APPENDIX - I
PRELIMINARY PHYTOCHEMICAL ANALYSIS

Alkaloids (Raaman, 2006)

The solvent free extract (50 mg) 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)

The plant extract (50 mg) was ground with 3 ml of distilled water and diluted with the same, 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.

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)

The plant extract (50mg) 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)

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

APPENDIX II
DPPH RADICAL SCAVENGING ASSAY
(Mensor *et al*., 2001)

Principle

Antioxidant activity of the phenolic compounds depends on their ability to scavenge the stable free radical, DPPH.

Reagents

1. Methanol-50 ml
2. DPPH (Diphenyl-2-picryl hydrazyl radical)-1mM in methanol

Procedure

Three ml of 1mM DPPH in methanol was added to 100µl of plant extract with concentrations ranging from 10µg to 100µg. DPPH solution with methanol was used as a positive control and methanol alone acted as a blank. When DPPH reacts with antioxidant in the sample, it was reduced and the color changed from deep violet to light yellow. This was measured at 517 nm. The percentage scavenging activity was calculated by the following formula.

$$\text{Scavenging activity (Per cent)} = \frac{A(control)-A(sample)}{A(control)} \times 100$$

APPENDIX-III
HYDROXYL RADICAL SCAVENGING ASSAY
(Elizabeth and Rao, 1990)

Principle

The hydroxyl radical scavenging activity was measured by studying the competition between deoxy ribose and the extracts for hydroxyl radicals generated with Fe^{3+}/Ascorbate, EDTA/ H_2O_2 system. TBARS is formed when the hydroxyl radicals attack deoxyribose, which can be quantified spectrophotometrically.

Reagents

1. Deoxy ribose (2.8mM)
2. $FeCl_3$ (0.1mM)
3. EDTA (0.1mM)
4. H_2O_2 (1mM)
5. Ascorbate (0.1mM)
6. KH_2PO_4-KOH buffer (20mM, pH 7.4)
7. TBA (1per cent)
8. HCl (25per cent)

Procedure

The reaction mixture contained deoxyribose (2.8mM), FeCl3 (0.1mM), EDTA (0.1mM), H_2O_2 (1mM), Ascorbate (0.1mM) and KH_2PO_4-KOH buffer (20mM,pH 7.4). 20µl of the plant extract was added such that the final volume was 1ml. The reaction mixture was incubated for I hour at 37°C. Deoxy ribose degradation was measured as TBARS by adding 0.5ml of TBA and 0.5ml of HCl. Boiled in a waterbath for 20 minutes, cooled and measured the absorbance at 532nm. The percentage scavenging activity was calculated by the following formula:

$$\text{Scavenging activity (Per cent)} = \frac{A(\text{control})-A(\text{sample})}{A(\text{control})} \times 100$$

APPENDIX - IV

SUPEROXIDE RADICAL SCAVENGING ASSAY

(McCord and Fridovich, 1968)

Principle

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

Reagents

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

Procedure

The assay tubes contained test sample (plant extract) with 0.2ml of EDTA, 0.1ml NBT, 0.05ml riboflavin and 2.55ml of phosphate buffer. The control tubes were also set up wherein DMSO was added instead of sample. The initial optical density of the solution were recorded at 560nm. After that, these tubes were placed in an area where they received uniform illumination for 30 minutes. Again the optical density was measured at 560nm. The difference in optical density before and after illumination is the quantum of superoxide production and the

percentage of inhibition by the test sample was calculated by comparing with the optical density of control. The percentage scavenging activity was calculated by the following formula:

$$\textit{Scavenging activity (Per cent)} = \frac{A(control)-A(sample)}{A(control)} \times 100$$

APPENDIX V

HYDROGEN PEROXIDE SCAVENGING ASSAY

(Ruch *et al.,* 1989)

Principle

The ability of the plant extracts to scavenge H_2O_2 was attributed to their phenolics which can donate electrons to H_2O_2 and neutralizing it to water and oxygen.

Reagents

1. Phosphate buffer (pH-7.2)
2. H_2O_2 in phosphate buffer (4mM)

Procedure

A solution of H_2O_2 (4mM) was prepared in phosphate buffer. Plant extracts at the concentration of 10mg/10µl was added to a H_2O_2 solution (0.6ml,40mM).The total volume was made upto 3ml.The absorbance of the reaction mixture was recorded at 230 nm.The blank solution contained phosphate buffer without H_2O_2. The percentage of H_2O_2 scavenged by the plant extract was calculated as follows:

$$\textit{Scavenging activity (Per cent)} = \frac{A(control)-A(sample)}{A(control)} \times 100$$

APPENDIX - VI

NITRIC OXIDE SCAVENGING ASSAY

(Green *et al*, 1982)

Principle

Aqueous solution of sodium nitroprusside spontaneously generates nitric oxide ($NO^{\bullet}$) at physiological pH, which interacts with O_2 to produce nitrite ions, which is measured colorimetrically at 546nm.

Reagents

1. Phosphate buffered saline (PBS) - pH 7.2

   NaCl-8.8g

   KCl-0.2g

   KH_2PO_4-0.2g

   Na_2HPO_4-1.15g Made up the volume to 1000 ml.

2. Sodium nitroprussside (100mM)

3. Griess reagent: 1per cent Sulphanilamide, 2 per cent orthophosphoric acid (H_3PO_4) and 1per cent Naphthyl ethylenediamine dihydrochloride.

Procedure

Three ml of reaction mixture containing sodium nitroprusside in PBS and the plant extract was incubated at 25°C for 150 minutes. Controls without test compound were kept in an identical manner. After incubation, 0.5ml of Griess reagent was added. The absorbance of the chromopore formed was read at 546nm.The percentage inhibition of nitric oxide generation was measured by comparing the absorbance values of control and those of test compounds (plant extract).

$$\text{Scavenging activity (Per cent)} = \frac{A(\text{control})-A(\text{sample})}{A(\text{control})} \times 100$$

APPENDIX VII
MTT ASSAY
(Loosdrecht *et al.*, 1994)

Principle

The 3(-4,5-dimethyl thiazol-2-yl) 2,5 dipthenyl tetrazolium bromide (MTT) measures the metabolic activity of the viable cells. The assay is non-radioactive and can be performed entirely in a microtiter plate. It is suitable for measuring cell proliferation, cell viability or cytotoxicity. The reaction between MTT and 'mitochondrial dehydrogenase' produces water-insoluble formazan salt. This method involves culturing the cells in a 96- well microtiter plate, and then incubating them with MTT solution for approximately 2 hours. During incubation period, viable cells convert MTT to a water- insoluble formazan dye. The formazan dye in the microtiter plate (MTP) is solubilised and quantified with an ELISA plate reader (650nm). The absorbance directly correlates with the cell number. This is applicable for adherent cells cultured in MTP.

Cell lines used

- Dalton's lymphoma ascites

Reagents

- RPMI-1640,(Himedia, Mumbai india)
- Trypsin-0.25per cent (Gibcos USA)
- FBS (Fetal bovine serum) (Gibcos USA)
- MTT 4mg/ml (Himedia)
- DMSO (Emerck India)
- Lysis buffer(15 per cent SLS in 1:1 DMF and water)
- Composition of RPMI; 9.54 g/lit, 10per cent FBS, 2000 mg sodium bicarbonate, 250µl each of penicillin (60mg/ml), streptomycin (100mg/ml), Amphotericin (200mg/ml)

Procedure

Increasing concentrations of AgMEGSS, AgMEGST, AgMEGSL, MEGSS, MEGST and MEGSL extracts were added to the cells and incubated at 37°c for 14 hrs in CO_2 incubator with 5per cent CO_2. The media was replaced with a fresh growth medium along with 20µl of 3(-4,5-dimethyl thiazol-2-yl) 2,5 dipthenyl tetrazolium bromide (MTT, sigma) MTT reagent was added to it. Again it was incubated for 4 hrs at 37°c. After incubation purple precipitate was clearly visible under the inverted microscope then the growth medium was removed and 200µl of 0.1 per cent 0.1N acidic isopropyl alcohol was added to the cells to dissolve the MTT formazon crystals. Then the covered plates were kept in the dark at 18-24 ^{o}c per overnight. The samples were drawn every 2 hrs and observed the reading at 570nm. If the reading is low returned the plate for incubation. Removed the plate cover and measured the absorbance of the wells including the blank at 570nm. The absorbance was recorded at 570nm immediately after the development of purple color. Each experiment was conducted in triplicate form. The average was calculated, and compared with the control test samples. The percentage growth inhibition was calculated using the following formula:

$$\text{Scavenging activity (Per cent)} = \frac{A(control)-A(sample)}{A(control)} \times 100$$

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 - 0.1per cent in PBS (pH 7.2)
2. PBS (pH 7.2)

    KCl - 40mg
    KH2PO4 - 20mg
    Na2HPO42H2O - 575mg
    NaCl - 900mg
    Distilled water - 100ml

Procedure

The DLA tumour cells propagated in the peritoneal cavity of the mice were taken and washed with saline Phosphate Buffered Saline thrice by centrifuging at low speed. 0.1ml containing $1x10^6$ cells was used for the *in vitro* assay. Various concentrations of the sample were incubated with DLA cell lines at 37^{0}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 = Dead cell count / (Dead cell count + Viable cell count) × 100

APPENDIX-IX
ESTIMATION OF CATALASE
(Luck, 1974)

Principle

The UV light absorption of H_2O_2 solution can be measured between 230 and 250 nm. On decomposition of H_2O_2 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_2PO_4 and 1.218g of $Na_2HPO_4.H_2O$ in distilled water and made up the volume to 1 litre.

2. H_2O_2 – Phosphate buffer

Diluted 0.16 ml of H_2O_2 (10per cent w/v) to 100ml with phosphate buffer. Freshly prepared and used. The absorbance of the solution should be about 0.5 at 240nm with a 1cm light path.

Procedure

Pipetted out 3.0 ml of H_2O_2 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 Δt required for a decrease in absorbance from 0.45 to 0.40 at 240 nm. This value was used for the calculation. If Δt 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-X
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. 45μM Methionine
3. 5.3μM Riboflavin
4. 84μM 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 µM Methionine, 5.3 µM Riboflavin, 84 µM NBT and 20 mM potassium cyanide. The tubes were placed in an aluminium foil lined box maintained at 25ºC 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.

APPENDIX-XI

ESTIMATION OF GLUTATHIONE S-TRANSFERASE

(Habig *et al*., 1974)

Principle

The enzyme activity was assayed by its ability to conjugate GSH with CDNB. The extent of conjugation causing a proportional change in the absorption at 340 nm was measured.

Reagents

1. 1mM 1-Chloro-2,4-dinitrobenzene (CDNB) in ethanol
2. 1mM Reduced glutathione (GSH)
3. 0.1M Phosphate buffer (pH 6.5)

Procedure

The assay was done at 25ºC under conditions giving activities linear with respect 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. 0.1 ml of both substrates (GSH and CDNB) were taken in 0.1M Phosphate buffer (pH 6.5) at room temperature to make a volume to 2.9 ml.

The reaction was initiated by adding 0.1 ml of liver homogenate to the reaction mixture. The readings were recorded against distilled water blank for a minimum of 3 minutes. The complete assay mixture without the enzyme (liver homogenate) served as the control to monitor non-specific binding of the substrate. Care was taken to ensure that the final concentration of ethanol in the mixture was always less than 4 per cent.

Calculation

Glutathione-S-Transferase (GST) activity was calculated using the extinction coefficient of the product formed (9.6mM/cm) and the values have been expressed as micromoles of CDNB conjugated/min/mg protein.

APPENDIX-XII
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 GSH for a specified time period. Then the remaining GSH was measured by the method of Ellman.

$$2GSH + H_2O_2 \xrightarrow{GPx} GSSG + 2\,H_2O_2$$

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_2O_2
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_2O_2 were added. The contents were mixed well and incubated at 37ºC 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 of Moron *et al*. (1979) as in Appendix – XVII. The activities were expressed as nmoles of GSH oxidised/min/mg protein.

APPENDIX-XIII
ESTIMATION OF GLUTATHIONE REDUCTASE
(David and Richard, 1983)

Principle

Glutathione reductase catalyzes the conversion of oxidized glutathione to reduced glutathione employing NADPH as a substrate. The amount of NADPH utilized is a direct measure of enzyme activity.

Reagents

1. 0.12M Phosphate buffer, pH 7.2
2. 15mM EDTA
3. 10mM Sodium azide
4. 6.3mM oxidized glutathione
5. 9.6mM NADPH

Procedure

The assay system contained 1 ml of 0.12M Potassium phosphate buffer, 0.1 ml of 15mM EDTA, 0.1 ml of 10mM Sodium azide, 0.1 ml of 6.3mM oxidized glutathione and 0.1 ml of enzyme source (liver homogenate) and water in the final volume of 2 ml. Kept for 3 minutes. Then 0.1ml of NADPH was added. The absorbance at 340nm was recorded at an interval of 15 seconds for 2 to 3 minutes. For each series of measurement controls were done that contained water instead of oxidized glutathione. The enzyme activity was expressed as milli moles of NADPH oxidized/min/mg protein

APPENDIX-XIV
ESTIMATION OF PROTEIN
(Shakir *et al*., 1994)

Principle

This method is based on the principle that different proteins contain different amounts of aromatic amino acid residues, which react with Folin-Ciocalteau reagent giving a blue color, which is read in a spectrophotometer at 750 nm.

Reagents

1. Alkaline copper sulphate
   2 per cent Na_2CO_3 in 0.1 N NaOH -100ml
   2 per cent Sodium potassium tartarate-1 ml
   2 per cent Copper sulphate-1 ml
2. Folin-Ciocalteau reagent stock
   2N Folin-Ciocalteau reagent was diluted with water (1:1 v/v)
3. Standard protein solution
   Standard BSA containing 20 mg/100 ml of 0.9per cent NaCl

Procedure

Into a series of clean dry test tubes pipetted out 0.2 to 1.0 ml of standard BSA solution corresponding to 40 to 200 µg of protein. Made up the volume to 1.9 ml with 0.1N NaOH. Added 1.0 ml of alkaline copper sulphate solution, mixed well and incubated for 3 minutes at 37ºC. Then added 0.5 ml of Folin ciocalteau reagent, mixed well and incubated for 3 minutes at 37ºC. The optical density was read at 750 nm in a spectrophotometer.

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 is measured at 620nm in a spectrophotometer.

Reagents

All the 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 µg/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. $FeCl_3$ solution: Dissolved 1.2 g of $FeCl_3.6H_2O$ 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 nother 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 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-XVII
ESTIMATION OF VITAMIN C
(Roe and Keuther, 1943)

Principle

Ascorbate is converted to dehydroascorbate by treatment with activated charcoal or bromine. Dehydroascorbic acid reacts with 2, 4 - dintrophenyl hydrazine (DNPH) to form osazones, which dissolves in sulphuric acid to give an orange coloured solution whose absorbance can be measured spectrophtometrically at 540 nm.

Reagents

1. 4 per cent TCA
2. 9N H_2SO_4
3. 2 per cent 2, 4 - dintrophenyl hydrazine (DNPH) – Dissolved 2 g of DNPH in 100 ml of 9 N H_2SO_4
4. 10 per cent thiourea
5. 85 per cent H_2SO_4
6. Stock standard solution: Dissolved 100 mg of ascorbic acid in 100 ml of 4 per cent TCA
7. Working standard: Diluted 10 ml of the stock solution to 100 ml with 4 per cent TCA

Procedure

The working standard of 0.2 to 1.0 ml containing 20 -100 µg of ascorbate respectively were pipette out in clean, dry test tubes, the volumes of which were also made up to 2.0 ml with 4 per cent TCA. DNPH reagent of volume 0.5 ml was added to all the tubes, followed by 2 drops of 10 per cent thiourea. The tubes were incubated at 37˚C for 3 hours. The osazones formed were dissolved in 2.5 ml of 85per cent H_2SO_4, in cold, drop by drop, with no appreciable rise in temperature. To the blank alone, DNPH reagent and thiourea were added after addition of sulphuric acid. After incubation for 30 minutes at room temperature, the absorbance was read spectrophotometrically at 540 nm. The content of ascorbic acid was calculated in the liver sample using the standard graph.

APPENDIX-XVIII
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.

$$2GSH\ H_2O_2 \xrightarrow{GPx} GSSG + 2H_2O$$

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% 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 color formed was read at 412nm in a spectrophotometer after 10 minutes. A standard curve of reduced glutathione was prepared using concentrations ranging from 10-50 µg of reduced glutathione in 5% TCA.

APPENDIX-XIX
ESTIMATION OF THE LEVEL OF 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. Tricholro 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 TBARS was

calculated using the extinction co-efficient 1.56 X 10^5 $M^{-1}cm^{-1}$. The results were expressed as nm of MDA / mg protein.

APPENDIX-XX

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, MGsS, AgMGsS, MGsT, AgMGsT, MGsL, AgMGsL, DLA+ MGsS, DLA + AgMGsS, DLA +MGsT, DLA+ AgMGsT, DLA +MGsL, DLA + AgMGsL and DLA were removed and histological studies were carried out to reveal the effect of selected medicinal plant *Gloriosa superba.*

The following steps were followed in the histological techniques.

- Liver homogenates are preserved in 10 per cent formalin solution for minimum 1 hour.
- Dehydration of liver homogenates were done by 3 changes of acetone (each 500ml).
- Cleaned the homogenate 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 58-60^0C.
- 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 haemotoxylin and eosin staining to evaluate the morphology and cellular composition.