Appendix I

ABTS radical scavenging assay

(Shirwaiker *et al*., 2006)

Principle

The ABTS radical scavenging cations were produced by reacting ABTS solution (7mM) with 2.45 mM ammonium persulphate. Absorbance was read at 745 nm spectrophotometrically and percentage inhibition was noted.

Reagents

1. ABTS solution: ABTS (2, 2'-azino-bis (3-ethyl benzo- thiazoline-6-sulfonic acid) (7 mM) with 2. 45 mM ammonium persulphate.

Procedure

The ABTS radical cations ($ABTS^{+}$) were produced by reacting ABTS solution (7mM) with 2.45 mM ammonium persulphate. The mixture was allowed to stand in the dark at room temperature for 12-16 hrs before use. TLC separated fractions 0.5ml was added to 0.3ml of ABTS solution and the final volume was made upto 1ml with ethanol. Absorbance was read at 745 nm and percentage inhibition was calculated by the following formula:

$$Scavenging \quad activity \quad (Percent\ ) = \frac{A[control\ ] - A[sample\ ]}{A[control\ ]} x100$$

Appendix II

DPPH radical scavenging assay

(Mensor *et al*., 2001)

Principle

The reduction of stable nitrogen-centered free radical DPPH by the antioxidants of plant extract via the process of hydrogen or electron donation causes the colour changes from violet to yellow. The colour change depends on their ability to scavenge DPPH which is measured at 518 nm.

Reagents

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

Procedure

The 3ml of 1mM DPPH in methanol solution was added to 100 µl of PBS containing 10-100µg of the plant extracts. DPPH solution with methanol was used as a

positive control and methanol acted as a control. When DPPH reacts with antioxidant in the sample, it was reduced and the colour changed from deep violet to light yellow. This was measured at 518 nm. The percentage scavenging activity was calculated by the following formula:

$$Scavenging \quad activity \ (Percent) = \frac{A_{518}[control] - A_{518}[sample]}{A_{518}[control]} x100$$

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 $Fe3^{+}$ / Ascorbate / EDTA / H_2O_2 system.

Reagents

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

Procedure

The reaction mixture contained deoxyribose (2.8 mM), $FeCl_3$(0.1 mM), EDTA (0.1 mM), H_2O_2 (1 mM), Ascorbate (0.1 mM) and KH_2PO-KOH buffer (20 mM, pH 7.4), 20 µl of the TLC separated fractions were added such that the final volume was 1 ml. The reaction mixture was incubated for 1 hour at 37°C. The deoxy ribose degradation was measured as TBARS by adding 0.5 ml of TBA and 0.5 ml of HCl boiling in a waterbath for 20 minutes, cooling and measured the absorbance at 532 nm. The percentage inhibition was calculated by the following formula:

$$Scavenging \quad activity \ (Percent) = \frac{A[control] - A[sample]}{A[control]} x100$$

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 (0.1 M containing 1.5 mg NaCN / 100 ml)
2. NBT (1.5 mM)
3. 0.12 mM riboflavin
4. 0.067 M phosphate buffer (pH7.8)

Procedure

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

$$Scavenging \quad activity \quad (Percent) = \frac{A[control] - A[sample]}{A[control]} x100$$

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 (4 mM)

Procedure

A solution of H_2O_2 (4 mM) was prepared in phosphate buffer (pH 7.2). H_2O_2 concentration was determined spectrophotometrically from its absorption at 230 nm was determined after 10 minutes against a blank solution containing phosphate buffer without H_2O_2. The scavenging activity of H_2O_2 by plant extract and the standard compounds was calculated using the formula:

$$Scavenging\ \ activity\ \ (Percent\ ) = \frac{A[control\ ] - A[sample\ ]}{A[control\ ]} x100$$

Appendix VI

Nitric oxide scavenging assay

(Green *et al.,* 1982)

Principle

The scavenging of nitric oxide is based the principle that aqueous solution of sodium nitroprusside spontaneously generates nitric oxide (NO) at physiological pH, which interacts with oxygen to produce the chromophore nitrite ion, which is measured colorimetrically at 546 nm.

Reagents

1. Phosphate buffered saline (pH 7.2)
2. Sodium nitroprusside (100 mM)
3. Griess reagent (1 percent sulfanilamide, 2 percent H_3PO_4, 0.01 percent Naphthyl ethylenediamine dihydrochloride)

Procedure

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

$$Scavenging\ \ activity\ \ (Percent\ ) = \frac{A[control\ ] - A[sample\ ]}{A[control\ ]} x100$$

Appendix VII

MTT assay

(Igarashi and Miyazawa, 2001)

Principle

The 2- (4, 4-dimethyl-2-tetrazoyl) - 2, 5-diphenyl-2,4 tetrazolium salt (MTT) is converted into its formazon derivative in living cells. The amount of formazon formed is a measure of the number of surviving cells. After solubilisation of the formazon in a suitable solvent the cell viability can be measured in a microtitre plate reader at 650nm.

Reagents

1. Complete medium
2. MTT- 3 mg / ml of saline
3. Isopropanol in 0.04 N HCl (acid- propanol)
4. PBS (Phosphate Buffered Saline)

Procedure

After removing the medium and serum from the treated cells, the cells incubated with 50 µl of MTT at 37^0C for 3 hours. After incubation 200 µl of PBS was added to all the samples. The liquid was then carefully aspirated. Then 200 µl of acid- propanol was added and left overnight in the dark. The absorbance was read at 650 nm in a microtitre plate reader (Anthos 2010, Austria). The optical density of the control cells were fixed to be 100% viability and the percent viability of the cells in the other treatment groups were calculated.

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% in PBS (pH 7.2)
2. KCl - 40 mg
3. KH_2PO_4 - 20 mg
4. $Na_2HPO_42H_2O$ -575 mg
5. NaCl - 900 mg

6. Distilled water – 100 ml

Procedure

The DLA tumor cells propagated in the peritoneal cavity of the mice were taken and washed with saline phosphate buffered saline thrice by centrifuging at low speed. 0.1 ml containing $1x10^6$ cells was used for the *in vitro* assay. Various concentrations of the sample were incubated with DLA cell lines at 37^0C 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.

$$Percent\ Cytotoxici\ ty = \frac{Dead\ cell\ count}{Dead\ cell\ count + Viable\ cell\ count} x100$$

Appendix IX

Estimation of Aspartate transaminase

(**Reitman and Frankel, 1957**)

Principle

The serum aspartate amino transaminase catalyses the reversible transfer of an amino group from aspartate to α-keto glutarate forming glutamate and oxaloacetate. AST catalyses the following reaction:

L -Aspartate + α - Keto glutarate $\xrightarrow{\text{AST (pH 7.4)}}$ Oxaloacetate + L - Glutamate

Oxaloacetate + 2, 4 DNPH $\xrightarrow{\text{Alkaline medium}}$ 2, 4 dinitrophenyl hydrazine (Brown)

Reagents

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

Procedure

0.5 ml 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 0.5 ml of 2, 4 - dinitrophenyl hydrazine (DNPH) reagent was added, mixed well and kept at room temperature for 20 minutes and 0.5 ml of 4 N 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 is defined as micromole of pyruvate formed /minute

Appendix X

Estimation of Alanine transaminase

(Reitman and Frankel, 1957)

Principle

The serum alanine amino transaminase catalyses the reversible transfer of amino group from L-alanine to alpha ketoglutarate with the formation of pyruvate and glutamate. The pyruvate so formed is allowed to react with 2 - 4 dinitrophenylhydrazine (DNPH) to produce 2, 4 - dinitrophenyl hydrazone derivative, which is measured photometrically.

L -Aspartate +α - Keto glutarate $\xrightarrow{\text{ALT (pH 7.4)}}$ Oxaloacetate + L – Glutamate

Oxaloacetate + 2, 4 DNPH $\xrightarrow{\text{Alkaline medium}}$ 2, 4 dinitrophenyl hydrazine (Brown)

Reagents

1. Tris buffer, pH 7.5 - 100 mMol/l
2. L- alanine – 500 mMol/l
3. 2- Oxoglutarate – 15 mMol/l
4. 2, 4 dinitrophenyl hydrazine reagent
5. Working sodium hydroxide (4N)

Procedure

0.5 ml of buffered substrate was incubated at 37^0C for 3 minutes and 0.1ml of serum was added, mixed well and incubated at 37^0C for 60 minutes. Then 0.5 ml of DNPH reagent was added, mixed well and kept at room temperature for 20 minutes and 0.5 ml of 4 N 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 ALT was expressed as U/L. One unit is defined as micromole of pyruvate formed /minute.

Appendix XI

Estimation of Alkaline phosphatase

(King, 1965)

Principle

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

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

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

3. Buffered substrate for use

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

4. Tricholoro acetic acid (20%)

5. Acid molybdate reagent

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

6. 1, 2, 4 - ANSA (1-amino-2-naphthol-6-sulphonic acid)

   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 5 ml of 20% sodium sulphite stoppered the bottle and shaken until it dissolved.

7. Stock Phosphate solution

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

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

Procedure

Pipetted out 6 ml of buffered substrate in test tube and placed it in water bath at 37^0C for few minutes. Added 0.3 ml of serum mixed well and incubated for 15 minutes. At the same time control and blank was also kept. For blank 0.3 ml of water was added to 6 ml buffered substrate. For control 0.3 ml of serum was added to 6 ml of distilled water. After that added 1.2 ml of 20% TCA and shaken well. 5 ml of filtrate was taken in separate test tubes. To blank and control added 0.8 ml of acid molybdate followed by 0.2 ml of ANSA. Mixed well and allowed it to stand for 10 minutes at 37^0C and the colour developed was read at 650nm.

Pipetted out 1.0 to 4.0 ml of standard solution and made up to 5 ml with distilled water. 0.8 ml of acid molybdate was added followed by 0.2 ml of ANSA. Standards were

also read at 650 nm. Alkaline phosphatase activity in serum was expressed as U/L. One unit is defined as micromole of phenol formed /minute.

Appendix XII

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 H_2O_2 by catalase, the absorption decreases with time. The enzyme activity can be calculated from this decrease.

Reagents

1. 0.067 M Phosphate buffer of pH 7.0

Dissolved 3.522 g of KH_2PO_4 and 1.218 g 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 (10% w/v) to 100 ml 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_2O_2 Phosphate buffer into the experimental cuvette and mixed in 0.01 - 0.04 ml sample with a glass rod flattened at one end. Noted the time Δt required for a decrease in absorbance from 0.45 to 0.4 at 240 nm. This value was used for the calculations. If Δt was greater than 60 seconds, then the measurements was repeated 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 Glutathione peroxidase

(Rotruck *et al.,* 1973)

Principle

A known amount of enzyme preparation was allowed to react with H_2O_2 in the presence of reduced glutathione (GSH) for a specified time period. Glutathione peroxidase converts GSH to oxidized glutathione. Then the remaining GSH was measured at 421 nm.

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

Reagents

1. 0.4 M Tris buffer, pH 7.0

2. 10 mM Sodium azide solution

3. 10 percent Trichloroacetic acid

4. 0.4 mM EDTA

5. 20 mM Hydrogen peroxide solution

6. 2 mM 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 and 0.1ml of hydrogen peroxide were also added. The contents were mixed well and incubated at 37°C for 10 minutes along with a tube containing the reagents except tissue homogenate. After 10 minutes, the reaction was arrested by the addition of 0.5 ml of 10 percent TCA, centrifuged and the supernatant was assayed for glutathione by the method of Moron *et al.* (1979) as in Appendix - XIX. The activities were expressed as nmoles of GSH oxidised/min/mg protein.

Appendix XIV

Estimation of Glutathione reductase

(David and Richard, 1983)

Principle

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

Reagents

1. 0.12 M Phosphate buffer, pH 7.2

2. 15 mM EDTA

3. 10 mM Sodium azide

4. 6.3 mM Oxidized glutathione

5. 9.6 mM NADPH

Procedure

The assay system contained 1 ml of 0.12 M Potassium phosphate buffer, 0.1 ml of 15 mM EDTA, 0.1 ml of 10 mM sodium azide, 0.1 ml of 6.3 mM 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 340 nm 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/minute/mg protein.

Appendix XV

Estimation of Superoxide dismutase

(Misra and Fridovich, 1972)

Principle

The superoxide dismutase uses the photochemical reduction of riboflavin as oxygen generating system and catalyses the inhibition of NBT reduction, the extent of which can be assayed spectrophotometrically.

Reagents

1. 50 mM potassium phosphate buffer, pH 7.8
2. 45 µM Methionine
3. 5.3 µM Riboflavin
4. 84 µM Nitro Blue Tetrazolium (NBT)
5. 20 mM 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 25°C and equipped with 15 W fluorescent lamps. Reduced NBT was measured spectrophotometrically at 600 nm after exposure to light for 10 minutes. The maximum reduction was evaluated in the absence of enzyme. Calculated the activity and expressed in unit / mg / protein. One unit of the enzyme activity was defined as the amount of enzyme giving 50 percent inhibition of the reduction of NBT.

Appendix-XVI

Estimation of Vitamin A

(Bayfield and Cole, 1994)

Principle

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

Reagents

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

1. Saturated TCA in chloroform

   15 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 clean, dry test tubes in the concentration range of 0-7.5 μg. The volumes in all the test 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 620 nm in a spectophotometer. The procedure was repeated for the sample tubes. Constructed a standard graph and read off the concentration in the samples. Vitamin A levels were expressed as μg/g tissue.

Appendix XVII
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 10 mg of α - tocopherol in 10 ml of absolute alcohol. 1 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 the 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 tubes, stoppered, mixed well and centrifuged. Transferred 1.0 ml of xylene layer into another stoppered tube and care should be taken not to include any ethanol or protein. Added 1.0 ml of 2, 2' dipyridyl reagent to each tube, stoppered and mixed. Pipetted out 1.5 ml of the mixtures into colorimeter cuvettes and read the extinction of the test and standard against the blank at 460 nm. Then, in turn with the blank, added 0.33 ml of ferric chloride solution. Mixed well and after exactly 15 minutes read test and standard against the blank at 520 nm. The amount of Vitamin E can be calculated using the formula,

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

Appendix- XVIII

Estimation of Vitamin C

(Roe and Keuther, 1953)

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% TCA
2. 9 N H_2SO_4
3. 2% 2, 4 - dintrophenyl hydrazine (DNPH) -Dissolved 2 g of DNPH in 100 ml of 9 N H_2SO_4
4. 10% Thiourea
5. 85% H_2SO_4
6. Stock standard solution: Dissolved 100 mg of ascorbic acid in 100 ml of 4% TCA
7. Working standard: Diluted 10 ml of the stock solution to 100 ml with 4% 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% TCA. DNPH reagent of volume 0.5 ml was added to all the tubes, followed by 2 drops of 10% thiourea. The tubes were incubated at 37˚C for 3 hours. The osazones formed were dissolved in 2.5 ml of 85% 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 XIX

Estimation of Reduced glutathione

(Moron *et al.,* 1979)

Principle

The reduced glutathione (GSH) is measured Edman's reaction with DTNB (5, 5'-dithio-2-nitro benzoic acid) to give a compound that absorbs at 412 nm.

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

Reagents

1. 0.2 M sodium phosphate buffer (pH 8.0)
2. 0.6 mM DTNB in 0.2 M phosphate buffer
3. 5 percent TCA
4. 25 percent TCA
5. Standard GSH solution (M.W. 307.33)

   Dissolved 10 mg of GSH in 100 ml of 5 percent TCA

Procedure

Liver tissues (0.1g) were homogenized in 5 percent TCA to get 20 per cent homogenate. 125 µl of 25% TCA was added to 0.5 ml of liver homogenate to precipitate the protein. The precipitated protein was centrifuged down at 1000 rpm 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 0.2 M sodium phosphate buffer (pH 8.0). 0.2 ml of freshly prepared DTNB solution (0.6 mM in 0.2 M phosphate buffer pH (8.0) was added to the tubes and the intensity of the yellow color formed was read at 412 nm 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 XX

Estimation of levels of TBARS

(Bishayee and Balasubramaniam, 1971)

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) – 0.25 N
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 nmol/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 2.0 ml 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 1.56×10^5 $M^{-1}cm^{-1}$. The results were expressed as nM of MDA / mg protein.

Appendix XXI

Histological Status

(Culling, 1974)

At the end of the treatment period, the liver of the normal mice treated with PBS, DMSO, Paraffin oil, Silymarin, MEFrB, MEFrF, MEFrL, MEFrB +DLA MEFrF +DLA, MEFrL +DLA and DLA were removed and histological studies were carried out.

The following steps were followed in the histological techniques

- Liver homogenates are preserved in 10 percent formalin solution for minimum 1 hour.
- Dehydration of liver homogenates was done by 3 changes of acetone (each 500 ml).

- Cleaned the homogenate form acetone by 3 changes of xylene (each 500 ml) for about 3 hours.
- Incubated the processed tissue bits in paraffin wax- 2 changes for 3-4 hours in an incubator at 58-60^{0}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 1-3 µm thick autopsy bit).
- Sections were taken on the glass slide.
- Sections on glass slide from wax by immersing in xylene.
- Sections were histochemically reacted with haemotoxylin and eosin staining to evaluate the morphology and cellular composition.

Appendix XXII

Antibacterial activity

(NCCLS, 1993)

Principle

The antimicrobials present in the plant extract are allowed to diffuse out into the medium and interact in a plate freshly seeded with the test organisms. The resulting zones of inhibition will be uniformly circular as there will be a confluent lawn of growth. The diameter of zone of inhibition can be measured in millimeters.

Reagents

1. Muller Hinton Agar Medium (1L): The medium was prepared by dissolving 33.9 g of the commercially available Muller Hinton Agar Medium (HiMedia) in 1000 ml of distilled water. The dissolved medium was autoclaved at 15 lbs pressure at 121°C for 15 minutes. The autoclaved medium was mixed well and poured onto 100 mm petriplates (25-30 ml/plate) while still molten.
2. Nutrient broth (1L): One litre of nutrient broth was prepared by dissolving 13 g of commercially available nutrient medium (HiMedia) in 1000 ml of distilled water and boiled to dissolve the medium completely. The medium was dispensed as desired and sterilized by autoclaving at 15 lbs pressure (121ºC) for 15 minutes.
3. Streptomycin disc (standard antibacterial agent).

Procedure

Petriplates containing 20 ml of Muller Hinton medium were seeded with 24 hr culture of bacterial strains. Wells were cut and 20 µl of the plant extracts were added. The

plates were then incubated at 37°C for 24 hours. The antibacterial activity was assayed by measuring the diameter of the inhibition zone formed around the well (NCCLS, 1993). Streptomycin disc was used as a positive control.

Appendix XXIII

Phytochemicals 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 20 ml. 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: 50 mg of the sample was dissolved in 5 ml 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 (50 mg) 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, 3 ml of chloroform layer was separated and 2 ml of 10% ammonia solution was added to it. Pink colour indicates the presence of glycosides.

Carbohydrates (Iyengar, 1995)

To 0.5 ml of the extract of the plant sample, 1 ml 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: 4 mg of the plant extract was treated with 0.5 ml of acetic anhydride and 0.5 ml 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 0.5ml of chloroform. Concentrated sulphuric acid was added slowly along the sides of the test tube. Red violet colour was observed for terpenoids

Appendix XXIV

Gas Chromatography and Mass Spectrometry

(Maciejewicz *et al.,* 2005)

Principle

As the name implies, it is actually two techniques that are combined to form a single method of analyzing mixtures of chemicals. Gas chromatography separates the components of a mixture and mass spectroscopy characterizes each of the components individually. By combining the two techniques, qualitatively and quantitatively evaluate a solution containing a number of chemicals.

Procedure

Chromatographic analysis of derivatized extracts were performed with a GC-MS Hewlett Packard 6890 Series II instrument equipped with an on-line injection system and mass -selective detector Model HP5973A. A capillary column HP-J fused silica column (30m x 0.25 mm, column thickness 0.25 m (5%)-biphenyl-(95%) dimethylsilioxane copolymer) was used. The carrier gas was at 1.21 ml/min constant flow. The compounds were separated by following method: isothermal at 100^{0}C for 5 min, 250^{0}C at 15^{0}C/min and finally isothermal at 280^{0}C for $1x10^{6}$ 10 minutes. Mass spectra were obtained in electron impact ionization at 70eV. The injection volume was 1 µl (split ratio 60:1). The identification of components was accomplished using computer searches in commercial libraries.

Appendix XXV

High Performance Thin Layer Chromatography

(Wagner and Bladt, 1996)

Principle

Separation may result due to the adsorption and partition depending upon the nature of adsorbents used on plates and the solvent system used for the development.

Procedure

Test solution preparation

The sample was centrifuged at 3000 rpm for 2 minutes and collected the supernatant liquid. This solution was used as test solution for HPTLC analysis.

Samples

The test solution (1 µl) and 3µl of standard solution was loaded as 6 mm band length in the 3 x 10 cm Silica gel $60F_{254}$ TLC plate using Hamilton syringe and CAMAG LINOMAT 5 instrument.

Spot development

The samples loaded plate was kept in TLC twin trough developing chamber (after saturated with solvent vapor) with respective mobile phase and the plate was developed in the respective mobile phase up to 90 mm.

Photo-documentation

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 captured the images at white light, UV 254 nm and UV366 nm.

Derivatization

The developed plate was sprayed with respective spray reagent and dried at 100^{0}C in hot air oven. The plate was photo-documented at daylight and UV 366nm using photo-documentation (CAMAG REPROSTAR 3) chamber.

Scanning

Before derivatization, the plate was fixed in scanner stage and scanning was done at 254 nm. The Peak table, Peak display and Peak densitogram were noted.

Appendix XXVI

Fourier Transform-Infrared Spectroscopy

(Mohd Nasir *et al.*, 2006)

Principle

Fourier Transform-Infrared Spectroscopy is very much helpful in examining the peak variation of amino groups and carboxylic groups. Some of the infrared radiation is absorbed by the sample and some of it is passed through (transmitted). The resulting spectrum represents the molecular absorption and transmission, creating a molecular fingerprint of the sample, which corresponds to the frequencies of vibrations between the bonds of the atoms making up the material. Because each different material is a unique combination of atoms, no two compounds produce the exact same infrared spectrum. In addition, the size of the peaks in the spectrum is a direct indication of the amount of material present.

Procedure

Infrared spectroscopy of Shimadzu Corporation of model IR prestige 21 was used. A drop of each extract was applied on a sodium chloride cell to obtain a thin layer. The cell was mounted on the IR and scanned through the IR region.