METHODOLOGY

3.0 METHODOLOGY

The present investigation aimed to evaluate the antioxidant potential and anticancer effects of fresh leaves of *Solanum nigrum.* These effects were evaluated using various *in vitro* and *in vivo* models.

The *Solanum nigrum* plants were grown in pots maintained in Kongunadu Arts and Science College Campus, Coimbatore, Tamil Nadu. Fresh leaves of the two varieties of *Solanum nigrum* i.e. the one bearing black berries indicated as BBL and other variety bearing red berries denoted as RBL were used for the analyses.

This research work was executed in four different phases. In the first phase, the antioxidant content of the two varieties of *Solanum nigrum* leaves, (BBL and RBL), were analyzed. The second phase included the evaluation of the antioxidant potential and the anticancer effects using a variety of *in vitro* experimental systems. In phase III, experiments were carried out to confirm the outcome of the second phase, using *in vivo* studies. The fourth phase included the identification of the nature of the phytochemicals and the major active components of the leaves using various separation and analytical techniques. The details of the methods used are given below.

CHEMICALS

In the present investigation, all the chemicals used were of analytical grade.

PHASE I

In the first phase, the two varieties of *Solanum nigrum* leaves, BBL and RBL, were analyzed for both enzymic and non-enzymic antioxidant contents.

The leaves were collected from the plants and used fresh for the estimation of each parameter. They were washed free of surface contaminants in running water and blotted dry with soft tissue paper.

DETERMINATION OF ENZYMIC ANTIOXIDANTS

The enzymic antioxidants assayed were superoxide dismutase (SOD), catalase (CAT), peroxidase (Px), glutathione peroxidase (GPx), glutathione reductase (GR), glucose 6-phosphate dehydrogenase (G6PD), ascorbic acid oxidase (AAO) and polyphenol oxidases (PPO).

ASSAY OF SUPEROXIDE DISMUTASE

The activity of superoxide dismutase was assayed by the method of Das *et al.* (2000).

PRINCIPLE

The method involves the generation of superoxide radical by photoreduction of riboflavin and its detection by nitrite formation from hydroxylamine hydrochloride. The nitrite reacts with sulphanilic acid to produce a diazonium compound, which subsequently reacts with naphthylamine to produce a red azo compound whose absorbance is measured at 543 nm.

REAGENTS

- Phosphate buffer (50mM, pH 7.4)
- L-methionine (20mM)
- Triton X - 100 (1% v/v)
- Hydroxylamine hydrochloride (10mM)
- EDTA (50 μM)
- Riboflavin (50 μM)
- Greiss reagent: Sulphanilamide (1%), phosphoric acid (2%) and naphthylethylene diamine dihydrochloride (0.1%).

PROCEDURE

Fresh BBL and RBL (0.5g) were weighed and homogenized with phosphate buffer, centrifuged at 2000g for 10 minutes and the supernatant was used as the enzyme source.

In this method, 1.4ml aliquots of the reaction mixture (75µl of L-methionine, 40µl of TritonX-100, 75µl of hydroxylamine hydrochloride, 100µl of EDTA and 1.11ml of phosphate buffer) were taken in test tubes. Then 100µl of the test sample was added to all the tubes followed by a brief preincubation at 37°C for 5 minutes. Then, 80 µl of riboflavin was added to all the tubes. The tubes were exposed for 10 minutes to 200W fluorescent lamps (Philips). The control tubes contained an equal amount of buffer instead of the sample. The sample and its respective control were run together. At the end of the exposure time, 1.0ml of Greiss reagent was added to each tube and the absorbance of the colour formed was measured at 543nm. One unit of enzyme activity was defined as the amount of SOD capable of inhibiting 50% of nitrite formation under the assay conditions. The activity of SOD is expressed as units/g leaf.

ASSAY OF CATALASE

The method of Sinha (1972) was followed to measure the activity of catalase.

PRINCIPLE

Catalase causes rapid decomposition of hydrogen peroxide to water and oxygen. The dichromate in acetic acid is reduced to chromic acetate when heated in the presence of H_2O_2 with the formation of perchloric acid as an unstable intermediate. The green colored chromic acetate produced can be measured spectrophotometrically at 610nm.

REAGENTS

- Phosphate buffer (0.01M, pH 7.0)
- Hydrogen peroxide (0.2M)
- Dichromate/acetic acid solution: The reagent was prepared by mixing 5% potassium dichromate with glacial acetic acid (1:3 by volume). This was again diluted in the ratio of 1:5 with water.

PROCEDURE

Exactly 0.5g of fresh BBL and RBL were ground separately with 2.5ml of phosphate buffer. The debris in the homogenate was removed by centrifugation at 2000g for 10 minutes at 4°C. The supernatant obtained was used for the assay of catalase.

The reaction mixture contained 1.0ml of buffer, 0.5ml of H_2O_2, 0.3ml of H_2O and 0.2ml of the enzyme was added to initiate the reaction. The tubes were incubated for 0, 30, 60 and 90 seconds respectively. The reaction was terminated by the addition of 2.0ml of acid reagent. To the control tube, the enzyme was added after the addition of the acid reagent. The tubes were then heated for 10 minutes in a boiling water bath and the green colour developed was read at 610nm. The activity of catalase is expressed in terms of μmoles of H_2O_2 utilized/minute/g leaf.

ASSAY OF PEROXIDASE

The activity of peroxidase was carried out according to the method of Reddy *et al.* (1995).

PRINCIPLE

In the presence of a hydrogen donor like pyrogallol, peroxidase converts H_2O_2 to water and oxygen. The oxidation of pyrogallol to a colored product called purpurogalli can be followed spectrophotometrically at 420 nm.

REAGENTS

- Phosphate buffer (0.1M, pH 6.5)
- Pyrogallol (0.05M in 0.1M phosphate buffer, pH 6.5)
- H_2O_2 (1% in phosphate buffer, pH 6.5)

PROCEDURE

Fresh BBL and RBL (0.5g) were extracted by homogenization into 2.5ml of phosphate buffer. The homogenates were centrifuged at low speed and the supernatant was used for the assay.

To 3.0ml of buffered pyrogallol solution, 0.5ml of H_2O_2 solution was pipetted out into a cuvette, mixed well and placed in a spectrophotometer. The change in the absorbance was noted for 2 minutes at an interval of 30 seconds to assay the non-enzymic oxidation of pyrogallol. To this, 0.1ml of enzyme extract was added and mixed well. The change in absorbance was again noted for every 30 seconds over a period of 2 minutes. The activity of peroxidase is expressed in terms of μmoles of pyrogallol oxidized / minutes /g leaf.

ASSAY OF GLUTATHIONE PEROXIDASE

The method of Rotruck *et al.* (1973) was followed to assay the activity of glutathione peroxidase (GPx).

PRINCIPLE

The assay is based on the reaction between the leftover glutathione in the reaction with DTNB (dithiobisnitrobenzoic acid) to form a compound that absorbs maximally at 412nm.

REAGENTS

- Sodium phosphate buffer (0.4M, pH 7.0)
- Sodium azide (10mM)

- Hydrogen peroxide (2.5mM)
- Reduced glutathione (4mM)
- Trichloroacetic acid (10%)
- Disodium hydrogen phosphate (0.3M)
- DTNB (0.04% in 1% sodium citrate)
- Reduced glutathione standard: 20mg of reduced glutathione was dissolved in 100ml of 5% TCA.

PROCEDURE

Fresh BBL and RBL (0.5g) were homogenized with 5.0ml of phosphate buffer, centrifuged at 5000g for 10 minutes and the supernatant was taken for the assay.

To 0.2ml of enzyme, 0.4ml of buffer, 0.1ml of sodium azide, 0.2ml of reduced glutathione, 0.1ml of H_2O_2 and 1.0ml of water were added to make up the volume to 2.0ml. The tubes were incubated at 37°C for 10 minutes. The reaction was terminated by the addition of 0.5ml of TCA and centrifuged. To 2.0ml of the supernatant, 3.0ml of disodium hydrogen phosphate solution and 1.0ml of DTNB reagent were added. The colour developed was read at 412nm. A blank was prepared with disodium hydrogen phosphate solution and 1.0ml of the DTNB reagent. Suitable aliquots of the standards were taken and treated in the same manner. The activity is expressed in terms of μmoles of glutathione utilized / minute/g leaf.

ASSAY OF GLUTATHIONE REDUCTASE

The method proposed by David and Richard (1983) was followed to determine the activity of glutathione reductase.

PRINCIPLE

Glutathione reductase catalyses the reduction of oxidized glutathione (GSSG) to reduced glutathione (GSH) employing NADPH as a substrate and is assayed by measuring the decrease in absorbance at 340nm.

REAGENTS

- Potassium phosphate buffer, (0.12M, pH 7.2)
- EDTA (15mM)
- Sodium azide (10mM)
- Oxidized glutathione (6.3 mM)
- NADPH (9.6mM)

PROCEDURE

Fresh BBL and RBL (0.5g) were homogenized in 5.0ml of buffer, centrifuged at 5000g for 10 minutes and the supernatant was used for the assay.

To 0.1ml of the leaf extracts, 1.0ml of potassium phosphate buffer, 0.1ml of EDTA, 0.1 ml of sodium azide, and 0.1ml of oxidized glutathione were added and the volume was made up to 2.0ml with water. The mixture was kept at room temperature for 3 minutes and 0.1ml NADPH was added. The absorbance at 340nm was recorded at intervals of 15 seconds for 2 to 3 minutes. For each series of measurement, controls were done that contained water instead of oxidized glutathione. One unit of GR is expressed as μmoles of NADPH oxidized/minute/g leaf.

ASSAY OF GLUCOSE 6 - PHOSPHATE DEHYDROGENASE (G6PD)

The method of Balinsky and Bernstein (1963) was adopted to assay G6PD.

PRINCIPLE

Glucose 6-phosphate dehydrogenase was assayed by measuring the increase in absorbance which occurs at 340nm due to NADP being reduced to NADPH. This reaction takes place when two electrons are transferred from glucose 6-phosphate to NADP in the reaction catalyzed by the enzyme glucose 6-phosphate dehydrogenase.

REAGENTS

- Tris-HCl buffer (0.1M, pH 8.2)
- NADP (0.2mM)
- $MgCl_2$ (0.1M)
- Glucose 6-phosphate (6mM)

PROCEDURE

Fresh BBL and RBL (0.5g) were ground with 5.0ml of the buffer, centrifuged at 5000g for 10 minutes and the supernatant was taken for the assay.

The reagents, 0.4ml of Tris-HCl buffer, 0.2ml of NADP, 0.2ml of magnesium chloride, and 1.0ml of water, were added to 0.2ml of enzyme in a cuvette. The reaction was started by the addition of 0.2ml of glucose 6-phosphate and the increase in the absorbance was measured at 340nm. The activity of the enzyme is expressed in terms of units/g leaf in which one unit is equal to the amount of enzyme that brought about a change in optical density of 0.01/minute.

ASSAY OF ASCORBATE OXIDASE

Ascorbate oxidase was assayed by the procedure of Oliver *et al.* (1967).

PRINCIPLE

Ascorbic acid oxidase activity was measured by assaying the change in the absorbance at 620nm, caused by the reaction of unutilized ascorbate with DCPIP.

REAGENTS

- Phosphate buffer (0.05M, pH 6.5)
- Phosphate buffer (500 µM, pH 6.8)
- Ascorbic acid (100 µM)
- Dicholorophenol indophenol (DCPIP) (0.4mM)

PROCEDURE

Fresh BBL and RBL (2.0g) were homogenized in 4.0ml of cold phosphate buffer (0.05M). The homogenate was centrifuged at 10,000g at 4°C for 10 minutes. The supernatant was used for the assay.

The assay mixture consisted of 1.0ml of ascorbic acid, 1.0ml of 500μM phosphate buffer and 2.0ml of the enzyme extract. The tubes were incubated at 37°C for 25 minutes. The reaction was stopped by the addition of 0.2ml of DCPIP. A control tube was set up without incubation and treated in a similar manner. The optical density was read at 620nm.

CALCULATION

The enzyme activity was calculated using the formula.

$$\text{Enzyme activity} = \frac{A \times TV}{t \times v}$$

Where A = absorbance of the sample after incubation minus the absorbance at '0' time.

TV = Total volume of the filterate

t = Time (minutes) of incubation with substrate

v = Total volume of filterate taken for incubation

The enzyme activity is expressed in terms of units/g leaf.

ASSAY OF POLYPHENOL OXIDASES

The activities of polyphenol oxidases were assayed by the method of Esterbauer *et al.* (1997).

PRINCIPLE

Phenol oxidases are copper proteins, which catalyse the aerobic oxidation of certain phenolic substrates to quinones, which are autooxidised to dark brown pigments known as melanins, which can be estimated spectrophotometrically at 495nm.

REAGENTS

- Tris - HCl (50mM, pH 7.2)
- Sorbitol (0.4M)
- NaCl (10mM)
- Phosphate buffer (0.1M, pH 6.5)
- Catechol (0.01M)

PROCEDURE

Fresh BBL and RBL (0.5g) were ground with Tris-HCl buffer, sorbitol (146mg) and NaCl (1.42mg) and was made upto 2.0ml, centrifuged at 2000g for 10minutes at 4°C and the supernatant was used for the assay.

Into a cuvette, 2.5ml of phosphate buffer and 0.3ml of catechol solution were added and the spectrophotometer was set to zero at 495nm. Then 0.2ml the enzyme extract was added to the same cuvette and the change in the absorbance was recorded every 30 seconds upto 5 minutes. One unit of either catechol oxidase or laccase is defined as the amount of enzyme that transforms one µmole of dihydrophenol to one µmole of quinone/minute.

The activity of PPO can be calculated using the formula.

Enzyme unit = K x (A / minute)

K for catechol oxidase = 0.272

K for laccase = 0.242

The enzyme activity is expressed as units / g leaf

DETERMINATION OF NON-ENZYMIC ANTIOXIDANTS

Non-enzymic antioxidants such as ascorbic acid, tocopherols, total carotenoids, glutathione (reduced), total phenols and chlorophyll, and the mineral antioxidants (Cu, Mn, Zn and Se) were estimated by the methods, elaborated below.

ESTIMATION OF ASCORBIC ACID

The ascorbic acid levels were estimated in the leaves of BBL and RBL by the method of Roe and Keuther (1943).

PRINCIPLE

Ascorbate is converted to dehydroascorbate by activated charcoal. Dehydroascorbate reacts with 2,4-dinitrophenyl hydrazine to form osazone, which dissolves in sulphuric acid to give an orange colored solution, whose absorbance can be measured at 540nm.

REAGENTS

- TCA (4%)
- Sulphuric acid (9N)
- 2,4-dinitrophenyl hydrazine reagent (2%)
- Thiourea (10%)
- Sulphuric acid (85%)
- Standard ascorbate solution (1mg/ml in 4% TCA)

PROCEDURE

Ascorbate was extracted into 4% TCA by homogenizing 1g of fresh leaves in it and the volume was made upto 10ml with 4% TCA. Particulate debris was removed by centrifugation. A pinch of activated charcoal was added, mixed vigorously using a cyclomixer and let stand for 5 minutes. The tubes were centrifuged again to pellet the charcoal particles. Aliquotes (0.5ml) of the supernatants were used for the estimation.

Aliquots ranging from 0.2-1.0ml of charcoal-treated standard ascorbate solution and the aliquots of the charcoal-treated vitamin extracts were made upto 2.0ml with 4% TCA. Then DNPH reagent (0.5ml) was added to each tube followed by 2 drops of thiourea solution. The contents were mixed and incubated at 37°C for 3 hours. The osazone crystals formed were dissolved by adding 2.5ml of 85% H_2SO_4 drop by drop while mixing, so as to avoid local heating. The tubes were

cooled on ice and the absorbance recorded at 540nm. From the standard curve constructed on an electronic calculator set to the linear regression mode, the concentrations of ascorbate in the samples were calculated and expressed as mg/g leaf.

ESTIMATION OF TOCOPHEROL

Tocopherol content was estimated in the leaves using Emmerie-Engel reaction as explained by Rosenberg (1992).

PRINCIPLE

The Emmerie - Engel reaction is based on the reduction of ferric to ferrous ions by tocopherol, which then forms a red colour with 2,2'-dipyridyl. Tocopherols and carotenes are first extracted with xylene and the extinction is read at 460nm to measure the carotenes. A correction is made for this after adding ferric chloride and read at 520nm.

REAGENTS

- Absolute alcohol
- Xylene
- 2,2'-dipyridyl (1.2g / L in n-propanol)
- Ferric chloride solution (1.2g/L in ethanol)
- Standard solution (D, L - α tocopherol, 10mg/L in absolute ethanol)
- Sulphuric acid (0.1N)

PROCEDURE

Fresh BBL and RBL (2.5g) were ground with a small volume of 0.1N sulphuric acid and the volume of the homogenate was made upto 50ml with 0.1N sulphuric acid. The contents of the flask were extracted overnight. At the end of the extraction period, the homogenate was shaken vigorously and filtered through Whatman No.1. filterpaper. Aliquots of the filterate were used for the estimation of tocopherol.

The leaf extracts (1.5ml), standard (1.5ml) and water (1.5ml) were pipetted out into four stoppered centrifuge tubes respectively. To all the tubes, 1.5ml the ethanol and 1.5ml of xylene were added, mixed and centrifuged. Then 1.0ml of xylene layer was transferred into another stoppered tube and 1.0ml of 2,2'-dipyridyl reagent was added and mixed. Then 1.5ml of the mixture was pipetted out into a spectrophotometer cuvette and the extinction was read at 460nm. Followed by the addition of 0.33ml of ferric chloride solution and after exactly 15 minutes, the absorbance of the red colour produced was read against blank at 520nm. The concentration of tocopherol in the samples were calculated using the formula,

$$\text{Tocopherol } (\mu g) = \frac{\text{Reading of the sample at 520nm} - \text{Reading of the sample at 460nm}}{\text{Reading of the standard at 520nm}} \times 0.29 \times 25$$

The results are expressed as µg of tocopherol / g leaf.

ESTIMATION OF TOTAL CAROTENOIDS

The total carotenoids were estimated by the method of Zakaria *et al.* (1979).

PRINCIPLE

The total carotenoids in the leaf samples were extracted in petroleum ether and estimated spectrophotometrically at 450nm.

REAGENTS

- Petroleum ether (40 to 60°C)
- Anhydrous sodium sulphate
- Alcoholic potassium hydroxide (12%)

PROCEDURE

Care was taken to ensure that all the steps subsequent to the saponification were carried out in the dark to avoid photolysis of carotenoids. About 5.0g of fresh BBL and RBL were homogenized and saponified with 2.5ml of 12% alcoholic potassium hydroxide in a water bath at 60°C for 30 minutes. The saponified extract

was transferred into a separating funnel and 10-15ml of petroleum ether (40-60°C) was added and mixed. The lower aqueous phase was transferred to another separating funnel and the upper petroleum ether containing the carotenoid pigment was collected. The extraction was repeated until the aqueous phase was colourless.

To the petroleum ether extract, a small quantity of anhydrous sodium sulphate was added to remove excess moisture, if any. The absorbance of the yellow colour was read at 450nm using the petroleum ether as a blank. The amount of total carotenoids, was calculated using the formula,

$$\text{Amount of total carotenoids} = \frac{A_{450} \text{ x volume of the sample x } 100 \text{ x } 4}{\text{Weight of the sample}}$$

ESTIMATION OF GLUTATHIONE (REDUCED)

The method of Moron *et al.* (1979) was followed to determine the amount of reduced glutathione.

PRINCIPLE

Reduced glutathione reacts with DTNB (5,5'-dithiobisnitrobenzoic acid) to give a yellow coloured product that absorbs maximally at 412nm.

REAGENTS

- TCA (5%)
- Phosphate buffer (0.2M, pH 8.0)
- DTNB (0.6mM in 0.2M phosphate buffer)
- Standard GSH (10mg of GSH) in 100ml of 5% TCA)

PROCEDURE

Fresh BBL and RBL (0.5g) were homogenized with 2.5ml of 5% TCA. The precipitated protein was centrifuged at 1000g for 10 minutes. Then 0.1ml of the supernatant was used for the estimation of GSH.

The supernatant (0.1ml) was made upto 1.0ml with phosphate buffer then 2.0ml of freshly prepared DTNB solution was added and the intensity of the yellow colour formed was read at 412 nm in a spectrophotometer after 10minutes. A standard curve of GSH was prepared. The values are expressed as μmoles of GSH/g leaf.

ESTIMATION OF TOTAL PHENOLS

The method proposed by Mallick and Singh (1980) was used to determine the total phenols in the leaves.

PRINCIPLE

Phenols react with phosphomolybdic acid in Folin - Ciocalteau reagent in alkaline medium to produce a blue coloured complex that absorbs maximally at 650nm.

REAGENTS

- Ethanol (80%)
- Folin - Ciocalteau reagent (1N)
- Sodium carbonate (20%)
- Standard (catechol 100μg/ml in water)

PROCEDURE

Fresh BBL and RBL (0.5g) were homogenized with 10X volume of 80% ethanol. The homogenate was centrifuged at 10,000g for 20 minutes. The residue was reextracted with 80% ethanol. The pooled supernatants were evaporated to dryness.

The residues were dissolved in a known volume of distilled water and 0.5ml of Folin-Ciocalteau reagent was added to an aliquot of this. After 3 minutes, 2.0ml of 20% sodium carbonate was added and the tubes were placed in a boiling water bath for exactly 1 minute. The absorbance was measured against a reagent blank at 650nm in a spectrophotometer. Standard catechol solutions corresponding to

2.0-10.0µg concentrations were also added with Folin-Ciocalteau reagent and sodium carbonate, and treated similarly. A standard curve was constructed using an electronic calculator on the linear regression mode, using which the concentrations of phenols in the samples were read. The values are expressed as mg phenols/g leaf.

ESTIMATION OF CHLOROPHYLL

The chlorophyll content was determined by the method proposed by Arnon (1949).

PRINCIPLE

The estimation of chlorophyll is based on the absorption coefficient at 663nm and 645nm.

REAGENT

Acetone (80%)

PROCEDURE

Fresh BBL and RBL (1.0g) were extracted with 20ml of 80% acetone. The tubes were centrifuged for 5 minutes at 5000g and the supernatants were transferred to volumetric flasks. The extraction was repeated until the residue was colourless. The supernatant was made upto 100ml with 80% acetone. The absorbance of the green colored solution was read at 645 and 663nm against 80% acetone blank. The total chlorophyll in the leaf was calculated using the formula,

$$\text{Total chlorophyll} = 20.2\,(A_{645}) + 8.02\,(A_{663}) \times \frac{V}{1000 \times W}$$

where V was the final volume of the extract and W was the weight of the leaves taken for extraction. The results are expressed as mg /g leaf.

QUANTIFICATION OF MINERALS

The antioxidant minerals such as Cu, Zn, Mn and Se were estimated by the following methods (De, 1989).

ESTIMATION OF COPPER

The copper was estimated by the neocuproine method.

PRINCIPLE

In neutral or weakly acidic solution (pH 3-9), Cu reacts with 2, 9-dimethyl-1, 10- phenanthroline (neocuproine) to form a complex which is extractable into the $CHCl_3$-CH_3OH mixture to produce a yellow colored solution. The latter can be measured spectrophotometrically at 457nm.

REAGENTS

- H_2SO_4
- HNO_3
- NH_2OH .HCl (1%)
- Neocuproine Reagent-100mg of 2,9-dimethyl 1,10-phenanthroline/100ml CH_3OH
- $CHCl_3$
- CH_3OH

PROCEDURE

To the sample (5g leaves) in a 250ml beaker, 1.0ml of concentrated H_2SO_4 and 5.0ml of concentrated HNO_3 were added. Then heated to SO_3 fumes. Concentrated HNO_3 (5.0ml) was added repeatedly and heated to fumes. It was cooled. Then redistilled water (80ml) was added to it and boiled. The solution was cooled and filtered into a 100ml volumetric flask. The volume was made upto 100ml. An aliquote of 50ml was pipetted out into a 125ml separating funnel and diluted to 100ml with redistilled water. Then 5.0ml of NH_2OH.HCl (1%) and 10ml of sodium citrate (40%) solutions were added and mixed thoroughly, the pH was

adjusted to 4.0-6.0. To this 10ml of neocuproine reagent was added and the contents were shaken for 30 seconds. The $CHCl_3$ extract was taken in a 25ml volumetric flask. The extraction of the aqueous layer with 10ml of $CHCl_3$ was repeated and the $CHCl_3$ extract was transferred into the same volumetric flask. The combined extracts were diluted to 25ml with CH_3OH and the contents were shaken well. The absorbance was measured at 450-460mm against a reagent blank carried through the same procedure.

ESTIMATION OF MANGANESE

Manganese was estimated by persulphate method.

PRINCIPLE

Manganese can be estimated by this method, based on oxidation of Mn^{+2} to MnO_4^- by $(NH_4)_2 S_2O_8$ in presence of $AgNO_3$.

REAGENTS

- HNO_3
- H_2SO_4
- Special Reagent-75g $HgSO_4$ + 200ml 85% H_3PO_4 + 35mg $AgNO_3$ in 1litre of 6N HNO_3
- $(NH_4)_2S_2O_8$

PROCEDURE

The samples (5g leaves) were digested with HNO_3-H_2SO_4 acids. To the digested sample in a 250ml conical flask, 5.0ml of the special reagent was added. The contents were diluted to 100ml with distilled water. Then 1.0g of $(NH_4)_2 S_2O_8$ was added and boiled for 1 minute. The contents were cooled to room temperature and the absorbance was measured at 525nm against a reagent blank.

ESTIMATION OF ZINC

Zinc was estimated by dithizone complex method.

PRINCIPLE

Zinc reacts with dithizone under optimum conditions and forms a red coloured dithizonate complex, measured colorimetrically at 535nm.

REAGENTS

- $KMnO_4$ (5%)
- NH_2OH .HCl (40%)
- Ammoniacal sulphite-cyanide solution - 350ml concentrated NH_4OH +3g KCN +10g Na_2SO_3 diluted to 1litre
- Dithizone solution - 125mg of dithizone in 500ml CCl_4

PROCEDURE

To the sample (5g of leaves) in a flask, 10.0ml of 5% $KMNO_4$ solution and 2–3ml of concentrated H_2SO_4 were added. The contents were refluxed for 4 hours and cooled. Then 40% $NH_2OH.HCl$ solution was added to reduce $KMNO_4$. Then the solutions were filtered and the volume was made up to 100ml.

To the 100ml of the made up solution, 30ml of ammoniacal sulphite-cyanide solution and dithizone solution were added. Then the solutions were extracted vigorously for 20 seconds and the absorbance was measured at 515nm against a blank.

ESTIMATION OF SELENIUM

Selenium was estimated by atomic absorption spectrophotometry.

PRINCIPLE

Selenium in the sample is converted into SeH_2 and aspirated into a ArH_2 flame, the absorption was measured the SeH_2 peak at 196nm.

REAGENTS

- Nitric acid (concentrated)
- Sulphuric acid (18N)

- Perchloric acid
- Hydrochloric acid (concentrated)
- Potassium iodide
- $SnCl_2$
- Zinc slurry - 50g zinc dust (200 mesh) in 100ml of deionized distilled water
- Selenium

PROCEDURE

To the sample (5g leaves) in a 150mL beaker, 10 ml of concentrated nitric acid and 12.0ml of 18N sulphuric acid were added. Then the samples were evaporated to SO_3 fumes. Small amounts of nitric acid was added from time to whenever the red brown fumes of NO_2 disappear in order to avoid loss of Se and maintain the oxidizing conditions. The contents were then cooled and 25ml of dionized distilled water and 1.0ml of $HClO_4$ was added. Again the samples were evaporated to SO_3 fumes and cooled. Then 40.0ml of concentrated HCl was added and made up the volume to 100ml with deionized distilled water.

The treated samples (25ml) were transferred into the reaction vessels. Then 1.0ml of 20% KI solution and 0.5ml of $SnCl_2$ solution were added. This was allowed to stand for 10 minutes. A medicine dropper was filled with 1.5ml of Zn-slurry which was kept in suspension with a magnetic stirrer. A stopper containing the medicine dropper was inserted into the side neck of the reaction vessel. The bulb was then squeezed to introduce the Zn-slurry into the sample. The SeH_2 peak was measured at 196nm.

PHASE II

In the second phase, various *in vitro* cell free systems and *in vivo* simulated *in vitro* models were used to study the antioxidant efficacy of BBL and RBL. Initially, the effect of BBL and RBL extracts on free radicals like DPPH, $SO^{\bullet}$ and $^{\bullet}OH$ and non-radical oxidants such as H_2O_2 and NO were analysed. Further analysis was carried out to find the extent of inhibition on lipid peroxidation,

protective effect on DNA damage and assessment of antioxidant status in goat liver slices in the presence and absence of the oxidants. Finally, the cytotoxic effect against cancer cell line was also carried out.

RADICAL SCAVENGING ACTIVITY OF *Solanum nigrum* LEAVES

The ability of the BBL and RBL extracts in scavenging the oxidative components was evaluated against DPPH stable free radical, superoxide and hydroxyl radicals, and the molecules like hydrogen peroxide and nitric oxide, in cell free systems.

DETERMINATION OF DPPH RADICAL SCAVENGING ACTIVITY

The efficacy of BBL and RBL to scavenge the stable free radical, the DPPH (2,2-diphenyl-1-picryl hydrazyl) was carried out by the method of Mensor *et al.* (2001).

PRINCIPLE

BBL and RBL were tested for their scavenging activity against the stable free radical (DPPH). The antioxidants are able to convert the purple colored DPPH into a yellow colored derivative in a concentration dependent manner. The colour developed can be measured at 518nm.

REAGENTS

- DPPH (0.3mM in methanol)
- Methanol

PROCEDURE

Methanolic solution of DPPH (0.5ml) was added to 20µl of BBL and RBL extracts (corresponding to 4mg) and 0.48ml of methanol and allowed to react at room temperature for 30 minutes. Methanol served as the blank and DPPH in methanol without leaf extracts served as the positive control. After 30 minutes, the absorbance was measured at 518nm and converted into percentage radical scavenging activity.

$$\text{Scavenging activity (\%)} = 100 - \frac{A_{518}\text{ (sample)} - A_{518}\text{ (blank)}}{A_{518}\text{ (blank)}}$$

DETERMINATION OF SUPEROXIDE PRODUCTION *in vitro*

The production of superoxide radical was determined by the method of Winterbourn *et al.* (1975).

PRINCIPLE

This method is based on the inhibition of the production of nitroblue tetrazolium formazone of the superoxide ion by the plant extracts and is measured spectrophotometrically at 560 nm.

REAGENTS

- Phosphate buffer (0.067M, pH 7.8)
- EDTA (0.1M containing 1.5mg NaCN / 100ml)
- Nitroblue tetrazolium (1.5mM)
- Riboflavin (0.12mM)

ASSAY

Leaf extracts (20%) were prepared in 0.067M phosphate buffer. The assay tubes contained 20µl of leaf extract, 0.2ml of EDTA, 0.1ml of nitroblue tetrazolium, 0.05ml of riboflavin and 2.55ml phosphate buffer. Control tubes were set up without the leaf extracts. The initial optical densities of the solutions were recorded at 560nm and the tubes were illuminated uniformly with a fluorescent lamp for 30 minutes. A_{560} was measured again and difference in optical density was taken as the quantum of superoxide production. The percentage inhibition by the leaf samples was calculated by comparing with the optical density of the control tubes.

DETERMINATION OF THE EXTENT OF INHIBITION OF H_2O_2

The ability of the leaf extracts to scavenge H_2O_2 was determined according to the method of Ruch *et al.* (1989).

PRINCIPLE

The H_2O_2 scavenging activity was measured in terms of a decrease in the absorbance at 230nm spectrophotometrically.

REAGENTS

- Phosphate buffer (0.1M, pH 7.4)
- H_2O_2 in phosphate buffer (4mM)

PROCEDURE

A solution of H_2O_2 was prepared in phosphate buffer. H_2O_2 concentration was determined spectrophotometrically from its absorption at 230nm. Plant extracts (1.0mg, 0.01ml) were added to H_2O_2 and incubated for 10 minutes. The absorbance at 230nm was determined against a blank containing phosphate buffer without H_2O_2. The percentage scavenging of H_2O_2 by leaf extracts and standard compounds was calculated using the formula.

$$\% \text{ scavenging of } H_2O_2 = \frac{A \text{ (control)} - A \text{ (sample)}}{A\text{(control)}} \times 100$$

DETERMINATION OF HYDROXYL RADICAL SCAVENGING ACTIVITY

Hydroxyl radical scavenging activity was determined by the method of Elizabeth and Rao (1990) using deoxyribose.

PRINCIPLE

Hydroxyl radical generated from deoxyribose by the action of Fe^{+2}/ ascorbate/ H_2O_2 system, produces thiobarbituric acid reactive substances (TBARS), which can be measured at 535nm in a spectrophotometer.

REAGENTS

- Deoxyribose (2.8mM)
- $FeCl_3$ (0.1mM)
- EDTA (0.1mM)
- H_2O_2 (1.0mM)
- Ascorbate (0.1mM)
- KH_2PO_4 – KOH buffer (20mM, pH 7.4)
- Thiobarbituric acid reagent

PROCEDURE

The reaction mixture (1.0ml) contained 0.1ml of deoxyribose, 0.1ml of $FeCl_3$, 0.1ml of EDTA, 0.1ml of H_2O_2, 0.1ml of ascorbate, 0.1ml of KH_2PO_4 – KOH buffer and 20µl of BBL and RBL leaf extracts (10mg) in a final volume of 1.0ml. Then the reaction mixture was incubated for 1 hour at 37°C. At the end of the incubation period, 1.0ml of TBA was added and heated in a boiling water bath for 20 minutes. The pink colour produced was measured at 535nm. The percent TBARS production for positive control (H_2O_2) was fixed as 100% and the relative percent TBARS was calculated for the other groups.

DETERMINATION OF INHIBITION OF NITRIC OXIDE GENERATION *in vitro*

Nitric oxide generation was measured by the method of 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 is estimated spectrophotometrically at 546nm.

REAGENTS

- Nitropruside (100mM)
- PBS (0.88% NaCl, 0.02% KCl, 0.02% KH_2PO_4 and 0.115% Na_2HPO_4) pH 7.2
- Griess reagent (1% sulphanilamide, 2% H_3PO_4 and 0.1% naphthylenediamine dihydrochloride)

PROCEDURE

The reaction mixture (3.0ml) containing 0.3ml of sodium nitropruside, 2.68ml of PBS and 20µl of BBL and RBL leaf extracts (20mg extract) was incubated at 25°C for 15 minutes. Control tubes were (100% generation) prepared without the leaf extracts. After incubation, 0.5ml of the reaction mixture was removed and 0.5ml of Griess reagent was added to it. The absorbance of the chromophore formed, indicative of the quantum of NO generated was read at 546nm.

In all the studies listed thus far in the Phase II of the study, *Solanum nigrum* leaves exhibited strong radical scavenging activities, the results of which are presented in the next chapter. Since oxidant assault to cells results in biomolecular damage, especially to lipids and DNA, the effect of *Solanum nigrum* leaf extracts on the damage to lipids and DNA were followed. The methods adapted are given below.

DETERMINATION OF THE EXTENT OF INHIBITION OF LPO IN LIVER HOMOGENATE BY THE *Solanum nigrum* LEAVES

In vitro lipid peroxidation was measured by the method of Okhawa *et al.* (1979) in the liver homogenate.

PRINCIPLE

The oxidizing agent, ferrous ions, induces lipid peroxidation (LPO) in goat liver hemogeneate and the LPO can be quantified as the extent of thiobarbituric acid substances (TBARS) formed.

REAGENTS

- Tris-buffered saline (TBS), (10mM Tris, 0.15M NaCl, pH 7.4)
- Ferrous sulphate - ($FeSO_4$ in TBS) (0.16mM) (prepared fresh)
- Thiobarbituric acid (TBA) – 1% (warmed to dissolve)
- Alcohol (70%)
- Acetone

ASSAY

BBL and RBL (0.5g) were weighed and homogenized in 1.0ml of cold TBS. Fresh goat liver was obtained from local slaughter house and washed free of blood. The fat deposits were removed, if any, and a 5% homogenate was prepared in ice cold TBS.

To 50µl of the goat liver homogenate, 50µl of the leaf extracts were added. Ferrous sulphate at a final concentration of 10µmoles was added in the assay medium to induce oxidation. The final volumes in the test tubes were made upto 500µl with TBS.

Control tubes were prepared for each sample, containing 50µl of leaf extract, 50µl of liver homogenate and TBS to make up the final volume to 500µl. Pro-oxidant ($FeSO_4$) was not added to the control tubes. A blank containing no plant extract, no lipid source (liver homogenate) but only ferrous sulphate and TBS to make the final volumes of 500µl was also prepared (100% oxidation). The experimental medium corresponding to autooxidation contained only the liver homogenate. All the tubes were incubated at 37°C for one hour.

Following the incubation period, 500µl of 70% ethanol was added to all the tubes to arrest the reaction. 1.0ml of TBA was added to all the tubes and kept in a

boiling water bath for 20 minutes. After cooling to room temperature, the tubes were centrifuged to clear the solution and the supernatants collected *in toto*. To the supernatant,500µl of acetone was added and the OD was measured at 535nm in a spectrophotometer. The percent inhibition was determined by comparing with the control.

EFFECT OF *Solanum nigrum* LEAVES ON DNA DAMAGE INDUCED BY H_2O_2

The assessment of the effect of BBL and RBL extracts on oxidative damage to DNA of different sources and molecular nature was analyzed. The types of DNA used were

- Linear (Phage) DNA - λ DNA
- Circular double stranded (bacterial, plasmid) DNA - pUC18
- High molecular weight haploid, genomic DNA *in vitro* (herring sperm DNA)
- High molecular weight genomic DNA in intact live cells (KB cells)

EFFECT OF *Solanum nigrum* LEAVES ON H_2O_2 INDUCED DNA DAMAGE

DNA damage was followed by the method elaborated by Chang *et al.* (2002) using λ phage DNA and pUC 18 plasmid DNA.

PRINCIPLE

Damage to DNA is reflected by a difference in the migration pattern on agarose gel, which can be followed as a measure of damage.

REAGENTS

- Tris-HCl buffer (50mM, pH 7.4)
- λ phage DNA / pUC 18 plasmid DNA (2µg in 5µl)
- $FeCl_3$ (500µM)
- Agarose (1%)
- Hydrogen peroxide (30%)

- Et Br (10mg / ml)
- Gel loading dye (0.25% bromophenol blue, 0.25% xylene cyanol, 50% glycerol)
- 50X TAE buffer (Tris base 24.2g, EDTA 18.612g, glacial acetic acid 5.7ml in a total volume of 100ml, pH 8.0).

PROCEDURE

The reaction was conducted in a total volume of 30µl containing 5µl of Tris-HCl buffer, 5µl of λ phage DNA / pUC 18 plasmid DNA, 5µl of extracts of BBL and RBL prepared in tris buffer, 10µl of H_2O_2 and 5µl of $FeCl_3$. The mixtures were incubated at 37°C for 15 minutes for pUC 18 DNA and 30 minutes for λ DNA. The reaction mixture was then placed in 1% agarose gel and run at 100 V for 15 minutes in a submarine gel electrophoretic apparatus. The DNA was visualized and photographed using an Alpha Digidoc digital gel documentation system.

ESTIMATION OF INHIBITION OF DNA DAMAGE USING HERRING SPERM DNA

The extent of damage caused in herring sperm DNA was measured by the method of Aeschlach *et al.* (1994).

PRINCIPLE

The extent of DNA damage caused to herring sperm DNA can be measured spectrophotometrically by the increase in absorbance at 532nm.

REAGENTS

- Tris-HCl buffer (10mM, pH 7.4)
- Hydrogen peroxide (30%)
- Magnesium chloride (5mM)
- Ferric chloride (50µM)
- EDTA (0.1M)
- TBA (1% w/v)

- HCl (25% v/v)
- L-ascorbic acid
- Herring sperm DNA (0.5 mg / ml of Tris-HCl buffer)

PROCEDURE

The reaction mixture contained 0.5ml of herring sperm DNA, 0.167ml of H_2O_2, 0.05ml of $MgCl_2$, 0.05ml of $FeCl_3$ and the leaf extract (10mg) or buffer to the same volume. The mixtures were incubated at 37°C for 1 hour. The reaction was terminated by the addition of 0.05ml of EDTA. The colour was developed by adding 0.5ml of thiobarbituric acid (TBA) and 0.5ml HCl followed by incubation at 37°C for 15 minutes. After centrifugation, the extent of DNA damage was measured by the increase in absorbance at 532nm.

DETERMINATION OF THE EXTENT OF DNA DAMAGE IN INTACT LIVE CELLS

The alkaline single cell gel electrophoresis assay (comet assay) was used to observe the extent of DNA damage in intact cells as proposed by Singh *et al.* (1988). Cultured oral carcinoma cells (KB cell line) were used to study the effect of leaf extract on DNA damage in the intact cells.

The KB cell line was purchased from the National Centre for Cell Sciences (NCCS), Pune, India. The cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS). Confluently grown cells were harvested by trypsinization (0.25%), and then collected into DMEM containing 10% FBS and the cells were held on ice till the assay. Before the assay, the cells were spun down at 2000 rpm for 5 minutes at 4°C in a microfuge and resuspended in 500µl of Hank's Balanced Salt Solution (HBSS).

REAGENTS

- HBSS
- Lysing solution (1M Tris, pH 8.0, 0.5M, EDTA, 2.5M NaCl, DMSO 10%, Triton X-100 1%)

- H_2O_2 (30%)
- Low melting point agarose (LMPA – 0.5%)
- Normal melting point agarose (NMPA – 1%)
- Alkaline electrophoresis buffer (10N NaOH, 0.2M EDTA, pH > 13)
- Neutralizing solution (1M Tris, pH 7.5)
- Et Br (5µg / ml)

PROCEDURE:

The plant sample (100mg) was homogenized in a microfuge tube using a micropestle with 100µl of HBSS. Then 20µl of the homogenate was used for the assay. KB cells were harvested by trypsinisation. 500µl of KB cells in HBSS was incubated in microfuge tubes with plant extracts in the presence and absence of H_2O_2.

The treated KB cells were incubated at 37°C for one hour. At the end of the incubation period, they were taken into 0.5% LMPA (melted and maintained at 37°C) by mixing equal volumes. The suspension (75µl) was layered carefully onto 1% NMPA coated glass slides and overlaid with a layer of LMPA without cells. After solidification, the 'minigels' were placed in cold lysing solution and incubated overnight at 4°C. The lysed cells were denatured in the alkaline electrophoretic buffer for 20 minutes and electrophoresed in the same buffer at 25 volts for 20 minutes. The slides were then neutralized in 1M Tris pH7.5 and stained with ethidium bromide. The slides were then scored for the presence of comet tails under oil immersion using a Nikon fluorescent microscope. Totally 100 cells per slide were scored and noted the frequency of DNA damage in each group.

EFFECT OF *Solanum nigrum* LEAVES ON ANTIOXIDANT STATUS OF THE MAMMALIAN LIVER SLICES

The influence of the leaf extract of *Solanum nigrum* on the enzymic and non-enzymic antioxidant status in the liver tissue was followed using an *in vitro* model of precision-cut liver slices. The model was very carefully designed to simulate *in vivo* intraperitonial exposure of oxidant. This study was formulated in

tune with the recommendations laid down by FRAME (Fund for the Replacement of Animals in Medical Experimentation) to minimize the use of live animals for experimenting and to develop a model system that would simulate *in vivo* conditions (www.frame-uk.demon.co.uk).

The goat liver was selected as the mammalian tissue to determine the antioxidant effect of leaf extracts in the presence and absence of the standard oxidizing compound (CCl_4). The dose of CCl_4 used was the same as the level used in *in vivo* studies by intraperitonial administration (2.0ml/kg tissue).

The liver was collected fresh from a local slaughter house immediately after the sacrifice of the animal. The tissue was quickly plunged into cold sterile HBSS and maintained at 4°C till the assay. Very thin (~1mm) slices were cut using sterile scalpel and the tissue (250mg) was taken in 1.0ml of sterile HBSS, in broad, flat-bottomed flaks. The necessary compounds (CCl_4 and / or leaf extract) were added and incubated at 37°C for one hour with mild shaking. Appropriate control groups were also set up. The standard oxidant CCl_4 was used at a concentration of 2.0ml/kg tissue.

To prepare the leaf extract, 100mg of leaves were taken and homogenized with 100µl of HBSS using a micropestle in a microfuge tube. The tubes were spun at 5000g for 3min and the homogenate free of debris was added at a dose of 20mg (20µl) per ml of HBSS (or 250mg of liver tissue)

After the incubation period, the tissues were homogenized in the same aliquot of the HBSS using a Teflon homogenizer and centrifuged to remove the debris. The supernatant was then used for the estimation of various parameters to assess the antioxidant potential.

The following groups were set up for all the assays

CONTROL GROUP	OXIDATIVE STRESS INDUCED GROUP
• Untreated control • BBL extract alone • RBL extract alone	• CCl_4 treated • CCl_4 + BBL extract • CCl_4 + RBL extract

The enzymic antioxidants assayed were superoxide dismutase, catalase, glutathione peroxidase, glutathione S-transferase, and glutathione reductase and glucose 6-phosphate dehydrogenase. The non-enzymic antioxidants determined were vitamin C, vitamin E, vitamin A and reduced glutathione. Lipid peroxidation was also measured in terms of MDA formed. For each parameter, the incubation of tissue slices with the oxidant and leaf extracts was carried out fresh on the day of the experiment.

ENZYMIC ANTIOXIDANT STATUS IN GOAT LIVER SLICES SUBJECTED TO OXIDATIVE STRESS

The activities of SOD, CAT, GPx, GR and G6PD were assayed using the protocols described in Phase I of this chapter. Instead of the plant source, the liver homogenate prepared after incubation was used as the enzyme source for the assays.

ASSAY OF GLUTATHIONE S-TRANSFERASE (GST)

The method of Habig *et al.* (1974) was used to determine the activity of glutathione S-transferase.

PRINCIPLE

The enzyme was assayed by its ability to conjugate GSH with 1-chloro-2, 4-dinitrobenzene (CDNB), the extent of conjugation causing a proportionate change in the absorption at 340nm.

REAGENTS

- CDNB in ethanol (1mM)
- Glutathione (1mM)
- Phosphate buffer, pH 6.5 (0.1M)

PROCEDURE

The assay was done at 25°C under conditions giving activities linear with respect to incubation time and protein concentrations for at least 3 minutes. The enzyme activity was determined by monitoring the change in the absorbance at 340nm in a spectrophotometer. 0.1ml of both substrates (GSH and CDNB) was taken in 0.1M phosphate buffer at room temperature to make a volume of 2.9ml. The reaction was started by adding 0.1ml of liver homogenate to this mixture. The readings were recorded against a distilled water blank for a minimum period 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%.

GST activity was calculated using the extinction co-efficient of the product formed ($9.6mM^{-1}cm^{-1}$) and the values have been expressed as nmoles of CDNB conjugated/minute/g tissue.

DETERMINATION OF NON-ENZYMIC ANTIOXIDANTS

The non-enzymic antioxidants determined in the tissue homogenate were ascorbic acid (Roe and Keuther, 1943), vitamin E (Rosenberg, 1992), reduced glutathione (Moron *et al.*, 1979) and vitamin A (Bayfield and Cole, 1980). The methods followed for the ascorbate, vitamin E and GSH were the same as those for leaf analysis (Phase I), wherein liver homogenates replaced the leaf homogenates.

ESTIMATION OF VITAMIN A

The level of vitamin A in the liver was estimated by the method described by Bayfield and Cole (1980).

PRINCIPLE

The colour produced by vitamin A, its acetate or palmitate with TCA is proportional in intensity to its concentration, which property is used for its spectrophotometric estimation.

REAGENTS

All reagents were prepared fresh; exposure of samples and reagents to light were avoided at all times.

- Saponification mixture (2N KOH in 90% alcohol)
- Petroleum ether (40^{o}-60^{o}C)
- Anhydrous sodium sulphate
- Chloroform
- TCA reagent (60% TCA in chloroform)
- Standard vitamin A (1.5mg vitamin A palmitate / 10ml of chloroform)

PROCEDURE

To 1.0ml of the liver slice homogenate, 1.0ml of saponification mixture was added. Then the tubes were gently refluxed for 20 minutes at 60^{o}C. The tubes were cooled, 20ml of water was added and mixed well.

Vitamin A was extracted twice with 10ml portions of petroleum ether (40-60^{o}C). Pooled the extracts and washed thoroughly with water. The layers were separated using separating funnels. Sodium sulphate was added to remove excess moisture. 1.0ml of the ether extract was then taken and evaporated to dryness at 60°C. The dried residue was dissolved in 1.0ml of chloroform.

Aliquots of the standards were pipetted out into a series of clean, dry test tubes in the concentration range of 0–7.5μg. The volumes in all the test tubes were made upto 1.0ml with chloroform. From a fast delivery pipette, 2.0ml of TCA reagent was added, rapidly mixing with the contents of the tubes. The absorbance was recorded immediately at 620nm in a spectrophotometer. The procedure was repeated for the sample tubes. A standard graph was constructed and the concentration in the samples was read off. Vitamin A content is expressed as μg/g tissue.

EXTENT OF LIPID PEROXIDATION IN LIVER SLICES

To establish antioxidant effect of the leaf extracts, the lipid peroxidation product was measured in terms of malondialydehyde formed.

ESTIMATION OF LIPID PEROXIDATION

The lipid peroxidation was measured by the method of Nichans and Samuelson, (1968).

PRINCIPLE

During the peroxidation of lipids, malondialdehyde is produced, which reacts with thiobarbituric acid (TBA) to generate a coloured product, which absorbs proportionately at 535nm.

REAGENTS

TCA-TBA-HCl reagent: The reagent mixture comprised of 15% (w/v) TCA, 0.375% (W/V) TBA and 0.25N HCl. The solution was heated mildly to assist the dissolution of the TBA.

PROCEDURE

To 1.0ml of the sample, 2.0ml of TCA-TBA-HCl reagent was added and mixed thoroughly. The solution was heated for 15 minutes in a boiling water bath. After cooling, the flocculent precipitate was removed by centrifugation at 1000g for 10minutes, the absorbance was determined at 535nm against a blank that contained all the reagents minus the sample. The results are expressed as nmoles formed/g tissue which were calculated using an extinction coefficient of the chromophore ($1.56X10^5 M^{-1} cm^{-1}$.).

In vitro CYTOTOXIC EFFECT OF THE LEAF EXTRACTS OF *Solanum nigrum* LEAVES ON KB ORAL CARCINOMA CELLS

To determine the cytotoxic effect of the leaf extracts, MTT (3-(4,5-dimethyl-thiazole-2-yl)-2,5-diphenyl tetrazolium bromide) assay was performed using KB cells by the method of Igarashi and Miyazawa (2001)

PRINCIPLE

MTT is converted by live cells into its formozan derivative. The surviving cell proportion can be determined by the amount of MTT formozan produced, which is measured in an ELISA reader after solubilization with a suitable solvent.

REAGENTS

- Phosphate buffered saline (PBS)
- MTT (3mg/ml in PBS)
- 2-propanol in 0.04N HCl
- H_2O_2 (200mM)

ASSAY

To 100µl of the incubated sample (350µl containing 10^6 KB cells, 87.5µl of H_2O_2, with or without 20µl (20mg of leaf extracts), added 50µl of MTT was added. Then the samples were incubated at 37°C for 3 hours with mild shaking. At the end of incubation period, 200µl of PBS was added to all the samples. The liquid was aspirated carefully after centrifugation at 10,000g for 3 minutes. The cells were resuspended in 200µl of 2-propanol containing 0.04 N HCl overnight in the dark. The absorbance was read at 650nm in an ELISA plate reader. By assuming the optical density of the control cells to be 100% the percent viability of the treated cells was calculated.

PHASE III

In the third phase, the antioxidant potential and the anticancer effects of BBL and RBL were evaluated in order to confirm the results of the *in vitro* studies, under *in vivo* conditions.

EVALUATION OF ANTIOXIDANT POTENTIAL OF *Solanum nigrum* LEAVES IN OXIDANT STRESSED RATS

Female albino rats belonging to Wistar strain were used as the experimental animals to determine the antioxidant effect. Oxidative stress was induced by ethanol-CCl_4. Ethanol was administered to the animals prior to CCl_4 administration, in order to stimulate the activity of cytochrome P450 2E1 (CYP2E1) which is involved in the conversion of CCl_4 into its corresponding highly reactive radical ($CCl_3^{\bullet}$).

Ethanol-induced oxidative stress appears to play a major role in mechanisms by which ethanol causes liver injury. Many pathways have been suggested to contribute to the ability of ethanol to induce a state of oxidative stress. One central pathway appears to the induction of CYP2E1 form of cytochrome P450 enzyme by ethanol.CYP2EI metabolizes and activates many toxicological substrates, including methanol, to more reactive toxic products. CYP2E1 is also an effective generator of reactive oxygen species such as superoxide radical and hydrogen peroxide in the presence of iron catalysts, produces powerful oxidants such as hydroxyl radicals. Thus the consumption of ethanol induces CYP2E1 which leads to the oxidative stress and also inactivates several antioxidants (Kessova and Cederbaum, 2003).

2E1, the isoform of cytochrome P450 metabolize CCl_4 also to the oxidative moiety $CCl_3\cdot$ (Gruebele *et al.,* 1996). The reactive metabolites such as trichloromethyl ($CCl_3\cdot$) and trichloromethyl peroxy ($CCl_3OO\cdot$) radicals emanated from CCl_4 initiate peroxidation of membrane unsaturated fatty acids. This lipid peroxidation of membrane seriously impairs its function and produces liver injury (Rajesh and Latha, 2004). Thus the pretreatment with ethanol resulting in CYP2E1 induction will result in maximal release of oxidative moieties even from a low sub-acute dose of CCl_4, in the liver.

Silymarin, which is a standard antioxidant, was also used to treat the animals in the present experimental set up in order to compare the efficacy of the extracts of BBL and RBL.

EXPERIMENTAL DESIGN

Adult female albino rats of Wistar strain weighing about 180 – 200g were obtained from Small Animal Breeding House of the Agricultural University, Thrissur. The animals were housed in large spacious cages maintained in controlled environment of temperature, humidity and light and dark cycles. They were fed with the standard pellet diet obtained from Hindustan Lever Ltd, Bangalore and water *ad libitum*. They were given a week's time to acclimatize with the laboratory conditions. The animal studies were cleared by the Institute of Animal Ethics Committee approved by CPCSEA(623/02/b/CPCSEA).

GROUPING OF ANIMALS

The animals were divided into 9 groups containing 6 animals in each group. The treatment groups were as follows:

CONTROL GROUPS (without ethanol + CCl_4)	**OXIDATIVE STRESSED GROUPS (with ethanol+ CCl_4)**
• Untreated control • Treated only with BBL extract. • Treated only with RBL extract. • Treated only with silymarin	• Ethanol + CCl_4 • Ethanol + BBL extract + CCl_4 • Ethanol + RBL extract + CCl_4 • Ethanol + Silymarin + CCl_4 • Ethanol alone treated group

The duration of the experimental period was 21 days. Alcohol was administered orally at a dose of 0.5ml of 50% alcohol per day per animal for 21 days. Freshly prepared crude aqueous extracts of BBL and RBL were administered by intragastric intubation at a dose of 250 mg/kg body weight/day for 21 days. The standard antioxidant, silymarin in paraffin oil was administered at a dose of 25mg/kg body weight/ day for 21 days. On the 21st day, a single CCl_4 injection was given subcutaneously at a dose of 0.5ml/kg body weight.

At the end of the experimental period (22nd day), after an overnight fast, the rats were mildly anesthetised using diethyl ether, and then the animals were sacrificed and dissected. The blood was collected by cardiac puncture and it was

allowed to clot and the serum was separated by centrifugation. The samples were stored at -80°C until the analyses.

The liver was removed and cleaned of blood by washing with sterile, ice cold saline. The tissue slices were blotted dry of blood and stored in cryovials containing 0.1M Tris- HCl buffer (pH 7.4) and stored at -80°C till analysis. All the parameters were analyzed within a week to avoid variations with time. A small portion of the liver was cut and fixed in 10% formalin for histopathological study.

Liver function marker enzymes and the lipid profile were analyzed in serum and the antioxidant status and lipid peroxidation products were evaluated in the liver.

ASSAY OF LIVER FUNCTION MARKER ENZYMES IN SERUM

The damage caused by different types of toxicants, infections etc and the protective effect rendered by various sources were identified by assaying the liver function marker enzymes namely aspartate transaminase (AST), alanine transaminase (ALT) and alkaline phosphatase (ALP). All these enzymes were assayed using kits which are available commercially (Dia Sys India Pvt. Ltd., Chennai).

ASSAY OF AST AND ALT

AST and ALT were assayed by the method of Bergmeyer *et al.* (1978).

PRINCIPLE

The enzymes AST and ALT catalyze the transfer of amino group from L-aspartate and L-alanine respectively to 2-ketoglutarate to yield, oxaloacetate / pyruvate along with L - glutamate.

Oxaloacetate/pyruvate can oxidize NADH to NAD^+ in the presence of malate dehydrogenase/lactate dehydrogenase. The conversion of NADH $+H^+$ to NAD^+ decreases the absorbance at 340nm, the rate of which is proportional to the AST/ALT activity.

REAGENTS

The reagent kit contained solution A (aspartate/buffer and enzyme/ coenzyme for AST and alanine/buffer and enzyme/coenzyme for ALT) and solution B (α-ketoglutarate). The working reagent was prepared by mixing 3.0 ml of solution A with 0.3 ml of solution B.

PROCEDURE

To 0.1ml of serum, 1.0 ml of the working reagent was added, mixed and incubated at 37°C for 60 minutes for AST and 30 minutes for ALT and the decrease in the absorbance was read at 340 nm. The enzyme activities were expressed as IU/L

ASSAY OF ALP

The activity of ALP was determined by the method of Schlebusch *et al.* (1974).

PRINCIPLE

ALP catalyses the hydrolysis of p-nitrophenyl phosphate to yellow coloured p-nitrophenol and phosphate at alkaline pH. The change in absorbance, measured at 415 nm, is directly proportional to the enzyme activity.

REAGENTS

The kit contained buffer and p-nitrophenyl phosphate (PNPP) substrate. The working reagent was prepared by mixing one vial of PNPP substrate with 5.0 ml of buffer.

PROCEDURE

To the working reagent (1.0ml), 20µl of serum was added and incubated for 1 minute. The increase in absorbance was measured at 415nm. The ALP activity is expressed as IU/L.

ESTIMATION OF LIPID PROFILE

Cholesterol and triglycerides levels were estimated in serum using the diagnostic kits purchased from Ark Diagnostics Pvt Ltd, Mumbai.

DETERMINATION OF CHOLESTEROL

Serum cholesterol was estimated by the method of Allain *et al.* (1974).

PRINCIPLE

Cholesterol esters are hydrolyzed by cholesterol ester hydrolase to free cholesterol and fatty acids. The free cholesterol produced and the pre-existing ones were oxidized by cholesterol oxidase to 3-cholestenone and H_2O_2. The H_2O_2 in the presence of peroxidase oxidizes the chromogen (4-amino antipyrene and phenol) to a red colored compound which can be read at 510 nm.

REAGENTS

The reagent kit contained solution 1 (buffer/enzyme/chromogen), solution 2 (phenol) and standard cholesterol (200 mg/dl). The working reagent was prepared by mixing equal volumes of solutions 1 and 2.

PROCEDURE

To 0.01ml each of the serum and the standard, 1.0 ml of the working reagent was added, mixed and kept at 37°C for 5 minutes. The colour developed was read at 510 nm against a reagent blank. The serum cholesterol is expressed as mg/dl.

ESTIMATION OF TRIGLYCERIDES

The triglycerides were estimated by the method of Bucolo and David (1973).

PRINCIPLE

Triglycerides are determined after enzymatic hydrolysis with lipases. Peroxidase catalyses the conversion of hydrogen peroxide, 4- amino antipyrine and N-ethyl-N-sulpho propyl-N-anisidine (ESPAS) to a purple colored quinoimine complex, which can be measured at 546 nm.

REAGENTS

The reagent kit contained triglycerides monoreagent and standard (200 mg/dl).

PROCEDURE

To 0.1 ml of the triglyceride monoreagent taken in three tubes marked as blank, standard and test, 0.01 ml of standard and serum were added in the respective tubes and incubated at 37°C for 10 minutes. All the tubes of the test and standards were read against blank at 546 nm. The values are expressed as mg/dl.

ANTIOXIDANT STATUS IN THE LIVER

The antioxidant contents (enzymic and non-enzymic) and the LPO products were determined in the liver of all experimental animals. The liver tissue was minced and 10% homogenate was prepared in 0.1M Tris - HCl buffer (pH 7.5).

The enzymic antioxidants analyzed were SOD, CAT, GPx and GST. The nonenzymic antioxidants (ascorbate, tocopherol, vitamin A and reduced glutathione) were also determined. The procedures used for the above assays were the same as those followed for the liver slice technique, which are elaborated in phases I and II. The contents of cytochromes b_5 and P450 were quantified in the liver homogenates.

The enzyme activities and LPO products were expressed as units/mg protein. Therefore, the protein content was also estimated in liver homogenates.

ESTIMATION OF CYTOCHROME b_5 AND CYTOCHROME P450

The content of cytochromes b_5 and P450 were estimated by the method of Omura and Sato (1964).

PRINCIPLE

The addition of sodium dithionite causes a rapid reduction of cytochrome b_5 which is characterized by an increase in absorbance at 427 nm. This property is used for its estimation.

The carbon monoxide (CO) adducts, formed by the reaction of reduced cytochrome P450 and CO, has an absorbance at 450 nm. This property enables the estimation of this cytochrome.

REAGENTS

- Phosphate buffer (0.1 M, pH 7.4)
- Sodium dithionite
- H_2SO_4 (concentrated)
- Formic acid
- KCl (1.15%)

PROCEDURE

The liver was quickly excised and washed throroughly with ice-cold isotonic KCl (1.15%). It was blotted dry, weighed and homogenized in isotonic KCl and centrifuged at 9500g for 15 minutes at 4°C in a refrigerated centrifuge. The supernant was further spun at 105,000g for 60 minutes at 4°C and the pellet (microsomal fraction) was used for the assay of cytochrome b_5 and cytochrome P450 contents.

The microsomes were suspended in isotonic KCl to obtain a protein concentration of approximately 10mg/ml. This suspension was further diluted with 0.1M phosphate buffer (pH 7.4), to achieve a protein concentration of approximately 2 mg/ml and mixed thoroughly, but gently.

The diluted suspension (3.0ml) was taken in each cuvette and the baseline was adjusted to zero at 427 nm. Addition of a few mgs of sodium dithionite to the sample cuvette resulted in a rapid increase in absorbance at 427 nm as a result of the reduction of cytochrome b_5. The cytochrome b_5 content was calculated by applying the millimolar extinction coefficient of $171 mM^{-1}cm^{-1}$. The results are expressed in terms of nmoles / mg protein.

After reading the optical density at 427nm, a few mg of sodium dithionite was added to the reference cuvette as well, and the contents of both cuvettes were

mixed and redistributed to obtain homogeneity. The baseline was adjusted to zero at 450nm. The contents of the sample cuvette were gassed gently for about 1 minute, with CO purged of oxygen (the CO was generated by the reaction of concentrated sulphuric acid with formic acid). This leads to the formation of CO adduct of cytochrome P450, which has an absorbance maximum at 450nm relative to 490nm. This was then converted to the concentration of cytochrome P450 using the extinction coefficient of 91 mM^{-1} cm^{-1}. The results are expressed in terms of nmoles/mg protein.

ESTIMATION OF TOTAL PROTEINS

The proteins were estimated by the method of Lowry *et al.* (1951).

PRINCIPLE

The blue colour developed by the reduction of phosphomolybdic-phosphotungstic components in the Folin-Ciocalteau reagent by amino acids, tyrosine and tryptophan, present in the protein, with the alkaline cupric tartarate can be measured at 660nm.

REAGENTS

- Sodium carbonate (2% in 0.1N NaOH) (solution 1)
- Copper sulphate (0.5% in 1% potassium sodium tartarate) (solution 2)
- Alkaline copper solution (mixed 50 ml of solution 1 with 1.0 ml of solution 2)
- Folin - Ciocalteau reagent (IN)
- Standard (5.0 mg BSA in 50 ml of 0.1N NaCl)

PROCEDURE

The working standard (0.2ml to 1.0ml) and 0.01ml of the enzyme samples were used for protein estimation. The volumes were made upto 1.0ml with distilled water in all the tubes. A tube with 1.0ml of distilled water served as the blank. To all the tubes 5.0ml of alkaline copper sulphate reagent was added, mixed well and allowed to stand for 30 minutes. Then 0.5ml of Folin-Ciocalteau reagent was

added, mixed well and incubated at 37°C for 3 minutes. The blue color developed was read at 660 nm.

LEVELS OF LIPID PEROXIDATION PRODUCTS IN THE LIVER

In order to asses the antioxidant potential of the crude aqueous extracts of BBL and RBL, the products formed due to lipid peroxidation were quantified. The liver homogenate was subjected to the same steps of TBARS quantification, as explained in phase II (for liver slices).

DETERMINATION OF HYDROPEROXIDES

The method proposed by Mair and Hall (1971) was adopted to estimate the hydroperoxides in the liver.

PRINCIPLE

Hydroperoxides are extracted into chloroform: methanol mixture under nitrogen to avoid aerial oxidation. They are then dissolved in acid chloroform and estimated spectrophotometrically after reaction with iodide and cadmium.

REAGENTS

- Chloroform : methanol (2:1)
- Acetic acid : chloroform (3:2)
- Potassium iodide (6.0g/ 50ml)
- Cadmium acetate (0.5g/ 100ml)

PROCEDURE

The liver homogenate (1.0ml) was mixed thoroughly with 5.0ml of chloroform: methanol mixture and shaken well. Then it was centrifuged at 1000g for 5 minutes to separate the two phases. The lower chloroform layer (3.0ml) was recovered carefully under a stream of nitrogen. To this, 1.0ml of acetic acid: chloroform mixture and 0.05ml of potassium iodide were added. The tubes were stoppered, mixed and kept in the dark at room temperature for 5minutes, followed

by the addition of 3.0ml of cadmium acetate. The solution was mixed and centrifuged at 1000g for 10minutes. The absorbance of the upper phase was read at 353nm against a blank containing the complete assay mixture except the tissue homogenate. The amount of hydroperoxides in the samples was calculated using the molar extinction coefficient of hydroperoxide, which is 1.73 $x10^4M^{-1}$ cm^{-1}. The values are expressed as nmoles/mg protein.

DETERMINATION OF CONJUGATED DIENES

The method of Buege and Aust (1978) was used to estimate the conjugated dienes.

PRINCIPLE

LPO is associated with rearrangement of the double bonds in the polyunsaturated fatty acids, leading to the formation of conjugated dienes, which absorb optimally at 233nm. The level of formation of conjugated dienes reflects the extent of LPO taking place.

REAGENTS

- Chloroform: methanol (2:1)
- Cyclohexane

PROCEDURE

The tissue homogenate (1.0ml) was mixed with 5.0ml of chloroform: methanol mixture. It was shaken well and centrifuged for 5 minutes at 1000g. The upper layer was removed by aspiration and 3.0ml of the lower layer was taken in a test tube and evaporated to dryness by placing in a water bath at 45°C under a stream of nitrogen. The residue left was dissolved after evaporation in 3.0ml of cyclohexane and the absorbance was read at 233nm against cyclohexane blank. The conjugated dienes have a molar extinction coefficient of $2.52x10^4$ $M^{-1}cm^{-1}$ which was used to calculate their concentrations. The values are expressed as nmoles/mg protein.

HISTOPATHOLOGICAL EXAMINATION OF THE LIVER

In order to support the biochemical analyses in the liver, the cellular and tissue architecture of the organ was observed. Small bits of tissues were fixed in 10% formalin immediately after autopsy. The procedure of Luna (1968) was followed for this study.

TISSUE PROCESSING

The tissues were placed in 10% formal saline (10% formalin in 0.9% sodium chloride) for 1 hour to rectify shrinkage due to higher concentration of formalin. They were then left overnight in running water after securing the mouths of the vessels with cotton guaze. The tissues were dehydrated in ascending grades of isopropanol by immersing in 80% isopropanol overnight followed by 100% isopropanol for one hour. The dehydrated tissues were cleared in two changes of xylene, one hour each. Then the tissues were impregnated with histology grade paraffin wax (melting point 58-60°C) at 60°C. The wax impregnated tissues were embedded in paraffin blocks using the same grade wax. The paraffin blocks were mounted and cut with rotary microtome at 6 micron thickness. The sections were floated in tissue floatation bath at 40°C and taken on a glass slide smeared with equal parts of egg albumin and glycerol. The sections were then melted in an incubator at 60°C and after 5 minutes allowed to cool.

TISSUE STAINING

The sections were deparaffinised by immersing in xylene for 10 minutes in a staining jar. The deparffinised sections were washed in 100% isopropanol and stained in Ehrlich's hematoxylin for 8 minutes. After staining in hematoxylin, the sections were washed in tap water and dipped in acid-alcohol (8.3% HCl in 70% alcohol) to remove excess stain. The sections were counterstained in 1% aqueous solution of eosin for 1 minute. The excess stain was washed in tap water and the sections were allowed to dry. Complete dehydration of the stained sections was ensured by placing the section in an incubator at 60°C for 5 minutes. The sections were cooled, and they were mounted in DPX mountant. The cell architecture in the liver was observed under high power objective in a microscope.

EVALUATION OF ANTICANCER EFFECT OF THE LEAVES OF *Solanum nigrum* IN DLA TUMOUR INDUCED MICE

The second part of this phase involved the evaluation of anticancer effect of the two varieties of *Solanum nigrum* leaves, the BBL and RBL, against Dalton's Lymphoma Ascites tumour induced in mice.

The results of the phase I and phase II showed that the leaves of BBL and RBL possess strong antioxidant activity. Therefore these effects were confirmed simultaneously in an *in vivo* experimental system using Swiss albino mice.

EXPERIMENTAL DESIGN

Female Swiss albino mice weighing about 18–22g were procured from the Small Animal Breeding House of the Agricultural University, Thrissur. The mice were maintained under standard laboratory conditions. They were fed with standard animal feed, the pellets supplied by M/s Hindustan Lever Ltd., Bangalore and water *ad libitum.*

PREPARATION OF LEAF EXTRACTS

Fresh leaves of BBL and RBL were collected, washed with water and the crude aqueous extracts were prepared in distilled water.

INDUCTION OF DALTON'S LYMPHOMA ASCITES TUMOUR

To induce tumor in the mice, DLA (Dalton's Lymphoma Ascites) cells were administered as a single injection at a dose of $1x10^6$ DLA cells/mouse intraperitoneally. Dalton's Lymphoma Ascites tumour cells were supplied by the Amala Cancer Institute, Amalanagar, Thrissur, Kerala, India.

TREATMENT GROUPS

The experimental animals were divided into six groups:

The first experimental setup, each group containing four mice, was observed for their lifespan and change in body weight. The second set of experimentswas maintained, each group containing six mice, to analyse the hematological parameters and lipid peroxidation in liver.

CONTROL GROUPS	DLA INDUCED GROUPS
• Control • BBL extract alone • RBL extract alone	• DLA • DLA+ BBL extract • DLA+RBL extract

The crude aqueous extracts of BBL and RBL were administered orally at a dose of 500mg/kg body weight, once per day for 21 days by intragastric intubation. DLA tumor induced animals received the treatment with leaf extracts from the next day of the tumour induction. The treatment was given for a period of 21 days.

At the end of 24 days, the mice (second set) were anesthetized using diethyl ether, and then the animals were sacrificed and dissected. The blood was drawn by cardiac puncture and collected in the tubes containing an anticoagulant (EDTA) and various hematological parameters (Hb, total RBC count, and total and differential WBC count) were analyzed in the blood.

The liver slices were blotted dry and were transferred to cryovials containing 0.1M Tris-HCl buffer (pH 7.4). The lipid peroxidation products were analyzed in the liver tissue.

DETERMINATION OF HEMATOLOGICAL PARAMETERS IN BLOOD

The hemoglobin (Hb) content, and RBC and WBC (total and differential) counts were determined in the whole blood using standard methodologies.

ESTIMATION OF HEMOGLOBIN

Hemoglobin was estimated by cyanmethhemoglobin method proposed by Drabkin and Austin (1932).

PRINCIPLE

The blood is allowed to react with solution of potassium cyanide and potassium ferricyanide (called Drabkin's solution). The reaction yields a product of stable colour-the cyanmethhemoglobin. The intensity of the colour is proportional to the concentration of hemoglobin.

REAGENTS

- Ferricyanide–Cyanide reagent (Drabkin's reagent)–(potassium ferricyanide (200mg), potassium cyanide (50mg) and potassium dihydrogen phosphate (140mg) in a litre of water).
- Cyanomethhemoglobin standard (Span diagnostic, Surat, India).

PROCEDURE

Blood (20µl) was added to 4.0 ml of the ferricyanide reagent. This was allowed to stand for 15 minutes and was read against a reagent blank at 540 nm. The standards were diluted in ferricyanide-cyanide solution to obtain a range of concentrations in the same manner. Blood hemoglobin level is expressed as g/dl.

ENUMERATION OF BLOOD CELL COUNTS

The blood cells were counted by the method of Chesbrough and McArthur, (1972).

ENUMERATION OF RED BLOOD CELLS

PRINCIPLE

The blood specimen is diluted (usually 200 times) with red cell diluting fluid which does not remove the white cells but allows the red cells to be counted under 400x magnification in a known volume of the fluid. Finally the number of cells in undiluted blood is calculated and reported as the number of red cells/mm^3.

REAGENTS

- RBC diluting fluid – (Trisodium citrate – 3.0g, distilled water – 99.0ml and formalin – 1.0ml)

PROCEDURE

The whole blood was taken into the RBC pipette exactly upto the 0.5 mark (Thoma pipette mark 101) and the diluting fluid (formal citrate solution) was

immediately drawn up to the mark 101. The pipette was rotated between the thumb and the forefinger. This gave a dilution of 1:200.

The cover glass was placed in position over the ruled area using gentle pressure. The suspension was mixed thoroughly by rotating the pipette for about a minute, holding it in a horizontal position, and finally shook at sidewise. The fluid was expelled from the stem of the pipette and filled the chamber immediately by holding the pipette at an angle of 45° and slightly touching the tip against the edge of the cover glass. There should not be any bubbles under the cover glass. Then the red corpuscles were allowed to settle for 2 to 3 minutes. The number of RBCs was counted in 180 small squares (4 squares of 16 at each four corners and one of 16 at centre). The cells touching the lower and right hand lines were not counted, but the cells touching the upper and left hand lines were counted. The cells counted are expressed as million cells /mm^3 blood.

CALCULATION

Number of RBCs / mm^3 = Number of cells counted in 5 squares x 10000

ENUMERATION OF WHITE BLOOD CELLS

PRINCIPLE

Blood is diluted with acid solution which removes the red cells by hemolysis and also accentuates the nuclei of the white cells; counting is done with a microscope under the low power (100X magnification) and knowing the volume of fluid examined and dilution of the blood, the number of white cells in undiluted whole blood is calculated and reported as the number of WBCs/mm^3.

REAGENTS

- WBC diluting fluid – (Turk solution) (Acetic acid – 3.0ml and distilled water – 97.0ml).

PROCEDURE

The whole blood was taken upto the mark 0.5 in WBC pipette and diluted upto the mark 11 with WBC fluid as described in RBC counting and filled the counting chamber in the same manner. Then the cells are allowed to settle for 3 minutes. The neubaur counting chamber was used to count the cells in the four corners and each of these 4 sq mm. areas is subdivided into 16 squares by using the low power objective and a medium ocular. While counting, the cells included were those touching the lines on the left and bottom. The difference between the two squares millimeter area should not be more than 10 WBCs. The white blood cells were expressed as thousand cells / mm^3 blood.

CALCULATION

Number of WBCs / mm^3 = Number of cells counted x 50

DETERMINATION OF DIFFERENTIAL COUNT

Differential count is the percent distribution of various white cells in the peripheral blood as determined from a blood smear stained with a polychromatic stain (Leishman stain, Wright's stain and others). Differential count is done with a stained blood smear under the microscope, using the oil-immersion objective (total magnification 1000x).

REAGENTS

- Leishman's Stain - To the Leishman's stain powder (0.2g) in a conical flask (250ml) with a graduation mark at 100ml, acetone free methanol (100ml) was added. The flask was warmed to 50°C with occasional shaking to dissolve the stain. When the stain was dissolved, the acetone free methanol was added to make the volume to 100ml. The solution was filtered and used for staining.
- Buffer water - The disodium hydrogen phosphate (3.76g) and potassium dihydrogen phosphate (2.10g) were dissolved in distilled water (1000ml) and the pH was maintained at 7.0.
- Methanol

PROCEDURE

A drop of well-mixed blood specimen (anticoagulated blood) was transformed to a clean greese free slide. The drop was placed approximately 1cm from the end and of about 5mm diameter.

The slide was placed on a flat table top with the drop of blood on the right. The left edges of the slide were held with the thumb and index finger of the left hand. Then the spreader slide was placed just in front of the drop of blood at an angle of 30^{o} to 45^{o} between the two slides. The spreader was drawn back until it touches the drop of blood. The blood was allowed to run along the edge of the spreader. The spreader was pushed to the end of the slide with a smooth quick movement. The blood smear was dried quickly by waving it rapidly in the air.

The slide was stained soon after making the smear. The smear was fixed with methanol (2 to 3 minutes). Then the smear was covered with diluted Leishman stain for 7-10 minutes. The stain was washed off with the buffered water. Finally, the fresh buffered water was added for 2 to 3 minutes to differentiate the film. The water adhering to the slide was removed and the slide was set in an upright position in a drying rack. Various types of white cells were identified on the basis of the characters as a result of staining with Leishman stain.

- **DETERMINATION OF LIPID PEROXIDATION**

The assay for lipid peroxidation was done following the method of Nichans and Samuelson (1968) explained already in phase II.

PHASE IV

From the previous chapters, it was found that the two varieties were exhibiting a marked antioxidant activity with different types of mechanism of action and also anticancer effects, both *in vitro* and *in vivo*. Following this, an attempt was made to identify the nature of the active components of the leaf extracts which may be responsible for the antioxidative and anticancer effects.

The various analyses carried out were preliminary phytochemical screening, thin layer chromatography (TLC), FT-IR, NMR and GC-MS techniques.

PRELIMINARY PHYTOCHEMICAL SCREENING

The preliminary phytochemical screening was done in the leaves, extracted serially with solvents of increasing polarity. The solvents used were petroleum ether, benzene, chloroform, ethylacetate and methanol. The leaves were collected, washed, blotted free of water and dried. The dried leaves were powdered in a mechanical grinder and sieved. The sieved fine powder was used for solvent extraction.

SOLVENT EXTRACTION

The leaf powder (75g) was successively extracted in cold with 375ml of each solvent (petroleum ether, benzene, ethyl acetate, chloroform, methanol and water) for 3 days. After extraction, each extract was filtered through a muslin cloth. The residue was air dried thoroughly before being subjected to extraction with the next solvent. The filtrates were evaporated to dryness at 40°C in a water bath and stored at 4°C. These extracts were subjected to phytochemical screening tests.

QUALITATIVE PHYTOCHEMICAL ANALYSIS

The extracts prepared serially in various solvents were tested for the presence of alkaloids, phenolics and flavonoids by the method of Khandelwal (2002).

DETECTION OF ALKALOIDS:

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

(b) Dragendroff's Test: A fraction of the extract was treated with Dragendroff's reagent (Indian Research Products, Chennai) and observed for the formation of reddish orange colored precipitate.

(c) Wagner's Test: A fraction of the extract was treated with Wagner's reagent (1.27g of iodine and 2g of potassium iodide in 100ml distilled water) and observed for the formation of reddish brown colored precipitate.

DETECTION OF PHENOLICS:

(a) Ferric Chloride Test: A fraction of the extract was treated with 5% $FeCl_3$ reagent and observed for the formation of a deep blue-black colour.

(b) Lead Acetate Test: A fraction of the extract was treated with 10% lead acetate solution and observed for the formation of white precipitate.

DETECTION OF FLAVONOIDS

(a) Aqueous NaOH Test: A fraction of the extract was treated with 1N aqueous NaOH reagent and observed for the formation of yellow-orange colouration.

(b) H_2SO_4 Test: A fraction of the extract was treated with concentrated H_2SO_4 and observed for the formation of orange colour.

(c) Schinodo's Test: A fraction of the extract was treated with a piece of magnesium turnings followed by a few drops of concentrated HCl and heated slightly. Observed for the formation of dark pink colour.

TLC ANALYSIS OF PHENOLIC AND ALKALOID COMPONENTS

Total phenolic and alkaloid components were fractionated from the leaves by the method of Harborne (1973) and Vitale *et al.* (1995) respectively. Then these fractions and crude aqueous extracts of fresh leaves were subjected to TLC analysis.

PREPARATION OF TOTAL PHENOLIC FRACTION

Fresh leaves (5g) were crushed using a mortar and pestle with 20ml of 80% ethanol at 80°C for 15 minutes. The extract was clarified by centrifugation and used for the analysis of phenols.

PREPARATION OF TOTAL ALKALOID FRACTION

To extract the alkaloids, 25g of leaves were extracted with ethanol: 28% NH_4OH (95.5:20), at room temperature overnight. The extract was filtered and concentrated under reduced pressure to a fumy residue, which was extracted twice with 1N HCl (10ml each) and filtered. Alkaloids were liberated at pH 9.8 by the addition of 0.7M Na_2CO_3, and extracted the solution with methylene chloride (3x5 ml). The organic extract was dried over anhydrous sodium sulphate to yield the total alkaloid fraction.

IDENTIFICATION OF PHENOLICS AND ALKALOIDS BY TLC

Phenolics and alkaloids were identified by the method of Harborne (1973). The extracted fractions were subjected to TLC on silica gel G60 F_{254} from E-Merck, India, as follows.

IDENTIFICATION OF PHENOLS

The total phenolic fraction of the leaves were chromatographed on TLC plates using toluene: ethyl formate: formate (50:40:10) as the solvent system. The presence of phenols on the plate was identified by spraying the plate after development with vanillin-sulphuric acid (2g in 100ml of 1N H_2SO_4). The R_f values of the spots obtained were calculated.

IDENTIFICATION OF ALKALOIDS

The total alkaloid fraction of the leaves were chromatographed on TLC plates using toluene: ethyl acetate: diethlamine (70:20:10) as the solvent system. The plate was then sprayed with Dragendroff's reagent. The R_f values of the spots were calculated.

FT- IR

Infrared light from a suitable source passes through a scanning Michelson inferometer and Fourier Transformation gives a plot of intensity versus frequency. When a sample is placed in the beam, it absorbs particular frequencies, so that their

intensities are reduced in the interferogram and the ensuing Fourier Transform is the infra red absorption spectrum of the sample.

NMR ANALYSIS

The powdered leaf material was also subjected to ^{1}H- NMR (Bruker, 200-mHz in $CdCl_3$, internal standard TMS). The chemical shift values were recorded as S values/ppm, relative to the TMS.

GC- MS SPECTRAL ANALYSIS

The powdered leaf material was analysed using a Shimadzu gas chromatography apparatus (model QP 5000 GC-MS) using a DB-S capillary column (30m) equipped with QP MS detector (EI, 70eV) with helium as a carrier gas at a flow rate of 1ml / minute.

The relative percentage amounts of compounds were calculated from the total peak areas from the apparatus software. The compounds were identified by computer searching, followed by matching the mass spectral data with those held in the database library.

STATISTICAL ANALYSIS OF THE DATA OBTAINED

All the parameters studied were subjected to statistical treatment using Sigma Stat Statistical Package (Version 3.1). One way ANOVA followed by post-hoc analysis using Fischer's LSD to all the parameters under study to test the level of statistical significance.

The values obtained for the various parameters analyzed in the four major phases and the salient observations made during the study are presented in the following chapter.