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## Appendices

### Appendix I

#### Phytochemical Analysis

##### 1. Test for alkaloids (Kumar *et al.*, 2009)

To the extract added 1 per cent HCl and drops of Mayer's reagent and Dragendorff's reagent. Organic precipitates indicate the presence of alkaloids in the sample (Wagner and Dragendorff's test).

##### 2. Test for flavonoids (Ayoola *et al.*, 2008)

5ml of dilute ammonia solution were added to a portion of aqueous filtrate of each plant extract followed by addition of Con. Sulphuric acid. A yellow coloration is observed which confirms the presence of flavonoids and it disappears on standing.

##### 3. Test for phenols (Raaman, 2006)

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

##### 4. Test for steroids (Edeoga *et al.*, 2005)

2ml of acetic anhydride was added to 0.5g of extract with 2ml of sulphuric acid. The color change from violet to blue or green indicated the presence of steroids.

##### 5. Test for saponins (Kumar *et al.*, 2009)

The extract with 20ml of distilled water was agitated in a graduated cylinder for 15minutes. The formation of 1cm layer of foam indicated the presence of saponins.

##### 6. Test for terpenoids (Ayoola *et al.*, 2008)

5ml of extract added 2 ml of chloroform and 2 ml of Con. Sulphuric acid to form a monolayer of reddish brown coloration of the interface was showed to form positive result for terpenoids (Salkowski test).

##### 7. Test for tannins (Edeoga *et al.*, 2005)

5ml of extract was added to few drops of 1 per cent lead acetate. A yellow precipitate indicated the presence of tannins.

##### 8. Test for glycosides (Raaman, 2006)

To 50 mg 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. Bomtrager's from the filtered hydrolysate, 3ml of chloroform layer was separated and 2ml of 1 per cent ammonia solution was added to it. Pink colour indicates the presence of glycosides.

#### 9. Test for amino acids (Kumar *et al.*, 2009)

To 2 ml of sample added 2 ml of Ninhydrin reagent and kept in water bath for 20 minutes. Appearance of purple colour indicated the presence of amino acids in the sample (Ninhydrin test).

#### 10. Test for proteins (Matsushita, 1993)

To 2ml of extract 1ml of 40 per cent sodium hydroxide solution and 1 to 2 drops of 1 per cent copper sulphate solution was added. A violet colour indicated the presence of peptide linkage of the molecule (Biuret test).

### Appendix II

#### DPPH radical scavenging activity

(Mensor *et al.*, 2001)

#### Principle

Disappearance of purple colour of radical DPPH by the antioxidants of the extract at 515nm in a spectrometer shows the scavenging ability.

#### Reagents

1. DPPH – 2, 2-diphenyl-2-picryl hydrazyl hydrate (0.3mM in methanol)
2. Methanol
3. Tris EDTA buffer, pH 7.2

#### Procedure

The leaf extract (10-100µg/ml) was prepared in Tris EDTA buffer added to 0.5ml of methanolic solution of DPPH and 0.48ml of methanol. The mixture was allowed to react at room temperature for 30minutes. Methanol served as the blank and DPPH in methanol, without the leaf extracts, served as the positive control. After 30 minutes of incubation, the discolouration of the purple colour was measured at 515nm in a spectrophotometer. The radical scavenging activity was calculated as follows:

$$\text{Per cent inhibition} = \frac{(\text{Control} - \text{test})}{\text{Control}} \times 100$$

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**Appendix III**  
**Superoxide radical scavenging activity**  
**(Winterbourne *et al.*, 1975)**

**Principle**

This assay is based on the inhibition of the production of pink colour nitro blue tetrazolium formazon on the superoxide ion by the plant extracts and is measured spectrophotometrically at 560 nm.

**Reagents**

1. EDTA (0.1M containing 1.5mg of NaCN)
2. Nitroblue tetrazolium (NBT – 1.5mM)
3. Riboflavin (0.12mM)
4. Phosphate buffer (0.067M, pH 7.6)

**Procedure**

Superoxide anions were generated in samples that contained in 3.0ml, 0.02ml of the leaf extracts (10-100µg/ml), 0.2ml of EDTA, 0.1ml of NBT, 0.05ml of riboflavin and 2.64ml of phosphate buffer. The control tubes were also set up where DMSO was added instead of the plant extracts. All the tubes were vortexed and the initial optical density was measured at 560nm in a spectrophotometer. The tubes were illuminated using a fluorescent lamp for 30minutes. The absorbance was measured again at 560nm. The difference in absorbance before and after illumination was indicative of superoxide anion scavenging activity.

$$\text{Per cent inhibition} = \frac{(\text{Control} - \text{test})}{\text{Control}} \times 100$$

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**Appendix IV**  
**Hydroxyl radical scavenging activity**  
**(Elizabeth and Rao, 1990)**

**Principle**

The principle of the assay is the quantification of 2'-deoxyribose degradation product, malondialdehyde, by its condensation with thiobarbituric acid at 532 nm.

**Reagents**

1. Deoxyribose (2.8mM)
2. Ferric chloride (0.1mM)
3. EDTA (0.1mM)
4. H<sub>2</sub>O<sub>2</sub> (1mM)
5. Ascorbate (0.1mM)
6. KH<sub>2</sub>PO<sub>4</sub>-KOH buffer (20mM, pH 7.4)
7. Thiobarbituric acid (1 per cent)

**Procedure**

The reaction mixture contained 0.1ml of deoxyribose, 0.1ml of FeCl<sub>3</sub>, 0.1ml of EDTA, 0.1ml of H<sub>2</sub>O<sub>2</sub>, 0.1ml of ascorbate, 0.1ml of KH<sub>2</sub>PO<sub>4</sub>-KOH buffer and 20µl of plant extracts in a final volume of 1.0ml. The mixture was incubated at 37°C for 1 hour. At the end of the incubation period, 1.0 ml of TBA was added and heated at 95°C for 20 minutes to develop the colour. After cooling, the TBARS formation was measured spectrophotometrically at 532 nm against an appropriate blank. The hydroxyl radical scavenging activity was determined by comparing the absorbance of the control with that of the samples. The percent TBARS production for positive control (H<sub>2</sub>O<sub>2</sub>) was fixed at 100 per cent and the relative percent TBARS was calculated for the extract treated groups.

$$\text{Per cent inhibition} = \frac{(\text{Control} - \text{test})}{\text{Control}} \times 100$$

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**Appendix V**  
**Hydrogen peroxide radical scavenging activity**  
**(Ruch *et al.*, 1989)**

**Principle**

The ability of the leaf extracts to scavenge hydrogen peroxide was assessed by the difference in the absorbance at 230nm.

**Reagents**

1. Phosphate buffer (0.1M, pH 7.4)
2. H<sub>2</sub>O<sub>2</sub> (40mM) in phosphate buffer

**Procedure**

A solution of H<sub>2</sub>O<sub>2</sub> (40mM) was prepared in phosphate buffer. Leaf extracts at the concentration of 10mg-100µg/ml were added to H<sub>2</sub>O<sub>2</sub> solution (0.6ml) and the total volume was made up to 3ml. The absorbance of the reaction mixture was recorded at 230nm in a spectrophotometer. A blank solution containing phosphate buffer, without H<sub>2</sub>O<sub>2</sub> was prepared.

The extent of H<sub>2</sub>O<sub>2</sub> scavenging of the plant extracts was calculated as:

$$\text{(Control - test)}$$

$$\text{Per centage scavenging of hydrogen peroxide} = \frac{\text{Control - test}}{\text{Control}} \times 100$$

**Appendix VI**  
**Nitric oxide radical scavenging activity**  
**(Green *et al.*, 1982)**

**Principle**

Sodium nitroprusside in aqueous solution, at physiological pH, spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions that are estimated spectrophotometrically at 546nm.

**Reagents**

1. Sodium nitroprusside (100mM)
2. Phosphate buffered saline (pH 7.4)
3. Griess reagent (1% sulphanilamide, 2% H<sub>3</sub>PO<sub>4</sub> and
4. 0.1% Naphthylethylene diamine dihydrochloride

### Procedure

The reaction was initiated by adding 2.0ml of sodium nitroprusside, 0.5ml of PBS, 0.5ml of leaf extracts (10-100µg/ml) and incubated at 25°C for 30 minutes. Griess reagent (0.5ml) was added and incubated for another 30 minutes. Control tubes were prepared without the extracts. The absorbance was read at 546 nm against the reagent blank, in a spectrophotometer.

$$\text{Per cent inhibition} = \frac{(\text{Control} - \text{test})}{\text{Control}} \times 100$$

### Appendix VII

#### Ferric reducing antioxidant power assay

(Benzic and Strain, 1996)

#### Principle

At low pH, reduction of ferric tripyridyl triazine (Fe III TPTZ) complex to ferrous form (which has an intense blue colour) can be monitored by measuring the change in absorption at 593nm. The reaction is non - specific, in that any half reaction that has lower redox potential, under reaction conditions, than that of ferric to ferrous half reaction will drive the ferrous (Fe III to Fe II) ion formation. The change in absorbance is therefore, directly related to the combined or “total” reducing power of the electron donating antioxidants present in the reaction mixture.

#### Reagents

##### FRAP Reagent

- a) Acetate buffer 300 mM pH 3.6: Weigh 3.1g sodium acetate trihydrate and add 16 ml of glacial acetic acid and make the volume to 1 L with distilled water.
- b) TPTZ (2, 4, 6-tripyridyl- s- triazine) (M.W. 312.34) 10 mM in 40mM HCl (M.W.36.46)
- c) FeCl<sub>3</sub>. 6H<sub>2</sub>O (M.W. 270.30) 20 mM

The working FRAP reagent was prepared by mixing a b & c in the ratio of 10:1:1 at the time of use.

Standard Ascorbic Acid (M.W. 176.13) 1000 µ M

**Procedure**

Sample (100µl) is mixed with 3 ml of working FRAP reagent and absorbance (593 nm) was measured at 0 minute after vortexing. Thereafter, samples are placed at 37°C in water bath and absorption is again measured after 4 minutes. Ascorbic acid standards (100µM-1000µM) were processed in the same way. Blank the analyzer/spectrophotometer with Blank -Measure the OD of standard and test at zero minute and again after four minutes at 593 nm. Results were calculated as follows:

FRAP value of Sample (µM) = (Change in absorbance of sample from 0 to 4 minute / Change in absorbance of standard from 0 to 4 minute) X FRAP value of standard (1000 µM) FRAP value of ascorbic acid is 2.

**Appendix VIII****Trypan blue exclusion assay****(Salomi and Panikkar, 1989)****Principle**

The viable cells exclude the dye and remained unstained, while non – viable cells take up the dye and are stained blue.

**Reagents**

1. Trypan blue - 0.1 per cent in PBS (pH 7.2)
2. PBS (pH 7.2)
  - KCl – 40mg
  - KH<sub>2</sub>PO<sub>4</sub>– 20mg
  - Na<sub>2</sub>HPO<sub>4</sub>. 2H<sub>2</sub>O - 575mg
  - NaCl - 900mg
  - Distilled water - 100ml

**Procedure**

The EAC tumour cells were propagated from the peritoneal cavity of mice and washed with phosphate buffered saline thrice by centrifuging at low speed. 0.1ml containing 1x10<sup>6</sup> cells was used for the *in vitro* assay. Various concentrations of the sample were incubated with EAC cell lines at 37<sup>0</sup>C for three hours. At the end of the

incubation period 0.1 ml trypan blue was added and layered the cells on the haemocytometer for counting. The dead cells were blue in colour and counted to calculate the percentage of dead cells.

Per cent Cytotoxicity =  $\text{Dead cell count} / (\text{Dead cell count} + \text{Viable cell count}) \times 100$

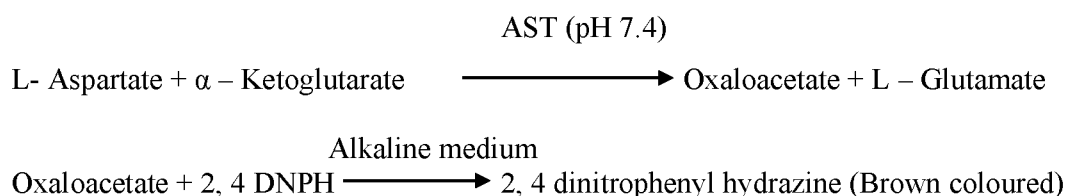
### Appendix IX

#### Estimation of aspartate transaminase (AST)

(Reitman and Frankel, 1957)

##### Principle

The AST converts L-aspartate and  $\alpha$ -ketoglutarate to oxaloacetate and L-glutamate. Formed oxaloacetate treated with 2, 4 dinitrophenyl hydrazine (DNPH) to produce derivative of hydrazine, which in an alkaline medium produces brown coloured complex, whose intensity is measured. AST catalyses the following reaction.



##### Reagents

1. Tris buffer, pH 7.5 – 100 mMol/L
2. L-aspartate – 500 mMol/L
3. 2-oxoglutarate – 15 mMol/L
4. Standard Pyruvate – 2 mMol/L
5. 2, 4 dinitrophenyl hydrazine reagent
6. Working sodium hydroxide (0.4N)

##### Procedure

0.5ml of buffered substrate was incubated at 37° C for 3 minutes and 0.1 ml of serum was added, mixed well and incubated at 37°C for 30 minutes. Then 0.5ml of 2, 4 – dinitrophenyl hydrazine (DNPH) reagent was added, mixed well and kept at room temperature for 20 minutes and 0.5ml of 0.4N working sodium hydroxide was added, and kept at room temperature for 10 minutes. Blank and standards were also processed in a similar way and the absorbance was measured spectrophotometrically at 505 nm. Activity

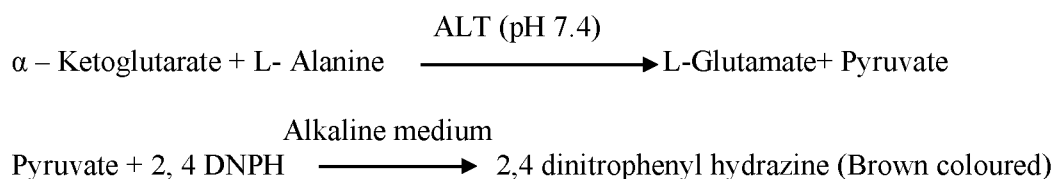
of AST was expressed as U/L. One unit as defined as micromole of pyruvate formed/minute.

### Appendix X

#### Estimation of alanine transaminase (ALT)

(Reitman and Frankel, 1957)

The ALT catalyses the transfer of amino group from L-alanine to  $\alpha$ -ketoglutarate with the formation of glutamate and pyruvate. Formed pyruvate was allowed to react with 2,4 dinitrophenyl hydrazine (DNPH) to produce 2, 4- dinitrophenyl hydrazone derivative, which is brown coloured in alkaline medium. The absorbance of this hydrazone derivative is proportionate to ALT activity.



#### Reagents

1. Tris buffer, pH 7.4– 100 mMol/ L
2. L-alanine - 500mMol/L
3. 2-oxoglutarate - 15mMol/L
4. 2, 4 dinitrophenyl hydrazine reagent
5. Working sodium hydroxide (0.4N)

#### Procedure

0.5ml of buffered substrate was incubated at 37°C for 3 minutes and 0.1ml of serum was added, mixed well and incubated at 37°C for 60 minutes. Then 0.5ml of DNPH reagent was added, mixed well and kept at room temperature for 20 minutes and 0.5ml of 0.4N working sodium hydroxide was added and kept at room temperature for 10 minutes. Blank and standards were also processed in a similar way and the absorbance was measured spectrophotometrically at 520 nm. Activity of ALT was expressed as U/L. One unit is defined as micromole of pyruvate formed/minute.

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**Appendix XI**  
**Estimation of alkaline phosphatase**  
**(King, 1965)**

**Principle**

Alkaline phosphatase was an enzyme which catalyses the splitting of phosphoric acid from certain monophosphoric esters. In this method disodium phenyl phosphate was hydrolyzed with the liberation of phenol and formation of sodium phosphate. The amount of phenol formed was estimated in a spectrophotometer at 650nm.

**Reagents**

## 1. Disodium phenyl phosphate (0.01M)

Dissolved 1.09g of disodium phenyl phosphate in water and made up to 500ml. Boiled, cooled and added little chloroform and kept in refrigerator (Solution A).

## 2. Sodium carbonate-sodium bicarbonate buffer (0.1M)

Dissolved 3.18g of anhydrous sodium carbonate and 1.68g of sodium bicarbonate in water and made up to 500ml (Solution B).

## 3. Buffered substrate for use

Equal volume of solution A and solution B was mixed which has pH of 10.

## 4. Trichloro acetic acid (20%)

## 5. Acid molybdate reagent

5g of ammonium molybdate dissolved in 5N sulphuric acid.

## 6. 1, 2, 4 – ANSA

0.25% of 1,2,4 – ANSA was prepared by adding 0.5g of dry powder ANSA to 190 ml of 15% sodium bisulphate and 5ml of 20% sodium sulphite stoppered the bottle and shaken until it dissolved.

## 7. Stock Phosphate solution

Dissolved 2.194g of pure potassium dihydrogen phosphate in water and made up to 500ml. Add few drops of chloroform (1mg/1ml of phosphate).

## 8. Working standard: Diluted 2 ml of stock standard to 500ml.

**Procedure**

Pipetted out 6 ml of buffered substrate in test tube and placed it in water bath at 37°C for few minutes. Added 0.3ml of serum mixed well and incubated for 15 minutes.

At the same time control and blank was also kept. For blank 0.3ml of water was added to 6ml buffered substrate. For control 0.3ml of serum was added to 6ml of distilled water. After that added 1.2ml of 20 per cent TCA and shaken well. 5ml of filtrate was taken in separate test tubes. To blank and control added 0.8ml of acid molybdate followed by 0.2ml of ANSA. Mixed well and allowed it to stand for 10 minutes at 37°C and the color developed was read at 650nm.

Pipetted out 1.0 to 4.0ml of standard solution and made up to 5ml with distilled water. 0.8ml of acid molybdate was added followed by 0.2ml of ANSA. Standards were also read at 650nm. Alkaline phosphatase activity in serum was expressed as U/L. The activity in tissue homogenate was expressed as micro mole of phenol liberated/min/mg protein.

## **Appendix XII**

### **Estimation of catalase (Luck, 1974)**

#### **Principle**

The UV light absorption of H<sub>2</sub>O<sub>2</sub> solution can be measured between 230 and 250 nm. On decomposition of H<sub>2</sub>O<sub>2</sub> by catalase, the absorption decreases with time. The enzyme activity could be arrived at from this decrease. This method is applicable only to enzyme solution, which absorb strongly at 240 nm.

#### **Reagents**

1. 0.067M Phosphate buffer of pH 7.0

Dissolved 3.522g of KH<sub>2</sub>PO<sub>4</sub> and 1.218g of Na<sub>2</sub>HPO<sub>4</sub>.H<sub>2</sub>O in distilled water and made up the volume to 1 litre.

2. H<sub>2</sub>O<sub>2</sub> – Phosphate buffer

Diluted 0.16 ml of H<sub>2</sub>O<sub>2</sub> (10per cent w/v) to 100ml with phosphate buffer. Freshly prepared and used. The absorbance of the solution should be about 0.5 at 240 nm with a 1cm light path.

#### **Procedure**

Pipetted out 3.0 ml of H<sub>2</sub>O<sub>2</sub> Phosphate buffer into the experimental cuvette and mixed in 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 0.40 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 the amount of enzyme required to decrease the absorbance at 240 nm by 0.05 units.

### **Appendix XIII**

#### **Estimation of superoxide dismutase**

**(Misra and Fridovich, 1972)**

##### **Principle**

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

##### **Reagents**

1. 50mM Potassium phosphate buffer, pH 7.8
2. 45 $\mu$ M Methionine
3. 5.3 $\mu$ M Riboflavin
4. 84 $\mu$ M NBT
5. 20mM Potassium cyanide

##### **Procedure**

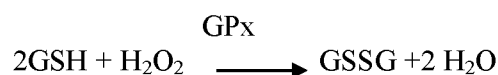
The incubation medium contained a final volume of 3 ml, 50 mM potassium phosphate buffer (pH 7.8), 45  $\mu$ M Methionine, 5.3  $\mu$ M Riboflavin, 84  $\mu$ M NBT and 20 mM potassium cyanide. The tubes were placed in an aluminium foil lined box maintained at 25°C and equipped with 15W fluorescent lamps. Reduced NBT was measured spectrophotometrically at 600nm after exposure to light for 10 minutes. The maximum reduction was evaluated in the absence of enzyme. Calculated the activity and expressed in units per mg protein. One unit of enzyme activity was defined as the amount of enzyme giving 50 per cent inhibition of the reduction of NBT.

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**Appendix XIV**  
**Estimation of glutathione peroxidase**  
**(Rotruck *et al.*, 1973)**

**Principle**

A known amount of enzyme preparation was allowed to react with hydrogen peroxide in the presence of reduced glutathione (GSH) for a specified time period. Glutathione peroxidase converts GSH to oxidised glutathione. Then the remaining GSH was measured at 421 nm.

**Reagents**

1. 0.4M Tris buffer, pH 7.0
2. 10mM sodium azide solution
3. 10 per cent Trichloroacetic acid
4. 0.4mM EDTA
5. 10mM H<sub>2</sub>O<sub>2</sub>
6. 2mM Glutathione solution

**Procedure**

To 2 ml of Tris buffer, 0.2 ml of EDTA, 0.1 ml of Sodium azide and 0.5 ml of tissue homogenate were added. 0.2 ml of glutathione followed by 0.1 ml of H<sub>2</sub>O<sub>2</sub> were added. The contents were mixed well and incubated at 37°C for 10 minutes along with a tube containing all the reagents except tissue homogenate. After 10 minutes, the reaction was arrested by the addition of 0.5 ml of 10 per cent TCA, centrifuged and the supernatant was assayed for glutathione by the method of Moron *et al.* (1979) as in Appendix – XIII. The activities were expressed as nmoles of GSH oxidised/min/mg protein.

**Appendix XV**  
**Estimation of Vitamin A**  
**(Bayfield and Cole, 1980)**

**Principle**

The colour produced by Vitamin A acetate or palmitate with TCA is proportional to its concentration, which was measured at 620 nm in a spectrophotometer.

**Reagents**

All reagents were prepared freshly. Exposure of sample and reagents to light was avoided.

1. Saturated TCA in chloroform

5 g clear TCA crystals were dissolved in 25 ml of alcohol-free chloroform, stored in dark.

2. Standard Vitamin A

1.5 mg Vitamin A palmitate was dissolved in 10 ml of chloroform.

**Procedure**

Aliquots of the standard were pipetted out into a series of clear, dry test tubes in the concentration range of 0-7.5µg. The volumes in all the tubes were made up to 0.1 ml with chloroform. From a fast delivery pipette, added 2 ml of TCA reagent rapidly, mixing with the contents of the tube. The absorbance was recorded immediately at 620nm in a spectrophotometer. The procedure was repeated for the sample tubes. Constructed a standard graph and read off the concentration in the sample. Vitamin A levels were expressed as mg/g tissue.

**Appendix XVI**  
**Estimation of Vitamin E**  
**(Rosenberg, 1992)**

**Principle**

Tocopherol can be estimated using Emmerie - 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 extinction 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: Dissolved 1.2 g of 2, 2' – dipyridyl in 1 litre of n – propanol
4. FeCl<sub>3</sub> solution: Dissolved 1.2 g of FeCl<sub>3</sub>.6H<sub>2</sub>O in 1 litre of ethanol. Kept in brown bottle
5. Standard solution: Dissolved 10mg of α - tocopherol in 10 ml of absolute alcohol. 91 mg of α– tocopherol is equivalent to 100 mg of tocopherol acetate.

**Procedure**

Into 3 stoppered centrifuge tubes (test, standard and blank), pipetted out 1.5 ml of each tissue 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. Added 1.5 ml of xylene to all the test tubes, stoppered, mixed well and centrifuged. Transferred 1.0ml of xylene layer into another stoppered tube and care should be taken not to include any ethanol or protein. Added 1.0ml of 2, 2' dipyridyl reagent to each tube, stoppered and mixed. Pipetted out 1.5ml of the mixtures into colorimeter cuvettes and read the extinction of the test and standard against the blank at 460nm. Then, in turn with the blank, added 0.33ml of ferric chloride solution. Mixed well and after exactly 15 minutes read test and standard against the blank at 520nm. The amount of Vitamin E can be calculated using the following formula and expressed as mg/g tissue.

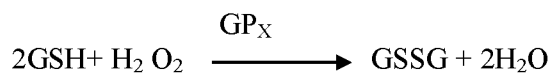
$$\text{Amount of tocopherol} = \frac{\text{Reading at 520 nm} - \text{Reading at 460 nm}}{\text{Reading of standard at 520 nm}} \times 0.29 \times 15$$

**Appendix XVII**

**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. 0.2M sodium phosphate buffer (pH 8.0)
2. 0.6 mM DTNB in 0.2 M phosphate buffer
3. 5 per cent TCA
4. 25 per cent TCA
5. Standard GSH solution (M.W. 307.33)  
Dissolved 10 µg of GSH in 100ml of 5 per cent TCA

**Procedure**

Liver tissues (0.1g) were homogenized in 5 per cent TCA to get 20 per cent homogenate. 125 µl of 25 per cent TCA was added to 0.5ml of liver homogenate to precipitate the protein. The precipitated protein was centrifuged down at 1000rpm for 10 minutes. The homogenate was cooled on ice and 0.1ml of the 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). 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 colour formed was read at 412nm in a spectrophotometer after 10 minutes. A standard curve of reduced glutathione was prepared using concentrations ranging from 2-10 nanmoles of GSH in 5 per cent TCA. The results were expressed as mean GSH of nmoles/gram of wet tissue.

**Appendix XVIII****Estimation on the level of malondialdehyde (MDA)  
(Nichans and Samuelson, 1968)****Principle**

The pink chromogen formed by the reaction of 2-thiobarbituric acid with breakdown products of lipid peroxidation malondialdehyde and other thiobarbituric acid reactive substances (TBARS) in acidic solution was read at 535 nm.

**Reagents**

1. Trichloro acetic acid (TCA) – 15%
2. Hydrochloric acid (HCl) – 0.25N
3. Thiobarbituric acid (TBA) – 0.38% in hot distilled water
4. TCA-TBA-HCl reagent-solution: 1, 2 and 3 were mixed freshly in the ratio of 1:1:1.

5. Stock standard – (MDA bis diethyl acetate) or (1, 1, 3, 3-tetra methoxy propane) was made upto 100ml with double distilled water.
6. Working standard-50 n mole/ml in double distilled water.

### Procedure

The tissue homogenate was prepared in Tris-HCl buffer (pH 7.5) 1.0 ml of the tissue homogenate was treated with 2.0ml of TBA-TCA-HCl reagent and mixed thoroughly. The mixture was kept in boiling water bath for 15 minutes. After cooling, the tubes were centrifuged for 10 minutes and the supernatant was taken for measurement. The absorbance of chromophore was read at 535 nm against the reagent blank. The amount of MDA was calculated using the extinction co-efficient  $1.56 \times 10^5 \text{ M}^{-1}\text{cm}^{-1}$ . The results were expressed as nmoles of MDA / mg protein.

## Appendix XIX

### Histological analysis of liver

(Chaves *et al.*, 2004)

At the end of the treatment period, the liver of the normal mice treated with PBS, DMSO, Paraffin oil, silymarin, ethanolic extract, PEGylated AgNPs, EAC + ethanolic extract and EAC + PEGylated AgNPs and EAC were removed and histological studies were carried out to reveal the effect of selected medicinal plant *Volkameria inermis*.

The following steps were followed in the histological techniques.

- ✓ Autopsy bits are preserved in 10 per cent formalin solution for minimum 1 hour.
- ✓ Dehydration of biopsy bits were done by 3 changes of acetone (each 500ml).
- ✓ Cleaned the bits from acetone by 3 changes of xylene (each 500ml) for about 3 hours.
- ✓ Incubated the processed tissue bits in paraffin wax- 2 changes for 3-4 hours in an incubator at 58-60°C.
- ✓ Embedded the tissue bits in paraffin wax after incubation in melted paraffin.
- ✓ The sections were cut from autopsy bit embedded in wax (Sections are 1-3µm thick autopsy bit).
- ✓ Sections were taken on the glass slide.
- ✓ Sections on glass slide were cleaned from wax by immersing in xylene.
- ✓ Sections were histochemically reacted with haematoxylin and eosin staining to evaluate the morphology and cellular composition.