APPENDIX I

Qualitative Analysis of Phytochemicals (Raaman, 2006)

Identification of Carbohydrates

Molisch's Test

To 2ml of aqueous extract, few drops of 20% α-naphthol in ethyl alcohol were added. Then about 1ml of concentrated sulphuric acid was added along the sides of the test tube. Reddish violet ring appeared at the junction of two layers indicated the presence of carbohydrates.

Identification of Amino acids and Proteins

Millon's Test

To 2ml of the filtrate added 5-6 drops of MiIlon's reagent was added. Formation of red precipitate indicated the presence of proteins and free amino acids.

Ninhydrin Test

To the filtrate lead acetate solution was added to precipitate tannins. It was then filtered and the filtrate was spotted on a paper chromatogram, sprayed with ninhydrin reagent and dried at 110^0 for 5 minutes. Appearance of violet spots indicated the presence of proteins and free amino acids.

Biuret Test

To the ammoniated alkaline filtrate 2-3 drops of 0.02% copper sulphate solution was added and formation of red colour indicated the presence of proteins and free amino acids.

Identification of Phenols

Ferric chloride Test

To 2ml of the extract, 2ml of ferric chloride solution was added and formation of deep bluish green solution indicated the presence of phenols.

Identification of Saponins/Saponin Glycosides

Sodium bicarbonate Test

To a few ml of the ethanolic extract, few drops of sodium bicarbonate was added and shaken well. Formation of honey comb indicated the presence of saponins.

Identification of Quinones/ Anthraquinones

Chloroform-Ammonia Test

To 0.5g of the plant sample, 10 ml of 5% sulphuric acid was added and allowed to boil. It was then filtered and while hot, to the filtrate 5ml of chloroform was added and heated on a boiling water bath. Two ml of the chloroform extract was mixed with 1ml of diluted 10% ammonia and the mixture was shaken. A pink-red color in the ammoniacal layer showed the presence of anthracene derivatives.

Borntrager's Test

About 50 ml of the extract was heated with 10% ferric chloride solution and 1ml of concentrated HCl. The extract was cooled, filtered and the filtrate was shaken with diethyl ether. The ether extract was further extracted with strong ammonia and pink or deep red coloration of aqueous layer indicated the presence of anthroquinone.

Identification of Alkaloids

Mayer's Test

A fraction of the extract was treated with Mayer's reagent (1.36g of mercuric chloride and 5g of potassium iodide in 100ml of distilled water) and observed for the formation of cream coloured precipitate indicates the presence of alkaloids.

Dragendorff's Test

To 1ml of the extract added 1ml of Dragendorff's reagent. Appearance of orange – red precipitate indicated the presence of alkaloids.

Wagner's Test

To a fraction of the filtrate a few drops of Wagner's reagent was added. Appearance of reddish-brown precipitate indicated the presence of alkaloids.

Identification of Flavonoids

The aqueous extract of the sample was reduced to dryness in a water bath. The residue was treated with dilute NaOH followed by addition of dilute HCl. A yellow solution with NaOH which turned colorless with dilute HCl confirmed the presence of flavonoids.

Pew Test

A piece of metallic Magnesium/ Zinc was added to 1ml of the extract, followed by addition of 2 drops of concentrated HCl. Formation of brown color confirmed the presence of flavonoids.

Identification of Tannin

Preparation of Extract

The plant material was suspended in methanol and allowed to stand overnight. It was refluxed for 4 hours. It was then filtered and the residue was washed with methanol. The filtrate was allowed to cool down, observed for any modification. This aliquot was used to assay tannins.

Braemer's Test

To 0.5 g of the methanolic/ethanolic extract, 10 ml of water was added and boiled. It was then filtered. To the filtrate, few drops of 10% $FeCl_3$ were added. A dark green, blue or brown color indicated the presence of tannin.

Identification of Volatile oils

Two ml of the extract solution was shaken with 0.1 ml diluted sodium hydroxide and a small quantity of dilute HCl. Formation of white precipitate indicated the presence of volatile oils.

Detection of Terpenoids

To 5ml of the extract, 2ml of chloroform was mixed and concentrated H_2SO_4 (3ml) was carefully added to form a layer. A reddish brown coloration at the interface indicated the presence of terpenoids.

APPENDIX II

Estimation of carbohydrate

(Anthrone *et al.*, 1999)

Reagents:

1. 2.5 N HCl
2. Anthrone reagent: Dissolve 200mg anthrone in 100ml of ice- cold 95% Sulphuric acid. Prepare fresh before use.
3. Standard Glucose: Stock- Dissolve 100mg in 100ml water.
4. Working Standard: 10ml of stock diluted to 100ml with distilled water. Store refrigerated after adding a few drops of toluene.

Procedure:

Weighing 100mg of the sample were taken in a boiling tube and hydrolyze it in a boiling water bath for 3 hours with 5ml of 2.5 N HCl and cool it to room temperature and neutralize it with solid sodium carbonate until the effervescence ceases. Finally make up to 100ml and centrifuge, the pellet was poured off and the supernatant was collected and take 0.5 and 1ml aliquots for analysis. Aliquots of standard glucose solution and supernatant solution were made up to 1ml distilled water and 4ml of anthrone reagent was added and heated for 8 minutes in a boiling water bath and allowed to cool rapidly. The dark green colour developed was read at 630 nm against reagent blank. A standard curve was constructed and the concentration of the glucose was calculated.

APPENDIX III

Estimation of protein

(Lowry *et al.*, 1951)

Reagents:

1. Solution A: 1% copper sulphate
2. Solution B: 2% sodium potassium tartarate
3. Solution C: 2% sodium carbonate in 0.1 N sodium hydroxide
4. Solution D: 1 ml of solution A + 1 ml of solution B and 100 ml of solution C were mixed just before use
5. Solution E: 1 N Folin Ciocalteu (mixed equal volume of commercially available reagent and water just before use)

6. Standard BSA: 50 mg of Bovine Serum Albumin (BSA) was dissolved in 50 ml of 0.1 N sodium hydroxide solution

Procedure:

Aliquots of standard protein solution and supernatant solution (obtained by dissolving a known amount of isolated solution in 0.1 N sodium hydroxide) were made up to1 ml with 0.1 N sodium hydroxide and shaken well to treat the protein with alkali. 3 ml of solution D was added, mixed well and incubated at 37˚C for 3 minutes. 0.3 ml of solution E was added, mixed well and incubated at 37˚C for 3 minutes again. The blue color developed was read at 670 nm against reagent blank. A standard curve was constructed and the concentration of the protein was calculated.

APPENDIX IV
Estimation of flavonoids
(Zhishen *et al.*, 1999)

Reagents

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

Procedure

0.1ml of methanolic extracts of plant sample was added to 0.3ml of distilled water. To this 0.03ml of 5% sodium nitrite was added to the tubes and incubated for 5 minutes. To this 1mM sodium hydroxide (0.2ml) was added and made up to 1ml with distilled water. The absorbance reading at 510nm 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 on the sample was calculated.

APPENDIX V
Estimation of total phenols
(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 650 nm.

Reagents

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

Procedure

Preparation of plant extract

Pre-weighed leaf sample (0.5 g) was ground in 5 ml of 80% ethanol. The homogenate was centrifuged at 10,000 rpm for 20 minutes. The supernatant was collected and the residue was re-extracted with 2.5 ml of 80% ethanol. After repeated centrifugation, the supernatants were collected and pooled. The ethanol was evaporated and the residue was dissolved in a known volume of distilled water and used for the estimation of phenol.

Estimation

Aliquots (0.2 to 2.0 ml) of the standard catechol solution were made up to 3 ml with distilled water. Folin-Ciocalteau (0.5 ml) reagent was added to each test tube. After 3 minutes, 2.0 ml of 20% sodium carbonate was added to each tubes. After mixing the tubes thoroughly, all the tubes were heated in a boiling water bath for exactly one minute and allowed to cool at room temperature. The blue colour developed was recorded at 650nm against a reagent blank. The concentration of phenols in the sample was calculated from the standard curve constructed on an electronic calculator set to the linear regression mode and expressed as mg phenols / g leaf.

APPENDIX VI

Estimation of alkaloids

Muthumani *et al* (2010)

Reagents

20% Acetic acid.

Chloroform

Anhydrous Na2SO4

Standard: 10mg of pure caffeine and dissolve in 25ml of 20% acetic acid A.R., dilute as aliquot a further 10 times with 20% acetic acid. This solution contains 40mg/ml

PROCEDURE

• Into four suitable separators were pipetted 1,2 and 3 of 40mg/ml standard solution

• The volume of each was made up to 5ml with 20% acetic acid

• To each separator 5 ml of acetate buffer and 1ml of methyl orange were added.

• After shaking for 10 sec.

• 5 ml of chloroform was added.

• The separators were stopped and shaken for 3 min.

• After standing for a few minutes chloroform layers were withdrawn into dry test tubes, dried with small amount of anhydrous Na_2SO_4

• Absorbance read on a spectrophotometer at 420nm using 10mm cells.

• From the reading standard curves was constructed.

CALCULATION

Express the amount in mg / g or 100 g sample.

APPENDIX – VII

HIGH PRESSURE THIN LAYER CHROMATOGRAPHY (HPTLC)

(Wagner *et al.*, 1996)

HPTLC is a valuable tool for the investigation of herbal products with respect to different aspects of their quality. The advantage of HPTLC over other techniques is that large number of samples can be simultaneously analyzed using small volume of mobile phase unlike HPLC, thus lowering analysis time and cost per analysis.

Instrumentation and Extraction of plant materials

The aqueous extract of the fruit samples were centrifuged at 3000rpm for 5 minutes. The supernatant was collected and used as test solution for HPTLC analysis. 5µl of the test solution and 5µl of standard solution was loaded as 5mm band length in the 4 x 10 Silica gel $60F_{254}$ TLC plate using a Hamilton syringe and CAMAG LINOMAT 5 instrument. The samples loaded plate was kept in TLC twin trough developing chamber (after saturation with solvent vapor) with respective mobile phase and the plate was developed in the respective mobile phase up to 90mm.

The developed plate was dried by hot air to evaporate solvents from the plate. The plate was kept in Photo-documentation chamber (CAMAG REPROSTAR 3) and the images were captured in visible light, UV 254nm and UV 366nm. After derivatization with

the appropriate reagents, the plate was photo-documented in visible light and UV 366nm mode using photo-documentation chamber. Finally, the plate was fixed in the scanner stage and scanning was done at UV 254nm. The peak table, peak display and peak densitogram of flavonoids were noted.

Flavonoid profile:

Quercetin was used as the reference standard for flavonoid analysis. The mobile phase used for development of flavonoids was toluene: acetone: formic acid: (4.5:4.5:1). For derivatization, the developed plate was sprayed with 1% ethanolic aluminium chloride reagent and dried at 100^{o}C for 10minutes in hot air oven.

Alkaloid profile:

Colchicine was used as the reference standard for alkaloid analysis. The mobile phase used for development of alkaloid was ethylacetate: methanol: water (10:1.35:1). For derivatization, the developed plate was sprayed with Dragendroff"s reagent and dried at 120^{0}C for 5 minutes in hot air oven.

Phenol profile:

Catachin was used as the reference standard for phenol analysis. The mobile phase used for development of phenol was toluene: chloroform: acetone (10:1.35:1). For derivatization, the developed plate was sprayed with 25% Folin-ciocalteau reagent and dried at 120^{0}C for 5 minutes in hot air oven.

APPENDIX VIII

ESTIMATION OF TOTAL ANTIOXIDANT ACTIVITY

Prieto *et al.*, (1999)

Principle:

This assay is based on the reduction of Mo (VI) to Mo (V) by the sample analyte and the subsequent formation of green phosphate / Mo (V) complex at acidic pH.

Reagents:

1. 0.6 M Sulphuric acid
2. 28 mM Sodium phosphate
3. 4 mM ammonium molybdate

4. Stock standard solution: Dissolved 100 mg gallic acid in 100 ml of methanol in a standard flask.
5. Working standard solution: Diluted 10 ml of stock solution to 100 ml with methanol. 1.0 ml of this solution contains 100 µg of gallic acid.

Procedure:

Different concentrations of working standard prepared in methanol ranging from 200-1000 µg/ml and 5,10, 15 mg/ml of the fruit extracts were pipetted into a series of test tubes and combined with 1ml of reagent solution (0.6M Sulphuric acid,28mM Sodium phosphate and 4 mM ammonium molybdate). The tubes were capped and incubated at 95ºC for 90 minutes, cooled to room temperature and the absorbance was recorded at 695 nm against blank.

APPENDIX – IX

Estimation of catalase

(Luck, 1974)

Principle

The UV light absorption of hydrogen peroxide solution can be easily measured between 230 and 250 nm. 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 (0.067 M pH 7.0)
   Dissolved 3.522 g of KH_2PO_4 and 7.268 g of $KHPO_4.2H_2O$ in distilled water and the volume was made up to 1 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 240 nm with 1 cm light path.

Procedure

Enzyme Extract

The sample was homogenized in a prechilled mortar and pestle with M/150 phosphate buffer (assay buffer diluted 10 times) at 1 - 4°C and centrifuged. The sediment was stirred with cold phosphate buffer, allowed to stand in the cold with occasional shaking and then

the extraction was repeated once or twice. The extraction should not take more than 24 hr. The combined supernatants were used for the assay.

Assay

Three ml of H_2O_2 Phosphate buffer was pipette out into the experimental cuvette. It was mixed well with 0.01 – 0.04 ml sample using the flattened end of a glass rod. The time required (Δt) for a decrease in absorbance from 0.45 to 0.40 (0.05 units) at 240 nm. This value was used for calculation. If Δt was greater than 60 seconds, the experiment was repeated with increased concentration of the sample.

The activity was calculated and expressed as units / mg protein. One enzyme unit was calculated as the amount of enzyme required to decrease the absorbance at 240 nm by 0.05 units.

Calculation

The concentration of $H_2O_2$and thereby the catalase activity was calculated using the extinction coefficient 0.036µ mole/ml.

APPENDIX – X

Estimation of peroxidase (Reddy *et al.*, 1995)

Principle

In the presence of the hydrogen donor pyrogallol, peroxidase converts H_2O_2 to water and oxygen. The oxidation of pyrogallol to the coloured product purpurogalli can be quantified spectrophotometrically at 430nm. The formation of the product is proportional to the activity of the enzyme and can be used as a measure of the same.

$$\text{Pyrogallol} + H_2O_2 \longrightarrow \text{Oxidized pyrogallol} + H_2O$$

Reagents

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

Procedure

One gram of the sample was mascerated with 5 ml (w/v) 0.1 M phosphate buffer (pH 6.5) in a homogeniser. The homogenate was then centrifuged at 300 g for 15 minutes.

The supernatant was used as the enzyme source. All the procedures were carried out at 0-5° C.

Three ml of 0.05 M pyrogallol solution and 0.5 to 1 ml enzyme extract was pipette out into a test tube. The spectrophotometer was adjusted to read '0' at 400 nm. To this, 0.5 ml of 1% H_2O_2 was added to the test cuvette. The change in absorbance for every 30 seconds upto 3 minutes was recorded.

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	= Y x (1000/300) Units

APPENDIX – XI

Assay of polyphenol oxidase
Esterbauer *et al.*, (1977)

Principle

Phenol oxidases are copper proteins, which catalyze the aerobic oxidation of certain phenolic substrates to quinines, which are auto-oxidized to dark brown pigments generally known as melanins, which can be estimated spectrophotometrically at 495 nm.

Reagents

1. Reaction medium - Tris-HCl (50mM, pH 7.2), sorbitol (0.4M), NaCl (10mM)
2. Catechol (0.01M)
3. Phosphate buffer (0.1M, pH 6.5)

Procedure

Preparation of enzyme extract

The enzyme extract was prepared by macerating 5 g of leaf tissue in 20 ml reaction medium containing tris-HCl. The homogenate was centrifuged at 2000 g for 10 minutes at 4ºC, the supernatant was used for the assay.

Assay

Both phosphate buffer (2.5 ml) and catechol solution (0.3 ml) was pipetted out into the experimental cuvette and the spectrophotometer was set at 495 nm. The sample (0.2 ml) was added to the same cuvette and the changes in absorbance were monitored for every 30 seconds up to 5 minutes. One unit of either catechol oxidase / laccase is defined as the amount of enzyme that transforms one µmole of dihydrophenol to one µmole of quinine/minute. The activity of PPO can be calculated using the formula,

$$\text{Enzyme unit} = K \times (\Delta A / \text{minute})$$

where, K for catechol oxidase = 0.272, K for laccase = 0.242

APPENDIX – XII
Estimation of superoxide dismutase
(Misra and Fridovich, 1972)

Principle

The assay of SOD is based on the inhibition of formation of NADH phenazine methosulphate - nitroblue tetrazolium formazon, the extent of which can be assayed spectrophotometrically at 560 nm.

Reagents

1. Sodium pyrophosphate buffer (0.025M, pH 8.3)
2. Phenazine methosulphate (PMS) (186µM)
3. Nitroblue tetrazolium (NBT) (300µM)
4. NADH (700µM)
5. Glacial acetic acid
6. n-butanol

Procedure

Preparation of enzyme extract - *Punica granatum* aril and rind (0.5g) were ground with 3.0 ml of sodium pyrophosphate buffer, centrifuged at 2000g for 10 minutes and the supernatant was used for the assay.

Assay

The assay mixture contained in a total volume of 3.0 ml, 1.2 ml of sodium pyrophosphate buffer, 0.1 ml of PMS, 0.3 ml of NBT, 0.2ml of enzyme preparations and 1.0 ml of water. NADH (0.2 ml) was added to start the reaction.

The assay mixture was incubated at 30° C for 90 seconds and the reaction was stopped by the addition of 1.0 ml of glacial acetic acid. To this mixture, n-butanol (4ml) was added and allowed to stand for 10 minutes and then centrifuged at 2000g for 5 minutes. The intensity of the chromogen in the butanol layer was measured at 560nm against butanol as blank. The system devoid of enzyme served as control. One unit of enzyme activity is defined as the amount of enzyme causing a 50% reduction in NBT oxidation/minute.

APPENDIX – XIII
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 540 nm.

Reagents

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

Procedure

About 1 g of the sample was homogenized in 4% TCA up to 10 ml and centrifuged at 2000 rpm for 10 minutes. To the supernatant obtained, a pinch of activated charcoal was added, shaken well and kept for 10 minutes. It was centrifuged once again and the charcoal residue was removed. 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 volume was made up 2.0 ml with 4% TCA. 0.2 to 1.0 ml 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 2.0 ml with 4% TCA. To this, 0.5ml of DNPH reagent was added to all the test tubes, followed by 2 drops of 10% thiourea solution and 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 temperatureand the absorbance was read spectrophotometrically at 540 nm. The content of ascorbic acid in the sample was calculated using the standard graph.

APPENDIX – XIV

Estimation of α-tocopherol (Emmeric -Engle method, 1938 as described by Rosenberg, 1992)

Principle

Tocopherol can be estimated using Emmerie – Engel reaction which is 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 extractives are read at 460 nm to measure carotenes. A correlation is made for these after adding ferric chloride and reading at 520 nm.

Reagents

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

Extraction of plant tissue

The sample was homogenized with water in a blender. 2.5 g of the homogenised sample was accurately weighed into a conical flask and 50 ml of 0.1 N H_2SO_4 was slowly added without shaking. The mixture was stoppered and allowed to stand overnight. On

the following day, the contents of the flask were shaken vigorously and filtered through Whatmann No. 1 filter paper discarding the initial 10 – 15 ml of filtrate. Aliquots of the filtrate were used for the estimation.

Procedure

Into 3 stoppered centrifuge tubes (test, standard and blank), 1.5 ml of extract, 1.5 ml of standard and 1.5 ml of water were added respectively. To the test and blank 1.5 ml of ethanol and to the standard, 1.5ml of water was added. Then, 1.5 ml xylene was added to all the test tubes, stoppered, mixed well and centrifuged. 1.0 ml of xylene layer was transferred into another stoppered tube, taking care not to include any other ethanol or protein. 1.0 ml of 2, 2'- dipyridyl reagent was added to each tube, stoppered and mixed. 1.5 ml of the mixture was pipette out into colorimeter cuvettes and the extinction of the test and standard was read against the blank at 460nm. For the blank, 0.33 ml of ferric chloride solution was added.

The amount of vitamin E can be calculated using the formula,

$$\text{Amount of tocopherols in } \mu g = \frac{\text{Reading at 520 nm} - \text{Reading at 460 nm}}{\text{Reading of standard at 520 nm} \times 0.29 \times 15}$$

APPENDIX-XV

Estimation of reduced glutathione

(Moron *et al.*, 1979)

Principle:

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

Reagents:

1. Phosphate buffer (0.2M, pH 8.0)
2. DTNB (0.6mM in 0.2M phosphate buffer)
3. TCA (5% and 25%)
4. Standard GSH (10mg/ml in 5% TCA)

Procedure:

A 20% homogenate was obtained by homogenizing 0.5g of the plant sample in 2.5 ml of 5% TCA. The homogenate was immediately acidified by adding 125ml of 25% TCA

to prevent aerial oxidation of glutathione. The precipitated protein was centrifuged at 1000rpm for 10 minutes. The homogenate was cooled on ice and 0.1ml of the supernatant was taken for the estimation. The supernatant was made up to 1 ml with 0.2M sodium phosphate buffer (pH 8.0). Two ml of freshly prepared DTNB solution was added to the tubes and the intensity of the yellow colour formed was read at 412 nm in a spectrophotometer after 10 minutes. A standard curve of GSH was prepared using concentrations ranging from 2-10 n moles of GSH in an electronic calculator set to the linear regression mode and the values of the samples were read off it. The values are expressed as n moles of GSH /g tissue.

APPENDIX – XVI
Determination of Thrombolytic Activity
(Prasad *et al.*, 2006)

Streptokinase

To the commercially available lyophilized streptokinase vial (15,00,000 IU) 5ml of sterilized distilled water was added and mixed properly. This suspension was used as a stock from which 100µl (30,000 IU) was used as a standard.

Procedure

Venous blood drawn from healthy volunteers (n=20) was transferred in different pre weighed sterile micro centrifuge tube (500µl/tube) and incubated at 37ºC for 45 minutes. After clot formation, serum was completely removed (aspirated out without disturbing the clot formed). Each tube with the clot was again weighed to determine the clot weight (clot weight = weight of clot containing tube – weight of tube alone). To the micro centrifuge tube containing clot, 100µl of streptokinase (30,000 IU) or various dilutions of the aqueous fruit extract was added. Water was also added to one of the tubes containing clot and this served as a negative thrombolytic control. All the tubes were then incubated for 90 minutes at 37^{o}C and the tubes were again weighed to observe the difference in weight after clot disruption. Differences obtained in weight taken before and after clot lysis was expressed as percentage of clot lysis. This test was repeated twenty times with different concentrations of the plant extracts in blood samples of twenty different healthy volunteers.

APPENDIX XVII

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.

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

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

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

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

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