# Appendices

## APPENDIX I

## QUALITATIVE ANALYSIS OF PHYTOCHEMICALS

**Alkaloids** (Raaman, 2006)

50 mg of solvent free extract was stirred with 1 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.

**Carbohydrates** (Iyengar, 1995)

To 0.5ml of the plant extract, 1ml of water and 5-8 drops of Fehling's solution was added, then heated on the flame and observed for brick red precipitate.

**Saponins** (Raaman, 2006)

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

**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 color was observed, which indicated the presence of steroids.

**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 color indicates the presence of phenolic compounds.

**Flavonoids** (Raaman, 2006)

Alkaline reagent test: 2 ml of aqueous solution of the plant extract was treated with 1 ml of 10% ammonium hydroxide solution. Yellow fluorescence indicated the presence of flavonoids.

**Protein** (Smith *et al.*, 1985)

To 1 ml of the extract few drops of Bradford's reagent was added and blue color product indicated the presence of protein.

**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 color indicates the presence of glycosides.

**Tannins** (Iyengar, 1995)

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

**Terpenoids** (Siddiqui and Ali, 1997)

4 mg of the plant 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 color was observed for the presence of 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 chloroform layer was pipette out in to another test tube and 1 ml of dilute ammonia was added. The resulting solution was observed for the color changes.

**Reducing sugar (Lengar, 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.

## APPENDIX II

## 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 (110µg/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 III

## ESTIMATION OF TANNINS

**(Vanillin-hydrochloride method)**

**(Robert, 1971)**

### Principle

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

### 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 colored.

2. **Standard solution:** Prepared standard catechin solution containing 1mg/ml methanol. Dilute this stock solution ten times, 10ml to 100ml (100µg/ml) concentration was used as working standard solution.
3. **Preparation of extract:** 1g of the ground sample in 50ml methanol. Mix occasionally by swirling. After 20-28 hours, centrifuge and collect the supernatant.

**Procedure**

Pipette out 1ml of the supernatant. To this 5ml of vanillin hydrochloride reagent was added and read in a spectrophotometer at 500nm after 20 minutes. 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 IV

## ESTIMATION OF PROTEIN

**(Lowry *et al.*, 1951)**

**Principle**

The aminoacid tyrosine and tryptophan present in the protein will react with the Folin-ciocalteau reagent. By the reduction of phosphomolybdic acid phosphotungstic components it will produce blue color. Also the color 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 0.1N NaOH
4. **Solution D:** Mixed just before use, 1ml of solution A, 1ml of Solution B and 100ml of Solution C
5. **Solution E:** 1N Folin-ciocalteau reagent

**Procedure**

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

## APPENDIX V

## ESTIMATION OF CATALASE ACTIVITY

**(Luck, 1974)**

**Principle**

The UV light absorption of hydrogen peroxide solution can be 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. But this method is applicable only to enzyme solution, which do not absorb strongly at 230 - 250 nm.

**Reagents**

1. **Phosphate buffer 0.067 M (pH 7.0):** Dissolved 3.522 g of $KH_2PO_4$ and 7.268 g of $K_2HPO_4.2H_2O$ in distilled water and made up the volume 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 0.5unit at 240 nm with 1 cm light path.

**Procedure**

**Enzyme extract**

The sample homogenized in a prechilled mortar and pestle with 0.067M phosphate buffer 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 24hours.The combined supernatants were used for the assay.

**Assay**

Pipetted out 3.0 ml of $H_2O_2$ Phosphate buffer into the experimental cuvette. Mixed with 0.01 - 0.04ml of sample with a glass rod flattened at one end. Noted the time $\Delta t$ required for a decrease in absorbance from 0.45 to 0.4 at 240 nm. This value was used for the calculation. If $\Delta t$ was greater than 60 seconds, then repeated the measurements with more concentrated solution of the sample. Calculated the activity and expressed in units per 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**

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

## APPENDIX VI

## 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 colored product called purpurogalli is measured calorimetrically.

Pyrogallol + $H_2O_2$ → Oxidized pyrogallol + $H_2O$

**Reagents**

1. Pyrogallol – 0.05 Unit phosphate buffer (pH 6.5)
2. 1% $H_2O_2$

**Enzyme extract**

Measured 1 gram of the sample with 5ml (w/v) 0.1 Unit phosphate buffer (pH 6.5) in a homogenizer. Centrifuge the homogenate at 300g for 15minutes. 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 0.1 to 0.5ml 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 cuvette. Recorded the change in the absorbance every 30 seconds up to 3 minutes.

**Calculations**

| | | |
|---|---|---|
| Change in absorbance/min | = | X |
| Weight of the plant material taken | = | 300mg |
| Volume of extract taken for the assay | = | 0.02ml of plant sample |
| Change in absorbance | = | X |
| Change in absorbance 1.5ml extract | = | (X/0.02ml) x 1.50-Y |
| (i.e.) Peroxidase activity in 300mg plant tissue | = | Y |
| Peroxidase activity/gram of Plant tissue | = | Y x (1000/300) Units. |

**APPENDIX VII**

**ASSAY OF SUPEROXIDE DISMUTASE**

**(Misra and Fridovich, 1972)**

**Principle**

Superoxide dismutase (SOD) uses the phytochemical reduction of riboflavin as oxygen generating system and catalyzes the inhibition of Nitro blue tetrazolium (NBT) reduction, the extent of which can be assayed spectrophotometrically at 600 nm.

**Reagents**

1. 50mM Potassium phosphate buffer
2. 45µM Methionine
3. 5.3µM Riboflavin
4. 84µM NBT
5. 20mM Potassium cyanide

**Procedure**

The incubation medium contained 300µl of each reagent (50mM potassium phosphate buffer, 45µM Methionine, 5.3µM Riboflavin, 84µM NBT and 20mM

potassium cyanide) To the test 300μl of sample was added and the final volume was made up to 3 ml with distilled water. The tubes were placed in an aluminium foil-lined box maintained at 25°C and equipped with 15W fluorescent lamps. Reduced NBT was measured spectrophotometrically at 600nm after exposure to light for 10 minutes. The maximum reaction was evaluated in the absence of enzyme. One unit of enzyme activity is defined as the enzyme reaction, which gives 50% inhibition of NBT reduction in one minute under the assay conditions and expressed as specific activity in units.

## APPENDIX VIII

## ESTIMATION OF POLYPHENOL OXIDASE ACTIVITY

**(Esterbauer *et al.*, 1977)**

### Principle

Polyphenol oxidases are copper proteins of wide occurrence in nature, which catalyses 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. 0.4M sorbitol
3. 10mM sodium chloride
4. 0.1M phosphate buffer (pH 6.5)
5. 0.01M catechol solution

### Procedure

### Enzyme extract

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

**Assay**

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

**Calculation**

| | |
|---|---|
| Enzyme units in the test | = K*(Δ/minutes) |
| K for catechol oxidase | = 0.272 |
| K for laccase | = 0.242. |

## APPENDIX IX

## ESTIMATION OF GLUTATHIONE-S-TRANSFERASE ACTIVITY

**(Habig *et al.*, 1974)**

**Principle**

GST conjugates with GSH and CDNB and the extent of conjugation causes a proportionate change in the absorption at 340nm, which can be followed spectrophotometrically.

**Reagents**

1. 1mM – 1-chloro 2,4-dinitrobenzene (CDNB) in ethanol
2. 1mM - Glutathione
3. 0.1M Phosphate buffer
4. 50mM Tris –HCl buffer (pH 7.2)

**Procedure**

**Enzyme extract**

The sample was homogenized with Tris–HCl buffer (pH7.2). The homogenated sample was centrifuged at 4°C for 30 minutes at 8500rpm.The supernatant was used as the enzyme source.

The estimation was done at 25°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. 0.1ml of both substrates GSH and CDNB was taken in 0.1M phosphate buffer (pH 6.5) at room temperature to make a volume of 2.9ml. The reaction was started by the addition of 0.1ml 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. It 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 moles and CDNB conjugated/minutes/g sample.

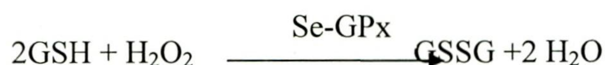
## APPENDIX X

## ASSAY OF GLUTATHIONE PEROXIDASE

**(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. Then the remaining GSH was measured by the method of Ellman.



$$2GSH + H_2O_2 \xrightarrow{\text{Se-GPx}} GSSG + 2\,H_2O$$

**Reagents**

1. 0.4M Tris buffer
2. 10mM sodium azide
3. 10% TCA
4. 0.4mM EDTA
5. 10mM $H_2O_2$
6. 2mM Glutathione

**Procedure**

To 2 ml of Tris buffer, 0.2 ml of EDTA, 0.1 ml of Sodium azide and 0.5 ml of enzyme extract were added in a test tube. 0.2 ml of glutathione followed by 0.1 ml of $H_2O_2$ were added to the mixture, mixed well and incubated at 37ºC for 10 minutes along with a tube containing all the reagents except sample. After 10 minutes the reaction was arrested by the addition of 0.5 ml of 10% TCA, centrifuged and the 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 VITAMIN C

**(Roe and Keuther, 1953)**

**Principle**

Ascorbate is converted to dehydroascorbate by treatment with activated charcol or bromine. Dehydroascorbic acid then reacts with 2, 4 dinitro phenyl hydrazine to form osazones, which dissolves in sulphuric acid to give an orange colored solution whose absorbance can be measured spectrophotometrically at 540nm.

**Reagents**

1. 4% TCA
2. 9N $H_2O$
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 4% TCA
7. Working standard: Diluted 1.0ml of the stock solution to 100ml with 4% TCA.

**Procedure**

1g of sample was ground and 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 volumes of the clear supernatants were noted. 0.5 and 1ml aliquots of this supernatant were taken for the assay.

The assay volume was made up to 2ml with 4% TCA. 0.2 to 1.0ml of the working standard solution containing 20 – 100μg of ascorbic acid respectively were pipetted out into clean dry test tubes, the volumes of which were also made up to 2.0ml with 4% TCA. Added 0.5 ml of DNPH reagents to all the test tubes, followed by 2 drop of 10% thiourea solution. Incubated at $37^0$C for 3 hours.

The osazones formed were dissolved in 2.5ml of 85% sulphuric acid, in cold, drop by drop, with no appreciable rise in temperature. To the blank DNPH reagent and thiourea were added after the addition of sulphuric acid. The tubes were incubated for 30minutes 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 XII
## ESTIMATION OF VITAMIN E
**(Rosenberg, 1992)**

**Principle**

Tocopherol can be estimated using Emmeric - Engel reaction which is based on the reduction of ferric ions by tocopherol, which then forms a red colour with 2, 2' - dipyridyl. Tocopherol and carotenes are first extracted with xylene and the extraction 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 reagent: Dissolved 1.2g of 2, 2'- dipyridyl in 1 litre n- propanol.
4. $FeCl_3$ solution: Dissolved 1.2 g of $FeCl_3$. $6H_2O$ in 1 litre ethanol. Kept in brown bottle
5. Standard solution: Dissolved 1g/ litre of α - tocopherol in absolute alcohol. 91 mg of α – tocopherol is equivalent to 100 mg 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 whatmann no.1 filter paper, discard the initial 10-15ml of fitrate. Aliquots of the filtrate were used for the estimation.

Into 3 stoppered centrifuge tubes (test, standard and blank), pipetted out 1.5 ml of extract, 1.5 ml of standard and 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. To all the tubes, added 1.5 ml of xylene, stoppered, mixed well and centrifuged. 1 ml of the xylene layer was transferred into another stoppered tube, excluding ethanol or protein. To each tube, 1.0 ml of 2, 2' - dipyridyl reagent was added, stoppered and mixed. Into the cuvettes, 1.5 ml of the mixture was pipette out and read the extinction of the test and the standard against blank at 460 nm. Then, beginning with the blank, added 0.33ml of ferric chloride solution. The amount of vitamin E can be calculated using the formula

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

**APPENDIX XIII**

**ESTIMATION OF POLYPHENOLS**

**(Malik 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 10000rpm 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 serial of test tubes. The volume was made up to 2.5ml with water. To all the tubes added 0.5ml 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. Construct 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 XIV

## ESTIMATION OF REDUCED GLUTATHIONE

**(Moron *et al.*, 1979)**

**Principle**

Reduced glutathione 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. 5% TCA
2. 0.2M Sodium phosphate buffer (pH 8.0)

**Procedure**

1g of the sample was homogenized in 5%TCA to give 20% homogenate. The precipitated protein was centrifuged at 1000rpm for 10 minutes. The homogenate was cooled on ice and 0.1 ml of the 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 the intensity of the yellow color formed was read at 412nm in a spectrophotometer after 10 minutes. A standard curve of reduced glutathione was prepared using concentrations ranging from 2 to 10 nanomoles of reduced glutathione in 5% TCA.

## APPENDIX XV

## 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. 28mM sodium phosphate
3. 4mM ammonium molybdate
4. Stock standard solution: Dissolved 100mg gallic acid in 100 ml of methanol in a standard flask
5. Working standard solution: diluted 10ml 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 200-1000µg/ml and 5, 10, 15 mg/ml of the plant extracts were pipetted into a series of test tubes and combined with 1ml of reagent solution (0.6 M sulphuric acid, 28mM sodium phosphate and 4mM ammonium molybdate). The tubes were capped and incubated at 95 °C for 90 minutes, cooled to room temperature and the absorbance was recorded at 695 nm against blank.

## APPENDIX XVI

## THROMBOLYSIS

**(Prasad *et al.*, 2007)**

### Specimen

Venous blood was drawn from the healthy volunteers (n=20).500μl of blood transferred to each of each of the previously weighed micro centrifuge tubes to form clots.

### Clavix

To the commercially available Clavix (75mg/ml) 1ml distilled water was added. This suspension was used as a positive control.

### Study design

Venous blood drawn from healthy volunteers (n=20) was transferred in different pre weighed sterile micro centrifuge tube (500μl/tube) and incubated at 37°C for 45 minutes. After clot formation, serum was completely removed (aspirated out without disturbing the clot formed). Each tube having clot was again weighed to determine the clot weight (clot weight=weight of clot containing tube-weight of tube alone).Each micro centrifuge tube containing clot was properly labeled and 1ml of Clavix which serves as a positive control, along with various dilution in aqueous plant extracts was added to the tubes. 1ml of water was also added to one of the tubes containing clot and this serves as a negative thrombolytic control. All the tubes were again weighed to observe the difference in weight after clot disruption. Differences obtain in weight taken before and after clot lysis was expressed as percentage of clot lysis. This test was repeated twenty times with all the two dilutions of the plant extracts in blood samples of twenty different healthy volunteers.

## APPENDIX XVII

## ESTIMATION OF TOTAL CHOLESTEROL

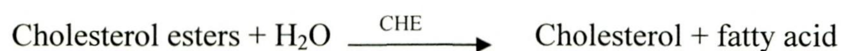
## CHOD-PAP METHOD

**(Allain *et al.*, 1974)**

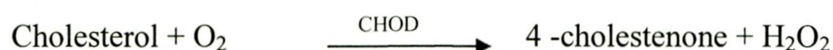
### Principle

Cholesterol esterase (CHE) hydrolyses cholesterol ester to free cholesterol is oxidized by the Cholesterol oxidase (CHOD) to 4-cholestenone and hydrogen peroxide,

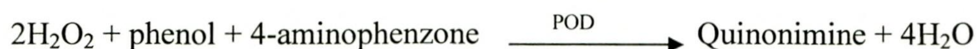
hydrogen peroxide formed reacts with 4-amino antipyrine and phenol in presence of peroxidase to produce pink colored compound called quinonimine dye.



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



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



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

### BRINE SHRIMP LETHALITY ASSAY (Oladimeji *et al.*, 2006)

Brine shrimp (Artemia Salina) were obtained by hatching brine shrimp eggs in artificial sea water (3.8% non ionized sodium chloride solution) for 48 hours. 200µl of the plant extracts of different concentration were added to 5ml of brine shrimp solution with 20 nauplii for each extracts in vials.

These vials were maintained at room temperature for 24 hours under the light and surviving 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,

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

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

## APPENDIX XIX

## HIGH PERFORMANCE 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 HPTLC analysis of methanolic extract plant samples was centrifuged at 3000rpm for 5 minutes. The supernatant was collected and used as test solution for HPTLC analysis. 2 µl of the test solution and 2µl 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 UV366nm. 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 and tannins 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°C for 10minutes in hot air oven.

**Tannin profile**

Tannic acid was used as the reference standard for tannin analysis. The mobile phase used for the development of tannin was Toluene: Ethyl acetate: Formic acid: Methanol (3:3:0.8:0.2). For derivatization, the developed plate was sprayed with 5% Ferric chloride reagent and dried at $100^0$ C for 10minutes in hot air oven.