCEDURE

Aliquots of standard protein solution (20-100µg), 0.1ml of liver homogenate were made up to 1ml with 0.1N NaOH and shook well to treat the protein with alkali. Added 3ml of solution D, mixed well and incubated at 37^0C for 3minutes. Added 0.3ml of solution E to each tube, mixed well and incubated further for 3minutes at 37^0C. read the color developed at 750nm against reagent blank.

Construct a standard curve and read of the concentration of protein in the aliquot taken. Calculated the concentration of protein in samples from the calibrated standard curve.

CALCULATIONS

Express the concentration of protein mg/g or 100g sample.

APPENDIX- VI

ESTIMATION OF ALBUMIN

(Rasanayagam *et al.*, 1986)

Albumin was estimation by dye finding method, using bromocresol green.

PRINCIPLE

Albumin in a buffer and medium finds with bromocresol green (BCG) and produce a green color whose absorbance in proportional to the albumin concentration.

REAGENTS

- Buffered dye reagent
  - Bromocresol green, buffer, pH3.8 preparative, surfactant.
- Albumin standard
  - 4g/ dl BSA (Bovine Serum Albumin)

PROCEDURE

Take 3 clean dry test tubes labeled as Blank (B), standard(S), test (T). Pipetted out 0.01ml serum into T and 0.01ml albumin standard s. To all these tubes added 2ml distilled H2O and 1ml of buffered dry reagent. Mixed well and measured immediately the absorbance of standard (S), and test (T) against Blank (B) on Photocolorimeter at 630nm.

CALCULATION

OD of T * Concentrated of Standard *100 / OD of Standard * Volume of Test *1000

APPENDIX -VII

ESTIMATION OF BILIRUBIN

Dangerfield and Finlayson (1953)

PRINCIPLE

Bilirubin reacts with diazonized sulfanilic acid in acidic medium to form azobilirubin, a purple colored complexes whose absorbance is propotional to bilirubin concentration. Direct Bilirubin, being water soluble is allowed to react with diazotized sulfanilic acid in the absence of an activator, while for total Bilirubin (Direct and Indirect) the diazotization is carried out in the presence of an activator.

REAGENTS

- Diazo A
- Diazo B
- Activator
- Artificial Standard (10mg %)

PROCEDURE

Taken 4 clean dry test tubes labeled as T_1, T_2 (total bilirubin) and D1, D2 (Direct bilirubin) and added 1.0ml of Diazo A and 0.1ml of Diazo B to T_1 and D_1. Added 1.0 ml of activator and made up the volume to 4.6 with distilled water, 0.2ml of serum was added to all the test tubes. Mixed well and the absorbance of D_1 and D_2 was measured exactly after 1 min at 540 nm against water. The test tubes T_1 and T_2 was kept in dark at room temperature for 5 min and read the absorbance at 540nm.

CALCULATIONS:

$$\text{Total Bilirubin in mg\%} = \frac{\text{A of } (T_1) - \text{A of } (T_2)}{\text{A of Standard}} \times 10$$

$$\text{Direct Bilirubin in mg\%} = \frac{\text{A of (D1)} - \text{A of (D2)}}{\text{A of Standard}} \times 10$$

APPENDIX – VIII

ESTIMATION OF TOTAL CHOLESTEROL

CHOD-PAP METHOD

(Allain *et al.*, 1974)

PRINCIPLE:

Cholesterol esterase (CHE) hydrolyses cholesterol ester to free cholesterol is oxidized by the Cholesterol oxidase (CHOD) to 4-cholestenone and hydrogen peroxide, hydrogen peroxide formed reacts with 4-amino antipyrine and phenol in presence of peroxidase to produce pink colored compound called quinonimine dye.

$$\text{Cholesterol esters} + H_2O \xrightarrow{\text{CHE}} \text{Cholesterol} + \text{fatty acid}$$

$$\text{Cholesterol} + O_2 \xrightarrow{\text{CHOD}} \text{4 -cholestenone} + H_2O_2$$

$$2H_2O_2 + \text{phenol} + 4\text{AP} \xrightarrow{\text{POD}} \text{Quinonimine} + 4H_2O$$

The intensity of the color formed is proportional to cholesterol concentration in the sample.

REAGENT:

Cholesterol standard: 200 mg/dl.

PROCEDURE:

Pipetted out into a clean dry test tube 1ml of cholesterol reagent and 20 µl of serum sample. Standards were prepared by adding 1ml of reagent and 20 µl of cholesterol standard. Mixed well and incubate at 37° C for 10 minutes. The absorbance of the samples and calibrator, were measured against the blank at 505 nm.

CALCULATION

$$\text{Total Cholesterol (mg/dl)} = \frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times 200$$

APPENDIX - IX

ESTIMATION OF HDL CHOLESTEROL

KIT METHOD

(Burstein *et al.*, 1970)

PRINCIPLE:

Chylomicrons, LDL and VLDL (low and very low density lipoproteins) are precipitated from serum by phosphotungstate in the presence of divalent cations such as magnesium. The HDL cholesterol remains unaffected in the supernatant and is estimated using cholesterol reagent.

$$\text{Serum / Plasma Chylomicrons)} \xrightarrow[Mg^{2+}]{\text{Phosphotungstate}} \underset{\text{(supernatant)}}{\text{HDL}} + \underset{\text{(Precipitate)}}{\text{(LDL + VLDL +}}$$

REAGENTS

- ❖ Cholesterol Reagent
- ❖ HDL Cholesterol Standard (25 mg/dl)
- ❖ Precipitating reagent

PROCEDURE:

STEP-I

PRECIPITATION OF LDL

Pipetted into a clean dry centrifuge tube 0.25 ml of serum and 0.5 ml of precipitating reagent. Mixed well and allowed to stand at room temperature for 10 minutes. Centrifuge for 10 minutes at 4000 rpm and separate clear supernatant. Use the supernatant for HDL-Cholesterol estimation.

STEP – II

ASSAY OF HDL CHOLESTEROL

Pipetted in a clean dry test tubes labeled blank (B), standard (S) and test for HDL-Cholesterol (T), 1.0ml of Cholesterol reagent. 0.05 ml of the supernatant from step- I was added to test (T). Mixed well. Incubated at 37°C for 10 minutes or (30°C) for 12minutes. The absorbance of the sample and calibrator were measured against the blank at 505nm.

CALCULATION

$$\text{HDL Cholesterol concentration (mg/dL)} = \frac{\text{Absorbance of Test}}{\text{Absorbance of standard}} \times 25 \times \text{dilution Factor}$$

APPENDIX – X

ESTIMATION OF LDL–C

Kit method (Rifai & Warnick, 1994)

PRINCIPLE

The LDL-C direct is a Homogeneous assay. When serum is mixed with R1, amphoteric surfactants protect LDL from enzyme reactions. CHE and CO reacts with non-LDL cholesterol, which is decomposed to water by catalase. R2 enables the conversion of LDL-C to hydrogen peroxide, which upon Oxidative condensation with HAD OS and 4AA yields a color complex. By measuring the absorbance of this blue colour complex produced, the LDL-C concentration in the sample can be calculated when compared with the absorbance of the LDL-C Calibrator.

REAGENTS:

- LDL-C Direct R1
- LDL-C Direct R2
- LDL-C Direct Calibrator

PROCEDURE:

In the clean test tube marked test added 0.5 ml of Reagent 1 and 0.005 ml of serum sample and in the another tube named calibrator added 0.005 ml of calibrator to 0.5 ml of Reagent1 and mixed well and incubated for 5 minutes at 370C. Then 0.15ml of Reagent 2 was added to both the tubes and mixed well and incubated for 5 minutes at 370C. The absorbance of calibrator and sample was measured at 600nm.

CALCULATION:

$$\text{LDL-C Concentration (mg/dl)} = \frac{\text{Absorbance of Sample}}{\text{Absorbance of Calibrator}} \times \text{Calibrator Concentration}$$

APPENDIX - XI

ESTIMATION OF TRIGLYCERIDES

KIT METHOD (Trinder, 1969)

PRINCIPLE:

Sample triglyceride incubated with Lipoprotein Lipase (LPL), liberate glycerol and free fatty acids glycerol is converted to Glycerol-3-Phosphate (G_3P) and Adenosine-5-diphosphate (ADP) by glycerol kinase and ATP. Glycerol-3-Phosphate (G_3P) is then converted by glycerol phosphate dehydrogenase (GPO) to Dihydroxy Acetone Phosphate (DAP) and hydrogen peroxide (H_2O_2). In the last reaction, hydrogen peroxide reacts with 4-amino phenazone (4-AP) and p-chloroform in presence of peroxidase (POD) to give a red colored dye.

$$\text{Triglycerides} + H_2O \xrightarrow{\text{LPL}} \text{Glycerol} + \text{free fatty acids}$$

$$\text{Glycerol} + \text{ATP} \xrightarrow{\text{glycerol kinase}} G_3P + \text{ADP}$$

$$G_3P + O_2 \xrightarrow{\text{GPO}} \text{DPA} + H_2O_2$$

$$H_2O_2 + \text{4-AP} + \text{p-chloro phenol} \xrightarrow{\text{POD}} \text{Quinone} + H_2O$$

The intensity of the color formed is proportional to the triglyceride concentration in the sample.

REAGENTS:

Standard: 200 mg/dl

PROCEDURE:

1ml of working reagent was added to each of 0.01ml of serum sample and 0.01 of standard taken in different tubes. Mixed and incubated at 37°C for 10 minutes. The absorbance of the sample and standard against blank was read at 546 / 670 nm.

CALCULATION

$$\text{Triglyceride concentration (mg/dl)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times 200$$

APPENDIX - XII

ESTIMATION OF ASPARTATE TRANSMINASE (AST)

KIT METHOD (Bradley, 1972)

PRINCIPLE

SGOT (AST) Catalyses the following reaction:

$$\text{L-Aspartate} + \text{2-Oxoglutarate} \xrightarrow{\text{AST}} \text{L-Glutamate} + \text{Oxaloacetate.}$$

$$\text{Oxaloacetate} + \text{NADH} \xrightarrow{\text{MDH}} \text{Malate} + \text{NAD}$$

$$\text{Sample pyruvate} + \text{NADH} \xrightarrow{\text{LDH}} \text{L-Lactate} + \text{NAD}$$

Oxaloacetate so formed is coupled with NADH to give malate. Then the sample pyruvate react with NADH and dehrogenate in the presence of Lactate dehydrogenase to give lactate, which gives brown colour in alkaline medium and this can be measured colorimetrically.

REAGENTS

SGOT Reagent

- 2-Oxoglutarate
- L-Aspartate
- MDH
- LDH
- NADH(yeast)
- Tris buffer (pH 7.8 at 250C)
- EDTA

Dilute 5 ml of SGPT Reagent to 20 ml with Aqua-4 water supplied in the kit.

PROCEDURE

To 1.0 ml of working reagent, 0.1 ml of serum sample was added and the absorbance of the sample was recorded at 1 min interval at 340 nm.

APPENDIX - XIII

ESTIMATION OF ALANINE TRANSAMINASE (ALT)

Kit method (Bradley, 1972)

PRINCIPLE

SGPT (ALT) Catalyses the following reaction:

$$\text{L-Alanine} + \text{2-Oxoglutarate} \xrightarrow{\text{ALT}} \text{Pyruvate} + \text{L-Glutamate}$$

$$\text{Pyruvate} + \text{NADH} \xrightarrow{\text{LDH}} \text{L-Lactate} + \text{NAD}$$

Pyruvate so formed is couple with NADH in the presence of Lactate dehydrogenase to give lactate, which gives brown colour in alkaline medium and this can be measured colorimetrically.

REAGENTS

SGPT Reagent:

- L-Alanine
- NADH
- LDH
- 2- Oxoglutarate
- Tris buffer (pH 7.5 at 25^0C)

Dilute 5 ml of SGOT Reagent to 20 ml with Aqua-4 water supplied in the kit

PROCEDURE

To 1.0 ml of working reagent, 0.1 ml of serum sample was added and recorded the absorbance of the sample at 1 min interval at 340 nm.

APPENDIX-XIV

ESTIMATION OF THIOBARBITURIC ACID REACTIVE SUBSTANCE (TBARS)

Nadigar *et al*, (1986)

PRINCIPLE

TBARS in TCA homogenate was determined by reacting them with Thiobarbituric acid. The color developed was measured at 540nm against dis. H2O.

REAGENT

- TCA 40%
- TCA 50%
- Thiobarbituric acid 0.67%: - 6.7 gm of Thiobarbituric acid was dissolved in distilled H2O and made up to 1000ml.

PROCEDURE

1. To test added 1ml of sample
2. To blank added 1ml of distilled H2O and to both added 2ml reagent.
3. Solution was heated in boiling water bath for 15mts cooled and centrifuged at 1000rpm for 540nm.

APPENDIX – XV

HISTOPATHOLOGICAL ANALYSIS OF TISSUES

(Culling, 1979)

The following steps were followed in Histopathlogical techniques

(1) Tissue samples obtained upon autopsy were preserved in 10% formalin solution for a minimum of one hour.

(2) Dehydration of the fixed tissue was done by giving three changes of acetone (each 500ml).

(3) Cleaning of tissue from acetone was effected by three changes of xylene (each 500ml) in a total duration of three hours.

(4) Incubation of processed tissue in melted paraffin was done by two changes 3-4 hours in an incubator maintained at 58°C

(5) Embedding of the tissue in paraffin wax was then done by immersing the tissue of molten paraffin and then cooling it to harden the paraffin.

(6) Sections of the paraffin embedded tissue were done using microtome adjusted to 1-3 μ thickness.

(7) The paraffin sections were carefully taken on glass slides.

(8) The sections were then cleaned from wax by immersing in xylene.

(9) The sections were stained with hematoxylin and eosin and screened to evaluate the morphology and cellular composition.

APPENDIX-XVI

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. 50μl of plant extract 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,

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

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

APPENDIX – XVII

THROMBOLYTIC ASSAY

Prasad *et al.*, (2007)

1. Leaf samples were prepared at different dilutions of 10,20,30,40 & 50µg in ethanol.
2. 6 ml of venous blood drawn from healthy human volunteers and was distributed into 6 different pre-weighed sterile microcentrifiuge tubes (1ml/tube).
3. Incubated the tubes for clot formation at 37^0c for 45 mins.
4. After clot formation, the serum was completely removed without disturbing the clot.
5. Each tube having the clot was again weighed to determine the clot weight (clot weight = weight of clot containing tube – weight of tube alone)
6. To each microcentrifuge tube containing the preweighed clot, added 1ml of the prepared leaf extract of the plant.
7. As positive control 1ml of Streptokinase and as negative control 1ml of ethanol was taken.
8. The tubes were then incubated for 90 min at 37^0c and observed for clot lysis.
9. After incubation the fluid released was removed and the tubes were again weighed to observe the difference in weight after clot distruption.
10. The difference obtained in weight taken before and after clot lysis was expressed as the percentage of clot lysis.

$$\textbf{Percentage of clot lysis} = \frac{\text{Weight of tube after clot formation} + \text{weight of tube after clot lysis}}{\text{Clot weight}}$$