3. METHODOLOGY

The present study focused on the responses evoked by *B. monnieri* under conditions of oxidative stress. The plan of work and the methodology adopted are presented in this chapter.

The work was carried out in four phases. In Phase I, different parts of *B. monnieri*, namely leaves, stolon and roots, were assessed for their antioxidant potential and free radical scavenging activity. The biomolecular protective effects and the apoptosis-modulating effects of the leaf extract in cell free and *in vitro* systems were studied in Phase II.

Phase III involved assessing the antioxidant status in precision-cut liver slices exposed to oxidative stress and confirming the results in experimental animals. In the final phase of the study, phytochemical analysis of the active component in *B. monnieri* leaves was carried out.

The layout of the study is presented below.

PHASE I

In this phase, the antioxidant status in different parts of *B. monnieri* was assessed. The leaves, stolon and roots of the plantlets were collected from the plants grown in pots in the University campus. They were washed thoroughly in running tap water in order to remove any dirt or soil particles adhered and blotted gently between folds of tissue paper to remove any water droplets. Both enzymic and non-enzymic antioxidants were analysed in all the three parts of the plant.

ENZYMIC ANTIOXIDANTS

The enzymic antioxidants analyzed in the parts of *B. monnieri w*ere superoxide dismutase, catalase, peroxidase, glutathione S-transferase and polyphenol oxidase.

ASSAY OF SUPEROXIDE DISMUTASE (SOD)

SOD was assayed according to the method of Kakkar *et al.* (1984).

PRINCIPLE

The assay of SOD is based on the inhibition of the formation of NADH-phenazine methosulphate-nitroblue tetrazolium formazon. The colour formed at the end of the reaction can be extracted into butanol and measured at 560nm.

REAGENTS

1. Sodium pyrophosphate buffer (0.025M, pH 8.3)
2. Phenazine methosulphate (PMS) (186µM)
3. Nitroblue tetrazolium (NBT) (300µM)
4. NADH (780µM)
5. Glacial acetic acid
6. n-butanol
7. Potassium phosphate buffer (50mM, pH 6.4)

PROCEDURE

PREPARATION OF ENZYME EXTRACT

The different samples, namely leaves, stolon and roots (0.5g), were ground with 3.0ml of potassium phosphate buffer, centrifuged at 2000g for 10 minutes and the supernatants were used for the assay.

ASSAY

The assay mixture contained 1.2ml of sodium pyrophosphate buffer, 0.1ml of PMS, 0.3ml of NBT, 0.2ml of the enzyme preparation and water in a total volume of 2.8ml. The reaction was initiated by the addition of 0.2ml of NADH. The mixture was incubated at 30°C for 90 seconds and arrested by the addition of 1.0ml of glacial acetic acid. The reaction mixture was then shaken with 4.0ml of n-butanol, allowed to stand for 10 minutes and centrifuged. The intensity of the chromogen in the butanol layer was measured at 560nm in a spectrophotometer (Genesys 10-S, USA).

One unit of enzyme activity is defined as the amount of enzyme that gave 50% inhibition of NBT reduction in one minute.

ASSAY OF CATALASE (CAT)

Catalase activity was assayed following the method of Luck (1974).

PRINCIPLE

The UV absorption of hydrogen peroxide can be measured at 240nm, whose absorbance decreases when degraded by the enzyme catalase. From the decrease in absorbance, the enzyme activity can be calculated.

REAGENTS

1. Phosphate buffer : 0.067 M (pH 7.0)
2. Hydrogen peroxide (2mM) in phosphate buffer

PROCEDURE

PREPARATION OF ENZYME EXTRACT

A 20% homogenate of the different parts of *B. monnieri* was prepared in phosphate buffer. The homogenate was centrifuged and the supernatant was used for the enzyme assay.

ASSAY

H_2O_2-phosphate buffer (3.0ml) was taken in an experimental cuvette, followed by the rapid addition of 40µl of enzyme extract and mixed thoroughly. The time required for a decrease in absorbance by 0.05 units was recorded at 240nm in a spectrophotometer (Genesys 10-S, USA). The enzyme solution containing H_2O_2-free phosphate buffer served as control.

One enzyme unit was calculated as the amount of enzyme required to decrease the absorbance at 240nm by 0.05 units.

ASSAY OF PEROXIDASE (POD)

The method proposed by Reddy *et al*. (1995) was adopted for assaying the activity of peroxidase.

PRINCIPLE

In the presence of the hydrogen donor pyrogallol or dianisidine, peroxidase converts H_2O_2 to H_2O and O_2. The oxidation of pyrogallol or dianisidine to a coloured product called purpurogalli can be followed spectrophotometrically at 430nm.

REAGENTS

1. Pyrogallol : 0.05 M in 0.1M phosphate buffer (pH 6.5)
2. H_2O_2 : 1% in 0.1M phosphate buffer, pH 6.5

PROCEDURE

PREPARATION OF ENZYME EXTRACT

A 20% homogenate was prepared in 0.1M phosphate buffer (pH 6.5) from the various parts of the plant, clarified by centrifugation and the supernatant was used for the assay.

ASSAY

To 3.0ml of pyrogallol solution, 0.1ml of the enzyme extract was added and the spectrophotometer was adjusted to read zero at 430 nm. To the test cuvette, 0.5ml of H_2O_2 was added and mixed. The change in absorbance was recorded every 30 seconds up to 3 minutes in a spectrophotometer (Genesys 10-S, USA). One unit of peroxidase is defined as the change in absorbance/minute at 430nm.

ASSAY OF GLUTATHIONE S-TRANSFERASE (GST)

Glutathione S-transferase was assessed by the method of Habig *et al*. (1974).

PRINCIPLE

The enzyme is assayed by its ability to conjugate GSH and CDNB, the extent of conjugation causing a proportionate change in the absorbance at 340nm.

REAGENTS

1. Glutathione (1mM)
2. 1-chloro-2,4-dinitrobenzene (CDNB) (1mM in ethanol)
3. Phosphate buffer (0.1M, pH 6.5)

PROCEDURE

PREPARATION OF ENZYME EXTRACT

The samples (0.5g) were homogenized with 5.0ml of phosphate buffer. The homogenates were centrifuged at 5000rpm for 10 minutes and the supernatants were used for the assay.

ASSAY

The activity of the enzyme was determined by observing the change in absorbance at 340nm. The reaction mixture contained 0.1ml of GSH, 0.1ml of CDNB and phosphate buffer in a total volume of 2.9ml. The reaction was initiated by the addition of 0.1ml of the enzyme extract. The readings were recorded every 15 seconds at 340nm against distilled water blank for a minimum of three minutes in a spectrophotometer (Genesys 10-S, USA). The assay mixture without the extract served as the control to monitor non-specific binding of the substrates.

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

ASSAY OF POLYPHENOL OXIDASE (PPO)

Catechol oxidase and laccase activities were estimated simultaneously by the method of Esterbauer *et al*. (1977).

PRINCIPLE

Phenol oxidases are copper containing proteins that catalyse the aerobic oxidation of phenolic substrates to quinines, which are autooxidized to dark brown pigments known as melanins. These can be estimated spectrophotometrically at 495nm.

REAGENTS

Tris-HCl (50mM, pH 7.2) containing sorbitol (0.4M) and NaCl (10mM)

Phosphate buffer (0.1M, pH 6.5)

Catechol solution (0.01M)

PROCEDURE

PREPARATION OF ENZYME EXTRACT

The enzyme extract was prepared by homogenizing 0.5g of plant tissue in 2.0ml of the extraction medium containing tris HCl, sorbitol and NaCl. The homogenate was centrifuged at 2000g for 10 minutes and the supernatant was used for the assay.

ASSAY

Phosphate buffer (2.5ml) and 0.3ml of catechol solution were added in the cuvette and the spectrophotometer was set at 495nm. The enzyme extract (0.2ml) was added and the change in absorbance was recorded for every 30 seconds up to 5 minutes in a spectrophotometer (Genesys 10-S, USA).

One unit of catechol oxidase or laccase is defined as the amount of enzyme that transforms 1µmole of dihydrophenol to 1µmole of quinone per minute.

The activity of PPO can be calculated using the formula

Enzyme units in the sample = $K \times (\Delta A/\text{minute})$

where, K for catechol oxidase = 0.272

K for laccase = 0.242

NON-ENZYMIC ANTIOXIDANTS

The non-enzymic antioxidants analyzed were ascorbic acid, α-tocopherol, total carotenoids, lycopene, reduced glutathione, total phenols, flavonoids and chlorophyll.

ESTIMATION OF ASCORBIC ACID

Ascorbic acid was analysed by the spectrophotometric method described by Roe and Keuther (1943).

PRINCIPLE

Ascorbate is converted into dehydroascorbate on treatment with activated charcoal, which reacts with 2,4-dinitrophenyl hydrazine to form osazones. These osazones produce an orange coloured solution when dissolved in sulphuric acid, whose absorbance can be measured spectrophotometrically at 540nm.

REAGENTS

1. TCA (4%)
2. 2,4-dinitrophenyl hydrazine reagent (2%) in 9N H_2SO_4
3. Thiourea (10%)
4. Sulphuric acid (85%)
5. Standard ascorbic acid solution: 100µg / ml in 4% TCA

EXTRACTION OF ASCORBIC ACID

Ascorbate was extracted from 1g of the plant sample using 4% TCA and the volume was made up to 10ml with the same. The supernatant obtained after centrifugation at 2000rpm for 10 minutes was treated with a pinch of activated charcoal, shaken vigorously using a cyclomixer and kept for 5 minutes. The charcoal particles were removed by centrifugation and aliquots were used for the estimation.

PROCEDURE

Standard ascorbate ranging between 0.2-1.0ml and 0.5ml and 1.0ml of the supernatant were taken. The volume was made up to 2.0ml with 4% TCA. DNPH reagent (0.5ml) was added to all the tubes, followed by 2 drops of 10% thiourea solution. The contents were mixed and incubated at 37°C for 3 hours resulting in the formation of osazone crystals. The crystals were dissolved in 2.5ml of 85% sulphuric acid, in cold. To the blank alone, DNPH reagent and thiourea were added after the addition of sulphuric acid. The tubes were cooled in ice and the absorbance was read at 540nm in a spectrophotometer (Genesys 10-S, USA).

A standard graph was constructed using an electronic calculator set to the linear regression mode. The concentration of ascorbate in the samples were calculated and expressed in terms of mg/g of sample.

ESTIMATION OF TOCOPHEROL

Tocopherol was estimated in the plant samples by the Emmerie-Engel reaction as reported by Rosenberg (1992).

PRINCIPLE

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

REAGENTS

1. Absolute alcohol
2. Xylene
3. 2,2'-dipyridyl (1.2g/L in n-propanol)
4. Ferric chloride solution (1.2g/L in ethanol)
5. Standard solution (D,L-α-tocopherol, 10mg/L in absolute alcohol)
6. Sulphuric acid (0.1N)

EXTRACTION OF TOCOPHEROL

The plant sample (2.5g) was homogenized in 50ml of 0.1N sulphuric acid and allowed to stand overnight. The contents of the flask were shaken vigorously and filtered through Whatman No.1 filter paper. Aliquots of the filtrate were used for the estimation.

PROCEDURE

Into 3 stoppered centrifuge tubes, 1.5ml of plant extract, 1.5ml of the standard and 1.5ml of water were pipetted out separately. To all the tubes, 1.5ml of ethanol and 1.5ml of xylene were added, mixed well and centrifuged. Xylene (1.0ml) layer was transferred into another stoppered tube. To each tube, 1.0ml of dipyridyl reagent was added and mixed well. The mixture (1.5ml) was pipetted out into a cuvette and the extinction was read at 460nm. Ferric chloride solution (0.33ml) was added to all the tubes and mixed well. The red colour developed was read exactly after 15 minutes at 520nm in a spectrophotometer (Genesys 10-S, USA).

The concentration of tocopherol in the sample was calculated using the formula,

$$\text{Tocopherols } (\mu g) = \frac{\text{Sample } A_{520} - A_{460}}{\text{Standard } A_{520}} \times 0.29 \times 0.15$$

ESTIMATION OF TOTAL CAROTENOIDS AND LYCOPENE

Total carotenoids and lycopene were estimated by the method described by Zakaria *et al*. (1979).

PRINCIPLE

Total carotenoids and lycopene can be extracted in the sample using petroleum ether and estimated at 450nm and 503nm respectively.

REAGENTS

1. Petroleum ether (40ºC - 60ºC)
2. Anhydrous sodium sulphate
3. Calcium carbonate
4. Alcoholic potassium hydroxide (12%)

PROCEDURE

The experiment was carried out in the dark to avoid photolysis of carotenoids once the saponification was complete. The sample (0.5g) was 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 to a separating funnel containing 10-15ml of petroleum ether and mixed well. The lower aqueous layer was then transferred to another separating funnel and the upper petroleum ether layer containing the carotenoids was collected. The extraction was repeated until the aqueous layer became colourless. A small amount of anhydrous sodium sulphate was added to the petroleum ether extract to remove excess moisture. The final volume of the petroleum ether extract was noted. The absorbance of the yellow colour was read in a spectrophotometer (Genesys 10-S, USA) at 450nm and 503nm using petroleum ether as blank. The amount of total carotenoids and lycopene was calculated using the formulae,

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

$$\text{Amount of lycopene} = \frac{3.12 \times A_{503} \times \text{Volume of the sample} \times 100}{\text{Weight of the sample}}$$

The total carotenoids and lycopene were expressed as mg/g of the sample.

ESTIMATION OF REDUCED GLUTATHIONE

Reduced glutathione was determined by the method of Moron *et al.* (1979).

PRINCIPLE

Reduced glutathione on reaction with DTNB (5,5'-dithiobis nitro benzoic acid) produces a yellow coloured product that absorbs at 412nm.

REAGENTS

1. TCA (5%)
2. Phosphate buffer (0.2M, pH 8.0)
3. DTNB (0.6mM in 0.2M phosphate buffer)
4. Standard GSH (10nmoles/ml of 5% TCA)

EXTRACTION OF GLUTATHIONE

A homogenate was prepared with 0.5g of the plant sample with 2.5ml of 5% TCA. The precipitated protein was centrifuged at 1000rpm for 10 minutes. The supernatant (0.1ml) was used for the estimation of GSH.

PROCEDURE

The supernatant (0.1ml) was made up to 1.0ml with 0.2M sodium phosphate buffer (pH 8.0). Standard GSH corresponding to concentrations ranging between 2 and 10 nmoles were also prepared. Two ml of freshly prepared DTNB solution was added and the intensity of the yellow colour developed was measured in a spectrophotometer (Genesys 10-S, USA) at 412nm after 10 minutes. The values are expressed as nmoles GSH/g sample.

ESTIMATION OF TOTAL PHENOLS

The amount of total phenols in the plant tissues was estimated by the method proposed by Mallick and Singh (1980).

PRINCIPLE

Phenols react with phosphomolybdic acid in Folin-Ciocalteau reagent to produce a blue-coloured complex in alkaline medium, which can be estimated spectro-photometrically at 650nm.

REAGENTS

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

PROCEDURE

The sample (0.5g) was homogenized in 10X volume of 80% ethanol. The homogenate was centrifuged at 10,000rpm for 20 minutes. The extraction was repeated with 80% ethanol. The supernatants were pooled and evaporated to dryness. The residue was then dissolved in a known volume of distilled water. Different aliquots were pipetted out and the volume in each tube was made up to 3.0ml with distilled water. Folin-Ciocalteau reagent (0.5ml) was added and the tubes were placed in a boiling water bath for exactly one minute. The tubes were cooled and the absorbance was read at 650nm in a spectrophotometer (Genesys 10-S, USA) against a reagent blank. Standard catechol solutions (0.2-1ml) corresponding to 2.0-10µg concentrations were also treated as above.

The concentration of phenols is expressed as mg/g tissue.

ESTIMATION OF FLAVONOIDS

The method proposed by Cameron *et al.* (1943) was used to extract and estimate flavonoids.

PRINCIPLE

Flavonoids react with vanillin to produce a coloured product, which can be measured spectrophotometrically.

REAGENTS

1. Vanillin reagent (1% in 70% sulphuric acid)
2. Catechin standard (110µg/ml)

EXTRACTION OF FLAVONOIDS

The samples (0.5g) were first extracted with methanol : water mixture (2:1) and secondly with the same mixture in the ratio 1:1. The extracts were shaken well and they were allowed to stand overnight. The supernatants were pooled and the volume was measured. This supernatant was concentrated and then used for the assay.

PROCEDURE

A known volume of the extract was pipetted out and evaporated to dryness. Vanillin reagent (4.0ml) was added and the tubes were heated in a boiling water bath for 15 minutes. Varying concentrations of the standard were also treated in the same manner. The optical density was read in a spectrophotometer (Genesys 10-S, USA) at 340nm. A standard curve was constructed and the concentration of flavonoids in each sample was calculated. The values of flavonoids were expressed as mg/g sample.

ESTIMATION OF CHLOROPHYLL

The chlorophyll content in the various parts of *B. monnieri* was estimated by the method of Witham *et al*. (1971).

PRINCIPLE

Chlorophyll is extracted in 80% acetone and the absorbance is measured at 645nm and 663nm. The amount of chlorophyll is calculated using the absorption coefficient.

REAGENT

Acetone (80%, prechilled)

PROCEDURE

Chlorophyll was extracted from 1g of the sample using 20ml of 80% acetone. The supernatant was transferred to a volumetric flask after centrifugation at 5000rpm for 5

minutes. The extraction was repeated until the residue became colourless. The volume in the flask was made up to 100ml with 80% acetone. The absorbance of the extract was read in a spectrophotometer (Genesys 10-S, USA) at 645 and 663nm against 80% acetone blank. The amount of total chlorophyll in the sample was calculated using the formula,

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

where,

V = final volume of the extract

W = fresh weight of the leaves

The values are expressed as mg chlorophyll/g sample.

The analysis of the enzymic and non-enzymic antioxidants in the leaves, stolon and roots of *B. monnieri* plant revealed that the leaves were the richest source of antioxidants. Hence, only the leaves were analyzed further in the study. In the next part of phase I, the leaves were extracted separately into methanol and chloroform, two solvents with differing polarity. A crude aqueous extract was also prepared and the three different extracts were analysed for their radical scavenging ability against a battery of free radicals and oxidants.

PREPARATION OF PLANT EXTRACTS

Fresh leaves of *B. monnieri* (Plate 1) were collected and 1g of them was homogenized in 10ml of the solvent. The organic extracts were dried at 60°C protected from light. The residue was weighed and dissolved in dimethyl sulfoxide (DMSO) to obtain the desired concentration. Aqueous extracts were prepared fresh.

EVALUATION OF THE RADICAL SCAVENGING EFFECTS OF *B. monnieri* LEAF EXTRACTS

The scavenging effects of *B. monnieri* leaf extracts were evaluated against DPPH, ABTS, hydrogen peroxide, superoxide, nitric oxide and hydroxyl radicals.

DPPH SCAVENGING EFFECTS

The ability of the leaf extracts to scavenge the DPPH radical was tested in a rapid dot-plot screening and quantified using a spectrophotometric assay.

PRINCIPLE

DPPH radical reacts with an antioxidant compound that can donate hydrogen, and gets reduced. DPPH, when acted upon by an antioxidant, is converted into diphenyl-picryl hydrazine. This can be identified by the conversion of purple to light yellow colour.

DOT-PLOT RAPID ASSAY

The rapid screening assay was performed by the method proposed by Soler-Rivas *et al*. (2000).

REAGENTS

1. TLC plates (silica gel 60 F_{254}-Merck)
2. DPPH (0.4mM) in methanol

PROCEDURE

Aliquots of plant extracts (3µl) were spotted carefully on TLC plates and dried for 3 minutes. The sheets bearing the dry spots were placed upside down for 10 seconds in a 0.4mM DPPH solution and the layer was dried. The stained silica layer revealed a purple background with yellow spots, which showed radical scavenging capacity.

DPPH SPECTROPHOTOMETRIC ASSAY

The scavenging ability of the natural antioxidants of the leaves towards the stable free radical DPPH was measured by the method of Mensor *et al*. (2001).

REAGENTS

1. DPPH – 2,2-diphenyl-2-picryl hydrazyl hydrate (0.3mM in methanol)
2. Methanol

PROCEDURE

The leaf extracts (20µl) were added to 0.5ml of methanolic solution of DPPH and 0.48ml of methanol. The mixture was allowed to react at room temperature for 30 minutes. Methanol served as the blank and DPPH in methanol, without the leaf extracts, served as the positive control. After 30 minutes of incubation, the discolouration of the purple colour was measured at 518nm in a spectrophotometer (Genesys 10-S, USA). The radical scavenging activity was calculated as follows:

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

ABTS SCAVENGING EFFECTS

The antioxidant effect of the leaf extracts was studied using ABTS (2,2'-azino-bis-3-ethyl benzthiazoline-6-sulphonic acid) radical cation decolourisation assay according to the method of Shirwaikar *et al*. (2006).

REAGENT

ABTS Solution (7mM with 2.45mM ammonium persulfate)

PROCEDURE

ABTS radical cations ($ABTS^{+}$) were produced by reacting ABTS solution (7mM) with 2.45mM ammonium persulphate. The mixture was allowed to stand in the dark at room temperature for 12-16 hours before use. Aliquots (0.5ml) of the three different extracts were added to 0.3ml of ABTS solution and the final volume was made up to 1ml with ethanol. The absorbance was read at 745nm in a spectrophotometer (Genesys 10-S, USA) and the per cent inhibition was calculated using the formula

$$\text{Inhibition } (\%) = \frac{(\text{Control} - \text{test}) \times 100}{\text{Control}}$$

HYDROGEN PEROXIDE SCAVENGING EFFECTS

The ability of the leaf extracts to scavenge hydrogen peroxide was assessed by the method of Ruch *et al*. (1989).

REAGENTS

1. Phosphate buffer (0.1M, pH 7.4)
2. H_2O_2 (40mM) in phosphate buffer

PROCEDURE

A solution of H_2O_2 (40mM) was prepared in phosphate buffer. Leaf extracts at the concentration of 10mg/10µl were added to H_2O_2 solution (0.6ml) and the total volume was made up to 3ml. The absorbance of the reaction mixture was recorded at 230nm in a spectrophotometer (Genesys 10-S, USA). A blank solution containing phosphate buffer, without H_2O_2 was prepared. The extent of H_2O_2 scavenging of the plant extracts was calculated as

$$\% \text{ scavenging of hydrogen peroxide} = \frac{(A_0 - A_1) \times 100}{A_0}$$

A_0 - Absorbance of control

A_1 - Absorbance in the presence of plant extract

MEASUREMENT OF SUPEROXIDE SCAVENGING ACTIVITY

The superoxide scavenging ability of the extracts was assessed by the method of Winterbourn *et al.* (1975).

PRINCIPLE

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

REAGENTS

1. EDTA (0.1M containing 1.5mg of NaCN)
2. Nitroblue tetrazolium (NBT – 1.5mM)

3. Riboflavin (0.12mM)
4. Phosphate buffer (0.067M, pH 7.6)

PROCEDURE

Superoxide anions were generated in samples that contained in 3.0ml, 0.02ml of the leaf extracts (20mg), 0.2ml of EDTA, 0.1ml of NBT, 0.05ml of riboflavin and 2.64ml of phosphate buffer. The control tubes were also set up where DMSO was added instead of the plant extracts. All the tubes were vortexed and the initial optical density was measured at 560nm in a spectrophotometer (Genesys, 10-S, USA). The tubes were illuminated using a fluorescent lamp for 30 minutes. The absorbance was measured again at 560nm. The difference in absorbance before and after illumination was indicative of superoxide anion scavenging activity.

MEASUREMENT OF NITRIC OXIDE SCAVENGING ACTIVITY

The extent of inhibition of nitric oxide radical generation *in vitro* was followed as per the method reported by Green *et al.* (1982).

PRINCIPLE

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

REAGENTS

1. Sodium nitroprusside (100mM)
2. Phosphate buffered saline (pH 7.4)
3. Griess reagent (1% sulphanilamide, 2% H_3PO_4 and 0.1% naphthylethylene diamine dihydrochloride)

PROCEDURE

The reaction was initiated by adding 2.0ml of sodium nitroprusside, 0.5ml of PBS, 0.5ml of leaf extracts (50mg) and incubated at 25°C for 30 minutes. Griess reagent (0.5ml) was added and incubated for another 30 minutes. Control tubes were prepared

without the extracts. The absorbance was read at 546nm against the reagent blank, in a spectrophotometer (Genesys 10-S, USA).

MEASUREMENT OF HYDROXYL RADICAL SCAVENGING ACTIVITY

The extent of hydroxyl radical scavenging from Fenton reaction was quantified using 2'-deoxyribose oxidative degradation as described by Elizabeth and Rao (1990).

PRINCIPLE

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

REAGENTS

1. Deoxyribose (2.8mM)
2. Ferric chloride (0.1mM)
3. EDTA (0.1mM)
4. H_2O_2 (1mM)
5. Ascorbate (0.1mM)
6. KH_2PO_4-KOH buffer (20mM, pH 7.4)
7. Thiobarbituric acid (1%)

PROCEDURE

The reaction mixture 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 plant extracts in a final volume of 1.0ml. The mixture was incubated at 37°C for 1 hour. At the end of the incubation period, 1.0 ml of TBA was added and heated at 95°C for 20 minutes to develop the colour. After cooling, the TBARS formation was measured spectrophotometrically (Genesys 10-S, USA) at 532nm against an appropriate blank. The hydroxyl radical scavenging activity was determined by comparing the absorbance of the control with that of the samples. The per cent TBARS production for positive control (H_2O_2) was fixed at 100% and the relative per cent TBARS was calculated for the extract treated groups.

PHASE II

The results of phase I, presented in the next chapter, showed that the leaves are the richest source of antioxidants when compared to stolon and roots. Among the three different extracts analysed, the methanolic extract of *B. monnieri* leaves possessed the maximum oxidative radical scavenging activity compared to the aqueous and chloroform extracts.

OXIDATIVE DAMAGE TO BIOMOLECULES

In phase II, an attempt was made to study the extent of oxidative damage to cellular biomolecules like membrane lipids and DNA and its protection by *B. monnieri* leaf extract in cell-free systems and intact cells. The effect of *B. monnieri* leaf extract on different types of cells subjected to oxidative stress was also studied. The methodology adopted is given below.

EVALUATION OF THE EFFECTS OF *B. monnieri* LEAF EXTRACTS ON OXIDANT INDUCED DAMAGE TO LIPIDS

Lipid peroxidation (LPO), a well-established mechanism of cellular injury, is used as an indicator of oxidative stress (Sangeetha *et al.*, 2010).

In the present study, the extent of lipid peroxidation was assessed in three different membrane models, namely goat RBC ghosts (plasma membrane lipids), goat liver homogenate (plasma membrane and intracellular lipids) and liver slices (intact cells).

PRINCIPLE

The extent of formation of thiobarbituric acid reactive substances (TBARS) from the damaged lipids by oxidizing agents can be used as a measure of damage to membrane lipids.

EVALUATION OF LPO IN RBC GHOSTS

REAGENTS

1. Isotonic KCl (1.15%)
2. Hypotonic KCl (0.5%)

3. Tris buffered saline (TBS) (10mM Tris, 0.15M NaCl, pH 7.4)
4. Ferrous sulphate (10µM)
5. Thiobarbituric acid (TBA) (1%)
6. Ascorbic acid (0.06mM)
7. Ethanol (70%)
8. Acetone

PREPARATION OF GOAT RBC GHOSTS

Goat blood (50ml) was collected fresh in a sterile container. The blood was immediately defibrinated using acid-washed stones. The defibrinated blood was transferred to another sterile container and diluted 1:1 with sterile isotonic KCl and transferred to the laboratory on ice. The RBCs were pelleted by centrifuging at 3000 x g for 10 minutes at 4°C. The supernatant was discarded and the pellet was washed thrice with isotonic KCl. The cells were then lysed at 37°C for one hour in hypotonic (0.5%) KCl. After lysis, the lysate was centrifuged at 5000 x g for 10 minutes at 4°C. The pellet obtained was washed repeatedly with hypotonic KCl until most of the haemoglobin was washed off and a pale pink pellet was obtained. The pellet was suspended in 1.5ml of TBS and 50µl aliquots were used for the assay, as described by Dodge *et al.* (1963).

PROCEDURE

The reaction mixture contained 50µl of RBC ghosts, 50µl of *B. monnieri* leaf extracts, 50µl $FeSO_4$ and 100µl of ascorbate in a total volume of 500µl, which was made up with TBS. A blank was prepared without the plant extract and lipid source, but containing only $FeSO_4$, ascorbate and TBS in a final volume of 0.5ml. An assay medium corresponding to 100% oxidation was prepared, which contained all the other constituents except the plant extract. The experimental medium corresponding to auto-oxidation contained only RBC ghosts. All the tubes were incubated at 37°C for one hour. After incubation, the reaction was arrested by adding 0.5ml of 70% alcohol to all the tubes. Then 1.0ml of TBA was added to all the tubes and heated in a boiling water bath for 20 minutes. After cooling to room temperature, the tubes were centrifuged and 0.5ml of acetone was added to the supernatant. The pink colour developed was measured at 535nm in a spectrophotometer (Genesys 10-S, USA).

ESTIMATION OF LPO IN GOAT LIVER HOMOGENATE

Goat liver was procured fresh from the slaughter house and washed free of blood using Tris-HCl buffer (40mM, pH 7.0). A 20% liver homogenate was prepared in the same buffer using a motorized Teflon homogenizer. The homogenate was clarified to remove debris and used as the membrane source for assessing LPO as per the method of Okhawa *et al*. (1979).

REAGENTS

1. KCl (30mM)
2. $FeSO_4$ (0.16mM)
3. Ascorbate (0.06mM)
4. TBA (1%)
5. Acetic acid
6. n-propanol:pyridine (15:1 v/v)

PROCEDURE

The reaction mixture containing 0.1ml of liver homogenate, 0.1 ml of KCl, 0.1ml of $FeSO_4$ and 0.1ml of ascorbate was incubated at 37°C for one hour in the presence (0.1ml corresponding to 50mg) and the absence (0.1ml of KCl) of extracts of *B. monnieri* leaves. To 0.4ml of the reaction mixture, 1.5 ml each of TBA and acetic acid were added and mixed well. The contents were heated in a boiling water bath for 20 minutes. After cooling, 1.0ml of distilled water and 5.0ml of n-propanol and pyridine mixture was added. After centrifugation, the pink coloured chromophore obtained was measured at 532nm in a spectrophotometer (Genesys 10-S, USA). The percentage inhibition of LPO was determined by comparing the results of the control and the test samples.

ESTIMATION OF LPO IN GOAT LIVER SLICES

The extent of LPO in goat liver slices was estimated by the method described by Nichans and Samuelson (1968).

REAGENTS

1. Phosphate buffered saline (PBS)
2. H_2O_2
3. TBA-TCA-HCl reagent (0.375% TBA, 15% TCA, 0.25N HCl)

PREPARATION OF GOAT LIVER SLICES

The goat liver was collected fresh from a slaughter house, plunged into cold sterile PBS and maintained at 4°C till use. Thin slices of 1mm thickness were cut using a sterile scalpel.

PROCEDURE

One gram of goat liver slice was taken in 4.0ml of sterile PBS in a broad flat-bottomed vessel. The oxidising agent H_2O_2 (500µM) and/or the *B. monnieri* leaf extract (500mg/kg tissue) were added and incubated at 37ºC with mild shaking for one hour. Appropriate controls were also set up.

After incubation, the goat liver slices were homogenized in the incubation medium using a Teflon homogenizer and the homogenate was used for the assay. The reaction was terminated by mixing 1.0ml of homogenate and 2.0ml of TBA-TCA-HCl reagent. The contents were incubated in a boiling water bath for 15 minutes and the pink colour developed was estimated at 535nm against a reagent blank, in a spectrophotometer (Genesys 10-S, USA).

EVALUATION OF THE EFFECTS OF *B. monnieri* LEAF EXTRACTS ON OXIDANT INDUCED DAMAGE TO DNA

DNA damage caused due to oxidative stress plays a critical role in carcinogenesis. During oxidative stress *in vivo* or when ROS react with DNA *in vitro*, several types of DNA damages occur, including strand breaks and base lesions (Staruchova *et al*., 2008).

The effect of the aqueous, methanol and chloroform extracts of *B. monnieri* leaves on the oxidant induced DNA damage was assessed in the present study both in cell-free systems and in intact cells.

The extent of DNA damage was assessed *in vitro* in commercially available DNA preparations from different hierarchies and in intact cells. The commercially available preparations included λ DNA (linear phage DNA), pUC18 DNA (circular plasmid DNA), herring sperm DNA (genomic haploid DNA) and calf thymus DNA (diploid eukaryotic DNA). The DNA from intact cells was from human peripheral blood cells.

ESTIMATION OF DNA DAMAGE IN λ DNA AND pUC18 DNA

The method described by Chang *et al*. (2002) was used to assess the DNA damage in λ DNA and pUC18 DNA.

REAGENTS

1. λ DNA
2. pUC18 DNA
3. Tris buffer (30mM, pH 7.4)
4. H_2O_2 (30%)
5. $FeCl_3$ (500μM)
6. Agarose (1%) in 1X TAE buffer
7. EtBr (10mg/ml)
8. Gel loading dye (0.25% bromophenol blue, 0.25% xylene cyanol, 50% glycerol)
9. 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 carried out in tris buffer (pH 7.4) at 37°C. $FeCl_3$ and H_2O_2 react with each other resulting in the generation of hydroxyl radicals. Each reaction mixture contained 5μl of tris buffer or pUC18 DNA / λDNA (2μg) and 5μl of tris buffer or plant extract. $FeCl_3$ (5μl) and 10μl of H_2O_2 were added to test samples and incubated at 37°C for 15 minutes for pUC18 DNA and 30 minutes for λ DNA. To the reaction mixture, 6μl of gel loading dye was added and electrophoresed in 1% agarose gel containing 3μg/ml EtBr, at 100V for 15 minutes. Gels were viewed under transilluminating UV light and photographed using an Alpha Digidoc digital gel documentation system (USA). The integrated density values (IDV) of the bands were recorded using the software of the Alpha Digidoc (Alpha Innotech, UK).

EVALUATION OF DNA DAMAGE IN HERRING SPERM AND CALF THYMUS DNA

The extent of damage to herring sperm DNA was measured according to the procedure of Aeschlach *et al.* (1994).

REAGENTS

1. Herring sperm DNA or calf thymus DNA (0.5mg/ml in 10mM Tris buffer pH 7.4)
2. H_2O_2 (30%)
3. $MgCl_2$ (5mM)
4. $FeCl_3$ (50µM)
5. EDTA (0.1M)
6. TBA (1%)
7. HCl (25%, v/v)

PROCEDURE

The assay mixtures (0.5ml) contained 0.05ml herring sperm DNA or calf thymus DNA, 0.167ml of H_2O_2, 0.05ml of $MgCl_2$, 0.05ml of $FeCl_3$ and leaf extracts (10mg) or buffer of the same volume. The contents were incubated at 37°C for one hour. The reaction was arrested by the addition of 0.05ml of EDTA. The colour was developed by the addition of 0.5ml of TBA and 0.5ml of HCl and incubating at 37°C for 15 minutes. After centrifugation, the extent of DNA damage was measured by an increase in absorbance at 532nm in a spectrophotometer (Genesys 10-S, USA).

DETERMINATION OF DNA DAMAGE IN INTACT CELLS

The extent of DNA damage within single cells was determined by the method of Singh *et al.* (1988). The cells used were human peripheral blood cells. Whole blood was used as the source of lymphocytes.

REAGENTS

1. HBSS (Hank's Balanced Salt Solution)
2. H_2O_2 (30%)
3. Low melting point agarose (LMPA – 0.5%)

4. Normal melting point agarose (NMPA – 1%)
5. Lysis solution (1M tris, pH 8.0, 0.5M EDTA, 2.5M NaCl, 10% DMSO, 1% triton X-100)
6. Alkaline electrophoresis buffer (10N NaOH, 0.2M EDTA, pH > 13)
7. Neutralizing solution (1M Tris, pH 7.5)
8. EtBr (5µg/ml)

PROCEDURE

Defibrinated whole blood (20µl) in 1ml of HBSS was treated with H_2O_2 (200µM) in the presence and the absence of extracts (20mg). The cells were incubated for 1 hour at 37°C. At the end of the incubation period, 150µl of the cell suspension was mixed with equal volume of 0.5% LMPA and maintained at 37°C. This suspension (75µl) was layered carefully onto microscopic slides coated with 1% NMPA and spread evenly using a coverslip (22 × 40 mm). The slides were placed on a slide tray maintained on ice. Once the agarose solidified, the coverslip was gently slid off and overlaid with a layer of LMPA without the cells. The slides were immersed in cold lysing solution and incubated overnight at 4°C. The lysed cells were then denatured in the alkaline electrophoresis buffer for 20 minutes. All the slides were placed in an electrophoresis chamber containing alkaline solution and the electrophoresis was carried out at 25V, 300mA for 20 minutes. The slides were removed and treated with neutralization buffer for 10 minutes (pH 7.5). The slides were stained with ethidium bromide, washed to remove the excess stain and scored for the presence of comet 'tails' under oil immersion in a Nikon fluorescence microscope (Japan). At least 100 cells/slide were scored and the frequency of DNA damage was noted from the number of comet-bearing cells.

EFFECT OF *B. monnieri* LEAF EXTRACT ON OXIDANT INDUCED APOPTOSIS

Oxidative stress is a state of imbalance between the generation of ROS and the level of antioxidant defense system. The oxidants formed as a natural by-product of enzymatic oxidase action, contributes to the background level of cellular oxidative stress. Excessive ROS are able to produce cellular membrane lipid peroxidation, lipid–protein interaction alteration, enzyme inactivation and DNA breakage, and in the end, to cause cell injury, apoptosis or necrosis. They can elevate oxidative stress beyond the protective

capacity of endogenous antioxidant defenses and induce apoptotic cell death (Liu *et al*., 2010a).

In the first part of the phase II analyses, of the three different extracts analysed, the methanolic extract of *B. monnieri* leaves showed the maximum effect in protecting the biomolecules from oxidative stress both in cell-free systems and in intact cells. Therefore, the methanolic extract alone was used to study the effect on the events of apoptosis in different types of cells such as *Saccharomyces cerevisiae*, primary chick embryo fibroblasts and cancer cell line. These cells were subjected to oxidative stress and the effect of the extract in counteracting this stress was assessed by analyzing characteristic apoptotic events.

Apoptosis is an active cell death defined by many morphological and biochemical events resulting in physiological cell loss from an organism. Cell shrinkage, nuclear condensation, DNA degradation into specific DNA fragments, membrane blebbing and the formation of small cell bodies surrounded by intact cell membranes are typical features of apoptosis.

The oxidant hydrogen peroxide was used to induce apoptosis in the yeast cells. The standard chemotherapeutic drug etoposide, which causes apoptotic cell death by inducing oxidative stress, was used in primary cultured chick embryo fibroblasts and Hep2 cells.

The treatment groups set up to study the events of apoptosis in the cell types were:

- Untreated control cells
- H_2O_2 / etoposide treated (positive control) cells
- Methanolic extract of *B. monnieri* leaves treated cells
- H_2O_2 / etoposide + methanolic extract of *B. monnieri* leaves treated cells.

The extent of cell death and the characteristic morphological and nuclear changes relative to apoptosis-related events were assessed in all the groups.

CULTURING OF YEAST CELLS

YPD medium (10g of yeast extract, 20g of peptone and 20g of dextrose, in 1000ml distilled water, pH 6.5) was prepared and sterilized by autoclaving after

aliquoting. The aliquots were cooled, stored at room temperature till use and were regularly checked for contamination.

Yeast cells were inoculated in the medium on the penultimate day of each assay and the flask was incubated in a temperature controlled orbital shaker at 30°C overnight. The medium was separated by centrifuging at 1000g for 15 minutes. The cells collected in the pellet were washed twice with saline. The pellet was then resuspended in saline. Aliquots containing 10^6 cells (counted using Neubauer ruling) were incubated for one hour at 30°C with or without H_2O_2 (200μM) and the leaf extract (20mg). A smear was made from the treated cells and used for various staining techniques, whereas the cells in suspension were used to determine the viability.

CULTURING OF CHICK EMBRYO FIBROBLASTS

REAGENTS

1. PBS (Phosphate buffered saline) - pH 7.4
2. Trypsin-EDTA (0.25% trypsin in 1mM EDTA)

PROCEDURE

An egg containing a live 8 or 9-day-old chick embryo was taken in a beaker with the blunt end up and swabbed with ethanol. The top of the egg was carefully punctured with the point of a pair of sterile scissors and a circle of shell was cut away carefully to expose the underlying membrane (the chorioallantois). With a second pair of sterile scissors, the chorioallantoic membrane was carefully cut and removed, to expose the embryo.

With the help of a sterile metal hook or a bent glass rod, the embryo was gently lifted by the neck, and placed in a 100mm Petri dish containing phosphate buffered saline. The embryo was washed several times with sterile PBS to remove all yolk and/or blood. It was then transferred to a clean dish containing PBS.

Using two sterile forceps, the head, limbs and viscera were removed. Care was taken to remove the entire limb by pulling at the proximal end. The remaining tissues of the embryo were placed in another sterile dish and washed with PBS. Then the embryo was finely minced with scissors. The minced tissue was transferred to a flask containing

PBS. After allowing the tissue pieces to settle, the PBS was removed with a sterile pipette. Two ml of trypsin was added and the solution was stirred gently for 15-20 minutes, at 37°C.

Fresh DMEM (PAA) containing 10% FBS (PAA) was used to resuspend the pellet. From the suspension, 20µl was taken to determine cell count and viability by trypan blue exclusion in a haemocytometer. The cells were then seeded in 25cm^2 plastic culture flasks containing DMEM + 10% FBS to a final concentration of 10^5 live cells / ml. The viability always ranged between 90-95%. The cultures were incubated at 37°C in 5% CO_2 and 95% humid atmosphere in a CO_2 incubator (Napco, UK).

CULTURING OF Hep2 CELLS

The Hep2 cell line was procured from the National Centre for Cell Science (NCCS), Pune, India. The cells were maintained in CO_2 incubator with 5% CO_2 and 95% humidity (Innoia CO-170, UK), supplemented with DMEM and 10% FBS. Penicillin and streptomycin (PAA) was also added to the medium to 1X final concentration from a 100X stock.

After the cells had attained confluent growth, the cells were trypsinized using trypsin-EDTA (PAA) and the required number of cells was seeded into sterile 6-well and 96-well plates for carrying out the various assays. Before the cells were seeded, a clean dry sterile cover slip was placed in each well of the 6-well plates. The plates were incubated in a CO_2 incubator in a 5% CO_2 and 95% humidity atmosphere.

Exposure to etoposide at a concentration of 200µM was given for 24 hours at 37°C. Plant extract was given at a concentration of 20mg. The cells were treated with the oxidant both in the presence and the absence of the leaf extract.

After treatment, the cover slip alone was removed, placed over a glass slide, sealed with Vaseline and used for various staining techniques. In the 96-well plates, the medium was removed and replaced with fresh medium and used for checking the viability of the cells.

EFFECT OF *B. monnieri* LEAF EXTRACT IN OXIDATIVE STRESS INDUCED APOPTOTIC EVENTS

The apoptotic events that occurred in the oxidative stress induced cells both in the presence and the absence of *B. monnieri* leaf extract were assessed by performing cytotoxicity assays and the morphological and nuclear changes were assessed by using various microscopic techniques.

CYTOTOXICITY ASSAY

The extent of cytotoxicity in the oxidant induced cells both in the presence and the absence of the leaf extract was determined by the MTT dye reduction assay as described by 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 by 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.

REAGENTS

1. PBS (phosphate buffered saline)
2. MTT – 3mg/ml in PBS
3. Isopropanol in 0.04N HCl (acid-propanol)

PROCEDURE

The treated cells (100 µl), were incubated with 50µl of MTT at 37°C for 3 hours. After incubation, 200µl of PBS was added to all the samples. The liquid was then carefully aspirated. Then 200µl of acid-propanol was added and left overnight in the dark. The absorbance was read at 650nm in a micro titer plate reader (Anthos 2020, Austria). The optical density of the control cells were fixed to be 100% viability and the per cent viability of the cells in the other treatment groups were calculated.

CELL VIABILITY ASSAY

The extent of survival in the cells induced with oxidative stress both in the presence and the absence of leaf extracts was studied by sulphorhodamine assay as proposed by Skehan *et al*. (1990).

PRINCIPLE

Sulphorhodamine B (SRB) is a bright pink aminoxanthene dye with two sulphonic groups. Under mildly acidic conditions, SRB binds to protein basic amino acids in TCA fixed cells to provide a sensitive index of cellular protein content, which is directly proportional to cell viability. The SRB assay provides a sensitive measure of drug-induced cytotoxicity and is useful in quantitating clonogenicity and is well suited to high volume, automated drug screening.

REAGENTS

1. PBS (phosphate buffered saline)
2. 40% TCA
3. Sulphorhodamine B - 0.4% in 1% TCA
4. 1% acetic acid
5. 10mM Tris (pH 10.5)

PROCEDURE

A portion of 350µl of ice cold 40% TCA was layered on top of the treated cells and incubated at 4^{o}C for one hour. The cells were then washed 5 times with 200µl of cold PBS. The buffer was removed, 350µl of SRB stain was added to each well and left in contact with the cells for 30 minutes at room temperature. The unbound dye was removed by washing 4 times with 350µl portions of 1% acetic acid. Then 10mM tris (350µl) was added to each well to solubilize the protein-bound dye and the plate was shaken gently for 20 minutes. The tris layer in each well was transferred to a new 96-well plate and the absorbance was read in a micro titre plate reader (Anthos 2020, Austria) at 492nm. The cell survival was calculated as the per cent absorbance compared to the control (untreated) cells.

APOPTOTIC MORPHOLOGICAL CHANGES IN THE CELLS

Cardinal morphological features of apoptotic cells include shrinkage, condensation of chromatin and cytoplasm, detachment of the cells from the neighbouring cells, fragmentation of the nucleus and membrane blebbing.

The morphological changes in the cells were followed in the presence and the absence of the leaf extract and/or the oxidant. The cells were fixed and stained with giemsa for 10 minutes and observed under the phase contrast microscope (Nikon, Japan) as explained by Chih *et al*. (2001).

REAGENTS

1. PBS (phosphate buffered saline)
2. Liquid giemsa stain (1:2 dilution in PBS)

PROCEDURE

The diluted giemsa stain (10µl) was added to the slide and spread by placing another cover slip over it. The cells were then observed for morphological changes using a phase contrast microscope (Nikon, Japan) at 400X magnification. The apoptotic ratio was calculated using the formula,

$$\text{Apoptotic ratio} = \frac{\text{Number of apoptotic cells}}{\text{Number of normal cells}}$$

NUCLEAR CHANGES DURING APOPTOSIS

Changes during the initial phase of apoptosis are associated with chromatin condensation around the nuclear membrane. These nuclear changes were observed in the cells in the presence and the absence of leaf extracts and / or oxidant by propidium iodide, ethidium bromide and DAPI staining.

PROPIDIUM IODIDE (PI) STAINING

The nuclear changes in the apoptotic cells were observed by PI staining as described by Sarker *et al*. (2000).

PRINCIPLE

Propidium iodide is a fluorescent molecule that intercalates into nucleic acids and stains the nuclear changes of apoptotic cells.

REAGENTS

1. PBS (phosphate buffered saline)
2. Acetone : Methanol (1:1)
3. Propidium iodide (PI) - 5µg / ml in PBS

PROCEDURE

The treated cells were washed with PBS to remove traces of medium and serum. The cells were permeabilized with 50µl of acetone : methanol (1:1) mixture at −20°C for 10 minutes. Then 10µl of PI was added, spread by placing a coverslip over it and incubated at 37°C for 30 minutes in the dark.

The apoptotic cells with fragmented nuclei were detected using the green filter of a fluorescence microscope (Nikon, Japan) at 400X magnification. The apoptotic ratio was calculated by the formula mentioned earlier.

ETHIDIUM BROMIDE (EtBr) STAINING

The method proposed by Mercille and Massie (1994) was followed to detect the nuclear changes in apoptotic cells, with minor modifications.

PRINCIPLE

Ethidium bromide intercalates into nucleic acids and can be used to visualize the nuclear changes in apoptotic cells.

REAGENTS

1. PBS (phosphate buffered saline)
2. Ethidum bromide- 50µg / ml in PBS

PROCEDURE

EtBr (10µl) was added to the treated cells and spread by placing a coverslip over it. The slides were incubated for 5 minutes at room temperature. The apoptotic cells with condensed chromatin and fragmented nuclei were counted by using fluorescent microscope (Nikon, Japan) using G-2A filter at 400X magnification. The apoptotic ratio was calculated as before.

DAPI STAINING

Apoptotic cells were detected with DAPI (4'-6'-diamidino-2-phenyl indole) staining technique as explained by Rashmi *et al*. (2003).

PRINCIPLE

DAPI forms fluorescent complexes with double-stranded DNA. The apoptotic nuclei (intensely stained, fragmented nuclei and condensed chromatin) can be scored in the dying cells by DAPI staining. The presence of nuclear apoptotic bodies and chromatin margination can also be observed after DAPI staining.

REAGENTS

1. PBS (Phosphate buffered saline)
2. Paraformaldehyde (3%) in PBS
3. Triton X-100 (0.2%) in PBS
4. DAPI - 1µg / ml in PBS

PROCEDURE

After the oxidant and/or plant extract treatment, the cells were fixed with 3% paraformaldehyde (50µl) for 10 minutes at room temperature. The fixed cells were then permeabilized with 0.2% Triton X-100 (50µl) for 10 minutes at room temperature and incubated for 3 minutes with 10µl DAPI after placing a coverslip over the cells to enable uniform spreading of the stain.

The apoptotic ratio was determined by counting the cells with condensed chromatin and fragmented nuclei under inverted fluorescent microscope (Moticam, Hong

Kong) using DAPI filter at 400X magnification. The apoptotic ratio was calculated as given earlier.

DNA FRAGMENTATION

The biochemical hallmark of apoptosis is the fragmentation of DNA at the linker regions between nucleosomes. DNA fragmentation that occurs during apoptosis breaks the chromosomal DNA to smaller fragments with 3' overhang. These fragments, when run on an agarose gel, form a typical laddering pattern after electrophoresis. This fragmentation, which generally occurs at the late phase of apoptosis, was analysed using agarose gel electrophoresis as proposed by Yin *et al.* (1994).

REAGENTS

1. Phosphate buffered saline
2. Lysis buffer (10mM Tris HCl, 10mM EDTA, 0.2%, Triton X-100, pH 7.5)
3. Buffer saturated phenol
4. Chloroform : Isoamylalcohol (24:1), prepared fresh
5. NaCl (4M)
6. Ice cold ethanol (70%)
7. RNase A (0.6 mg/ml)
8. Agarose (NMPA) (2%)
9. TAE buffer (50X, pH 8.0) -Tris 2M, glacial acetic acid 5.7%, sodium EDTA 0.1M
10. TE buffer (10mM tris, pH 8.0 and 1mM EDTA)
11. Ethidium bromide (10mg / ml)

PROCEDURE

Cells were treated with the oxidant and/or plant extract for one hour. The tubes were centrifuged after incubation and washed twice with cold PBS. The cell pellet was lysed in 1ml lysis buffer. After 10 minutes on ice, the lysate was centrifuged at 13,000g for 10 minutes at 4°C. The nucleic acids in the supernatant were extracted with phenol-chloroform-isoamyl alcohol in the ratio of 25:24:1. For every 500µl of aqueous phase, 37.5µl of 4M NaCl was added and the nucleic acids were precipitated with two volumes

of ice cold ethanol at -20°C overnight. The pellet was then rinsed with ice cold 70% ethanol, air dried and dissolved in 20µl of TE buffer.

After digestion of RNA with RNase A (0.6 mg/ml) at 37°C for 30 minutes, the samples were electrophoresed in 2% agarose gel with TAE buffer. The DNA was visualized with ethidium bromide stain under UV transilluminator and documented using Alpha Digidoc gel documentation system (Alpha Innotech, USA).

PHASE III

EVALUATION OF THE ANTIOXIDANT POTENTIAL OF *B. monnieri* LEAVES

Having seen the effect of *B. monnieri* leaves in cell-free systems and intact cells and also on the events of apoptosis in different types of cells, it was felt crucial to analyse the antioxidant potential of the plant both under *in vitro* and *in vivo* conditions.

EFFECT OF *B. monnieri* LEAVES IN AN *in vitro* MODEL EXPOSED TO OXIDATIVE STRESS

During the last two decades, substantial efforts have been made towards the development and international acceptance of alternative methods to safety studies using laboratory animals. Animal testing should cause as little suffering to animals as possible, and those animal tests should only be performed where necessary.

In order to minimize the use of live animals, in the present study, precision cut liver slices were used as an *in vitro* model. Precision-cut liver slices are an appropriate model of *in vitro* systems for many reasons, including simplicity and ease of preparation, retention of normal organ architecture, and the ability to obtain multiple slices from each organ (Catania *et al*., 2007).

The liver slices were subjected to oxidative stress and the effect of the methanolic extract of *B. monnieri* leaves in counteracting this stress was assessed by analyzing the antioxidant status.

The layout of the tissue preparation and the treatment with oxidant and/or the plant extracts were the same as explained earlier (for LPO in intact cells). The oxidant used was H_2O_2 at a final concentration of 500µM. The slices exposed to different treatments were incubated for one hour at 37°C with mild shaking.

After incubation, a homogenate was prepared in PBS, held on ice and the antioxidant status was analyzed. For each parameter, the incubation of tissue slices with oxidant and plant extracts was carried out separately and fresh on the day of the experiment.

ANTIOXIDANT STATUS IN GOAT LIVER SLICES EXPOSED TO OXIDATIVE STRESS

ENZYMIC ANTIOXIDANTS

The enzymic antioxidants that were analyzed were SOD, CAT, peroxidase, glutathione S-transferase and glutathione reductase. The methodology adopted for assessing the activities of SOD, CAT, POD and GST was the same, which is explained in phase I of the study. Liver homogenate was used as enzyme source for the assays instead of plant extracts.

ASSAY OF GLUTATHIONE REDUCTASE

Glutathione reductase was assayed by the procedure adopted by David and Richard (1983).

PRINCIPLE

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

REAGENTS

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

PROCEDURE

The assay system contained 1ml of phosphate buffer, 0.1ml of EDTA, 0.1ml of sodium azide, 0.1ml of oxidized glutathione and 0.1ml of enzyme source and the volume

was made up to 2ml with distilled water. The tubes were incubated for 3 minutes and 0.1ml of NADPH was added. The absorbance was read at 340nm in a spectrophotometer (Genesys 10UV) at every 15 seconds interval for 2-3 minutes. For each series of measurement, controls were set up that contained water instead of oxidized glutathione. The enzyme activity was expressed as μmoles of NADPH oxidized/minute/g liver tissue.

DETERMINATION OF NON-ENZYMIC ANTIOXIDANTS

The non-enzymic antioxidants determined in the tissue homogenate were ascorbic acid, vitamin E, reduced glutathione and vitamin A.

The procedures for the estimation of ascorbate, vitamin E and reduced glutathione were the same as those used in phase I study, except that the liver homogenate was the sample used for the assay.

ESTIMATION OF VITAMIN A

Vitamin A was estimated by the method of Bayfield and Cole (1980).

PRINCIPLE

The assay is based on the spectrophotometeric estimation of the colour produced by vitamin A acetate or palmitate with TCA.

REAGENTS

1. Saponification mixture (2N KOH in 90% alcohol)
2. Petroleum ether (40°-60°C)
3. Anhydrous sodium sulphate
4. Chloroform
5. Vitamin A palmitate
6. TCA reagent (60% TCA in chloroform) – prepared fresh.

PROCEDURE

All procedures were carried out in the dark to avoid the interference of light. Liver homogenate (1.0ml) was mixed with 1.0ml of saponification mixture and refluxed for 20 minutes at 60°C in the dark. The tubes were cooled and 20ml of water was added and

mixed well. Vitamin A was extracted twice with 10ml of (40°-60°C) petroleum ether. The two extracts were pooled and washed thoroughly with water. Anhydrous sodium sulphate was added to remove excess moisture. An aliquot of the extract (1.0ml) was taken and evaporated to dryness at 60°C. The residue was dissolved in 1.0ml chloroform. Standards (vitamin A palmitate) of concentrations ranging from 0-7.5µg were pipetted out into a series of test tubes.

The volume in all the tubes was made up to 1.0ml with chloroform. TCA reagent (2.0ml) was added rapidly, mixed and the absorbance was read immediately at 620nm in a spectrophotometer (Genesys 10UV). The same procedure was repeated for the sample tubes also. Vitamin A content was expressed as µg/g tissue.

ASSESSING THE ANTIOXIDANT POTENTIAL OF *B. monnieri* LEAVES *in vivo*

After assessing the antioxidant potential of *B. monnieri* leaf extract in the *in vitro* model (goat liver slices), it was felt imperative to conduct the studies on experimental animals. This was because, under *in vivo* conditions, several physiological and biochemical events influence the absorption, distribution, metabolism and elimination of the compounds under study, which may, in turn, affect their efficacy. The layout of the study was as follows.

EXPERIMENTAL DESIGN

Healthy male Wistar albino rats aged 6-8 weeks were procured from Small Animal Breeding Station, Trissur. The animals were randomly divided into seven groups of six animals each after an acclimatization period of two weeks. They were fed with standard pellet diet and 10% alcohol in drinking water *ad libitum*. The protocol was approved by the Institutional Animal Ethics Committee (623/02/b/CPCSEA).

TREATMENT GROUPS

The oxidant used to induce stress *in vivo* was CCl_4, which is metabolized to the oxidative moiety by the cytochrome P450 2E1 (CYP2E1) isoenzyme. In the animals treated with oxidant, a 20 day pretreatment with alcohol (10%) in drinking water was given to induce CYP2E1 activity. CCl_4 was administered as a single subcutaneous injection (2.0ml/kg body weight) diluted 1:1 in paraffin oil on the 21st day. The

methanolic extract of *B. monnieri* leaves was prepared such that the final concentration was 500mg/ml. The methanol was evaporated and the residue was resuspended in water for gavage feeding. The plant extract was administered at a dose of 500mg/kg body weight. The experimental design was as follows:

Group I	:	Control
Group II	:	Alcohol
Group III	:	Alcohol + CCl_4
Group IV	:	Alcohol + methanolic extract of *B. monnieri* leaves
Group V	:	Alcohol + methanolic extract of *B. monnieri* leaves + CCl_4
Group VI	:	Alcohol + Silymarin
Group VII	:	Alcohol + Silymarin + CCl_4

Silymarin (a standard hepatoprotective antioxidant) was given at a dose of 25mg/kg body weight/day. Both the plant extracts and silymarin were administrated by gastric intubation (gavage) for 21 days. After the treatment period of 21 days, the animals were sacrificed on the 22^{nd} day by cervical dislocation.

The animals were quickly dissected and blood was collected by cardiac puncture. Serum was separated by centrifugation at 2000g at room temperature and plunged in cryovials at -85°C (Ilshin, Korea).

The liver and kidney were immediately excised and stored in 0.1M ice cold tris-HCl buffer (pH 7.4) in cryovials at -85°C till the assay. Serum samples were analyzed for liver marker enzymes and lipid profile and the tissues were examined for their antioxidant status.

ASSAY OF SERUM MARKER ENZYMES

The marker enzymes for hepatic damage, namely aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP) and γ-glutamyl transpeptidase (γ-GT), were estimated in the serum. All these enzymes were assayed using kits procured from Span Diagnostics Limited, Sachin, India.

ASSAY OF AST AND ALT (Bergermeyer *et al.*, 1978)

PRINCIPLE

This assay is based on the principle that AST and ALT catalyse the transfer of amino group from L-aspartate/L-alanine to α-ketoglutarate to yield oxaloacetate/pyruvate respectively. Oxaloacetate/pyruvate can oxidise NADH to NAD^+ in the presence of malate dehydrogenase/lactate dehydrogenase. The decrease in absorbance at 340nm in a spectrophotometer (Genesys 10-S, USA) due to the oxidation of NADH is monitored kinetically and is proportional to AST/ALT activity.

REAGENTS

1. Reagent 1 – Buffer

Tris (80 mmol/l pH 7.8)

L-aspartate or L-alanine (240 mmol/L)

MDH ≥ 600 U/L

LDH ≥ 600 U/L

2. Reagent 2 – Substrate

2-oxoglutarate (12 mmol/L)

NADH (0.18 mmol/L)

3. Working reagent

Four parts of reagent 1 were mixed with one part of reagent 2.

PROCEDURE

To 100µl of serum, 1000µl of working reagent was added. The tubes were mixed well and the absorbance was read after 60 seconds and the change in absorbance was measured for 2 minutes at 340nm in a spectrophotometer (Genesys 10-S, USA). AST/ALT activity is expressed as IU/L.

ASSAY OF ALP (Schlebusch *et al.*, 1974)

PRINCIPLE

At alkaline pH, ALP catalyzes the hydrolysis of p-nitrophenyl phosphate to yellow coloured p-nitro phenolate and phosphate; the change in absorbance measured at 415nm is directly proportional to the enzyme activity.

REAGENTS

1. p-nitrophenyl phosphate (PNPP)
2. Buffer

The working reagent was prepared by mixing one vial of PNPP substrate with 5.0ml buffer.

PROCEDURE

To 20µl of serum, 1.0ml of working reagent was mixed and after one minute, the increase in absorbance was measured at 415nm in a spectrophotometer (Genesys 10-S, USA). The ALP activity is expressed as IU/L.

ASSAY OF γ–GT (Persijn and van der Slik, 1978)

PRINCIPLE

γ-GT catalyzes the transfer of amino group between L-γ-glutamyl-3-carboxy-4-nitroanilide and glycylglycine to form L-γ-glutamyl glycylglycine and 5-amino-2-nitrobenzoate, which is measured as an increase in absorbance proportional to the γ-GT activity in the sample.

REAGENTS

1. Buffer
2. Substrate (L-γ-glutamyl-3-carboxy-4-nitroanilide)
3. Working reagent : The substrate tablet was dissolved in 2.2ml of buffer

PROCEDURE

Working reagent (1.0ml) was incubated at assay temperature (37°C) for one minute and 0.1ml of sample was added. The contents were mixed well and the initial absorbance was read at 405nm in a spectrophotometer (Genesys 10-S, USA) after one minute and the absorbance reading was repeated after every 1, 2 and 3 minutes. The mean absorbance change per minute was calculated (ΔA/minute) and enzyme activity is expressed as IU/L.

ESTIMATION OF LIPID PROFILE

The levels of cholesterol, triglycerides, free fatty acids and phospholipids were estimated in the serum samples. The kits used for these assays were purchased from Span Diagnostics Ltd., Sachin, India.

ESTIMATION OF CHOLESTEROL (Allain *et al.*, 1974)

PRINCIPLE

Cholesterol esters are hydrolysed to cholesterol and fatty acids. The cholesterol is oxidized by cholesterol oxidase to form cholesten-3-one and H_2O_2. This H_2O_2 oxidizes 4-amino antipyrene and phenol to a red coloured compound, which can be measured at 505nm.

REAGENTS

1. Reagent 1

1. Buffer (50 mmol/L pH 6.7)
2. Cholesterol oxidase ≥ 50 U/L
3. Cholesterol esterase ≥ 100 U/L
4. Peroxidase ≥ 3 IU/L
5. 4-amino antipyrine (0.4 mmol/L)

2. Reagent 2

Cholesterol standard (200 mg/dL)

PROCEDURE

To 1.0ml of cholesterol reagent, 10µl of serum and standards were added separately. The contents were mixed well and incubated at 37°C for 10 minutes. The absorbance of the standard and sample was measured against reagent blank at 505nm within 60 minutes. The serum cholesterol is expressed as mg/dl.

ESTIMATION OF TRIGLYCERIDES (Bucolo and David, 1973)

PRINCIPLE

Triglycerides are hydrolysed by lipases to glycerol and free fatty acids. Glycerol is converted to H_2O_2 and dehydroxyacetone phosphate. H_2O_2 combines with 4-chlorophenol to form a pink coloured complex, whose absorbance is measured at 500nm.

REAGENTS

1.	Buffer	Magnesium chloride	9.8 mmol/L
		PIPES	100 mmol/L
		Chloro-4-phenol	3.5 mmol/L
2.	Enzymes	Lipase	≥ 1000 IU/L
		POD	≥ 1700 IU/L
		Glycerol 3 phosphate oxidase	≥ 3000 IU/L
		Glycerol kinase	≥ 600 IU/L
3.	4-amino-antipyrine (PAP)		0.5 mmol/L
4.	Adenosine triphosphate Na (ATP)		1.3 mmol/L
5.	Standard	Glycerol	2.8 mmol/L
		Triglycerides	200 mg/dL

PROCEDURE

To three tubes, namely blank, calibrator and assay tubes, 300µl of buffer was added and 3µl of sample was added only to the assay tube. The contents were mixed vigorously and allowed to stand for 10 minutes at 37°C. The absorbance was recorded at 546nm in a spectrophotometer (Genesys 10-S, USA) against blank. The values are expressed as mg/dL.

ESTIMATION OF FREE FATTY ACIDS (Falhot *et al.*, 1973)

PRINCIPLE

Lipids are solubilized in the presence of phosphate buffer, and mixed with high density copper reagent (pH 8.1). The copper soap remaining in the upper organic layer is determined spectrophotometrically with diphenylcarbazide at 550nm.

REAGENTS

1. Phosphate buffer (33 moles/L, pH 6.4)
2. Extracting solvent (chloroform : hexane : methanol in the ratio 5:5:1)
3. Copper reagent (500nmoles/L each of $CuSO_4$, triethanolamine, sodium hydroxide, sodium chloride, pH 8.1)
4. Standard palmitic acid (2mM/L)
5. Diphenyl carbazide

PROCEDURE

To 0.1ml of serum, 1.0ml of phosphate buffer, 6.0ml of extracting solvent and 2.5ml of copper reagent were added and mixed well. The tubes were mixed vigorously for 90 seconds and left for 15 minutes. The contents were centrifuged and 3.0ml of the upper layer was transferred to another tube containing 0.5ml of diphenyl carbazide and mixed. Standard palmitic acid was treated with copper reagent and diphenyl carbazide. The absorbance was measured at 550nm in a spectrophotometer (Genesys 10-S, USA) against phosphate buffer as blank. The serum free fatty acids are expressed as mg/dL.

ESTIMATION OF PHOSPHOLIPIDS (Zilversmit and Davis, 1950)

PRINCIPLE

Phospholipids are digested with sulphuric acid and the inorganic phosphorus formed reacts with ammonium molybdate and aminonaphthol sulphonic acid (ANSA) to form a blue colour, which is measured at 680nm.

REAGENTS

1. Sulphuric acid (5N)
2. Concentrated HNO_3
3. Ammonium molybdate (2.5%)
4. ANSA (0.1%)
5. Standard phosphate (KH_2PO_4 to give a final concentration of 8µg of phosphorus per ml)

PROCEDURE

To 0.1ml of serum, 1.0ml of 5N H_2SO_4 was added and digested in a digestion flask until a light brown colour was formed. Concentrated HNO_3 (2-3 drops) was added and digested until the solution became colourless. Then 1.0ml of water was added and heated in a boiling water bath for 5 minutes. It was followed by 1.0ml of ammonium molybdate and 0.1ml of ANSA and the volume was made up to 10ml with distilled water. Standards were also treated similarly and the absorbance was measured at 680nm in a spectrophotometer (Genesys 10-S, USA) within 10 minutes. The serum phospholipids are expressed as mg/dL.

ANTIOXIDANT STATUS IN THE LIVER AND THE KIDNEY OF OXIDANT CHALLENGED RATS

The antioxidant status was assessed in the liver and the kidney of the experimental rats. A known weight of the tissue was homogenized in 0.1M ice cold tris-HCl buffer (pH 7.5) to give a 10% homogenate and used as the source of antioxidants.

The enzymic antioxidants analysed were SOD, CAT, POD, GST and GR. The non-enzymic antioxidants analysed were vitamin C, vitamin E, vitamin A and reduced glutathione. The procedures for the assay of the enzymic and the non-enzymic antioxidants were the same as those adopted for phase I and phase II studies. Protein thiols were also estimated. The DPPH scavenging activity by the liver of experimental animals was analyzed by DPPH assay as elaborated in Phase I of the study.

The enzyme activities are expressed as specific activity (units / mg protein). The protein content of the enzyme extracts was estimated using the protocol proposed by Lowry *et al.* (1951).

DETERMINATION OF PROTEIN THIOLS

Sedlack and Lindsey (1968) have outlined the procedure for the estimation of protein thiols.

PRINCIPLE

The sulphydryl groups in tissues can be determined using Ellman's reagent. In this method, DTNB is reduced by SH group to form one mole of 2-nitro-5-mercapto benzoic acid per mole of SH.

REAGENTS

1. Tris-EDTA (0.2M, pH 8.0)
2. DTNB (0.1M)
3. Methanol
4. Standard glutathione (50mg/100ml of 0.2M Tris-EDTA, pH 8.0)

PROCEDURE

PREPARATION OF HOMOGENATE

The tissue (1.0g) was homogenized with 5ml of 0.2M Tris-EDTA (pH 8.0).

ASSAY

The reaction mixture contained 0.2ml of the homogenate, 1.5ml of buffer (0.2M tris, pH 8.0, containing 0.2M EDTA) and 1.0ml of DTNB and made upto 10.0ml with absolute methanol. Two tubes containing a reagent blank without sample and another without DTNB were stoppered and allowed to stand for 15 minutes with occasional shaking at room temperature. The reaction mixture was then centrifuged at 3000g for 15 minutes and absorbance measured at 420nm in a spectrophotometer (Genesys 10-S, USA). The level of total thiols minus glutathione levels gives the amount of protein thiols in the tissue. Protein thiols are expressed as mg/g tissue.

ASSAY OF LIPID PEROXIDATION

The extent of lipid peroxidation was estimated according to the method of Okhawa *et al.* (1979).

PRINCIPLE

Malondialdehyde (MDA) formed from the breakdown of polyunsaturated fatty acids serves as a convenient index for the determination of the extent of peroxidation reaction. MDA, a product of lipid peroxidation reacts with TBA (thiobarbituric acid) to give a pink coloured product having absorption maxima at 535nm.

REAGENTS

1. TCA (10%)
2. TBA (0.1M)
3. Phosphate buffer (0.12M, pH 7.2)

PROCEDURE

A 20% liver homogenate was prepared in phosphate buffer (pH 7.2). To 0.5ml of the homogenate, 1.0ml of TCA and 1.0ml of TBA were added and mixed thoroughly. The

mixture was heated in a boiling water bath for 20 minutes. The tubes were centrifuged at 1000g for 10 minutes and the absorbance was read at 535nm in a spectrophotometer (Genesys 10-S, USA) against a blank containing all the reagents except the homogenate. The MDA equivalents of the samples were calculated using the extinction coefficient $1.56 \times 10^5\ M^{-1}cm^{-1}$.

ESTIMATION OF PROTEIN (Lowry *et al.*, 1951)

PRINCIPLE

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

REAGENTS

1. Sodium carbonate (2% in 0.1N NaOH)
2. Copper sulphate (0.5% in 1% potassium sodium tartarate)
3. Alkaline copper solution: 50ml of solution 1 was mixed with 1ml of solution 2.
4. Folin-Ciocaltaeu reagent (1N)
5. Standard (100µg BSA/mL)
6. Tris buffer (0.1M, pH 7.5)

PROCEDURE

The enzyme preparation from each assay was used as the protein source for estimation.

Standards corresponding to 0.2 to 1.0ml (20-100µg) were pipetted out into a series of test tubes and 10µl of the enzyme extract was used for the estimation. The volume was made up to 1.0ml in all the tubes with distilled water. To this, 5.0ml of solution 3 was added, mixed well and incubated at 37°C for 3 minutes. Then 0.5ml of Folin-Ciocalteau reagent was added, mixed well and incubated at 37°C for 3 minutes. The blue colour developed was read at 660nm in a spectrophotometer (Genesys 10-S, USA).

PHASE IV

The results of the first three phases of the study revealed that *B. monnieri* leaf extract possessed good antioxidant potential and was able to scavenge free radicals. The extract was also able to overcome oxidative stress in cell free systems, *in vitro* models and *in vivo* systems. Hence, in the final phase of the study, phytochemical analysis was carried out to identify the active principle responsible for the protection against oxidative stress induced damage.

PRELIMINARY PHYTOCHEMICAL SCREENING

The methanolic extract of the leaves of *B. monnieri*, was screened for the presence of phytochemicals according to 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 chlorate and 5g of potassium iodide in 100ml distilled water) and noted for a cream coloured precipitate.

b) **Dragendroff's test:** A fraction of the extract was treated with Dragendroff's reagent and observed for the formation of reddish orange 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 of distilled water) and observed for the formation of reddish brown precipitate.

DETECTION OF PHENOLIC COMPOUNDS

a) **Ferric chloride test:** A fraction of the extract was treated with 5% $FeCl_3$ solution and observed for the formation of deep blue 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:** To a fraction of the extract, 1N aqueous NaOH was added and observed for the formation of yellow-orange colour.

b) **Concentrated H_2SO_4 test:** To a small fraction of the extract, concentrated H_2SO_4 was added and observed the orange colour formed.

c) **Schinodo's test:** To a small fraction of the extract, a piece of magnesium turnings was added, followed by concentrated HCl and then heated slightly and the formation of dark pink colour was observed.

DETECTION OF SAPONINS

a) **Foam test:** A fraction of the extract was vigorously shaken with water and observed for persistent foam.

b) Haemolytic test: A fraction of the extract was added with a drop of blood placed on a glass slide and observed for the haemolytic zone.

EXTRACTION OF ALKALOID, PHENOLIC, FLAVONOID AND SAPONIN FRACTIONS

TOTAL ALKALOID FRACTION

Fresh leaves of *B. monnieri* (5g) were extracted with 20ml of ethanol:28% NH_4OH, (95:5) 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. The solution was extracted with methylene chloride (3×5ml). The organic extract was dried over anhydrous sodium sulphate to yield the total alkaloid fraction.

TOTAL PHENOL FRACTION

Fresh leaves (1g) were crushed using a mortar and pestle and extracted with 20ml of 80% ethanol at 80°C for 15 minutes. The extract was clarified by centrifugation and used for the analysis of phenols.

FLAVONOID FRACTION

The phenolic extract was further extracted with petroleum ether (3×5ml), when the flavonoids were present in the aqueous fraction.

SAPONIN FRACTION

Fresh leaves (20g) were crushed, transferred to a conical flask, and 200ml of 20% aqueous ethanol was added. The sample was heated over a hot water bath for 4 hours with continuous stirring at 55°C. The mixture was filtered and re-extracted with another 200ml of 20% ethanol. The combined extracts were reduced to 40ml over a water bath at about 90°C. The concentrate was transferred into a 250ml separating funnel, 20ml of diethyl ether was added and shaken vigorously. The aqueous layer was recovered and the ether layer was discarded. The extraction was repeated twice with the addition of n-butanol. The combined n-butanol extract was washed twice with 10ml of 5% NaCl. The remaining solution was heated in a water bath, evaporated and dried in an oven. The saponin content was calculated as gram percentage.

All the four fractions were then subjected to TLC analysis. Aliquots (3µl) of the extracts were spotted onto the TLC plates and subjected to separation using different solvent mixtures specific for the components as described by Harborne (1973).

TLC OF ALKALOIDS, PHENOLICS, FLAVONOIDS AND SAPONINS

The extracted fractions were subjected to Thin Layer Chromatography on silica gel G60 F_{254} plates (EMerck). The alkaloid fraction was developed with CH_2Cl_2 : ethanol : 28% ammonium hydroxide (85:14:1) and sprayed with Dragendroff's reagent. Phenolics were separated with acetic acid : chloroform (45:5) and flavonoids with n-butanol : acetic acid : water (4:1:5) and both were detected with vanillin-H_2SO_4 (10% vanillin in ethanol : concentrated sulphuric acid in 2:1 ratio) spray reagent. The saponin fraction was developed with chloroform: methanol: water (13:7:2) and detected with 10% H_2SO_4.

The R_f values of the spots were calculated as the ratio of the distance travelled by the solute to that by the solvent front.

SPECTRAL ANALYSIS

A preliminary absorption spectral analysis was done by a survey scan of the methanolic extract of *B. monnieri* in a nanospectrophotometer (Optizen, Korea). The instrument was set to scan mode and the absorption spectrum was obtained in the range of 190nm – 1100nm.

HPTLC ANALYSIS

The methanol residue (100mg) of *B. monnieri* leaves was dissolved in methanol (1ml) and centrifuged at 3000rpm for 5 minutes. The supernatant was collected and used for HPTLC analysis. The test sample (3µl) was loaded as an 8mm band in the 5 × 10 Silica gel G60 F_{254} plate using a Hamilton syringe and CAMAG INOMAT 5 instrument. After saturation with the solvent vapour, the TLC plate loaded with test and the reference was kept in a TLC twin trough developing chamber with the respective mobile phase (given below) and developed up to 90mm.

The developed plates were dried in hot air oven to evaporate the solvents from the plates. The plates were kept in a photo-documentation chamber (CAMAG REPROSTAR 3) and the images were captured in white light, UV 254nm and UV 366nm. After derivatization with the appropriate reagents (as given below), the plates were photo-documented at daylight for alkaloids, phenols, flavonoids and saponins, at UV 366nm for flavonoids. The peak table, peak display and peak densitogram of alkaloids, phenolics, flavonoids and saponins were noted.

HPTLC PROFILE OF ALKALOIDS

The mobile phase used was ethylacetate : methanol : water (10:1.35:1). The developed plates were sprayed with Dragendroff's reagent followed by ethanol sulphuric acid. Then the plates were heated at 120°C for 5 minutes in a hot air oven. Nicotine was used as the reference and the presence of alkaloids was confirmed by the appearance of bright orange coloured zones in the daylight mode.

HPTLC PROFILE OF PHENOLICS

The mobile phase used was toluene : chloroform : acetone (4: 2.5: 3.5). After development, the plate was sprayed with 25% aqueous Folin-Ciocalteau reagent and

heated at 120°C for 5 minutes in a hot air oven. Quercetin was used as the reference standard for the analysis of phenolics. The presence of phenolics was confirmed by the appearance of blue or blue-grey coloured zones at daylight.

HPTLC PROFILE OF FLAVONOIDS

The mobile phase used was ethylacetate : butanone : formic acid : water in the ratio of 5:3:1:1. The plate was sprayed with 1% ethanol aluminium chloride reagent and heated at 120°C for 5 minutes in a hot air oven. Rutin was used as the reference standard for flavonoid analysis. The presence of flavonoids was confirmed by the appearance of yellow and yellow-green fluorescence at UV 366nm.

HPTLC PROFILE OF SAPONINS

The mobile phase used was chloroform : acetic acid : methanol : water in the ratio of 6.4 : 3.2 : 1.2 : 0.8. The plate was sprayed with anisaldehyde sulphuric acid reagent and dried at 110°C for 3 minutes in a hot air oven. Saponin was used as the reference standard. The presence of saponins was confirmed by the appearance of blue or yellowish brown coloured zones in visible light.

HPLC ANALYSIS

HPLC analysis was conducted with a Shimadzu chromatograph equipped with photodiode array detector and a 250mm reverse phase column. Shade dried *B. monnieri* leaves were powdered and dissolved in appropriate volume of HPLC grade methanol and injected into the apparatus. The sample analysis of the powdered leaf sample was performed at room temperature, in the wavelength of 205nm. The total run time was 20 minutes at 1000 psi and the mobile phase used was 0.2% phosphoric acid and methanol in the ratio of 65:35.

GC-MS ANALYSIS

The methanol extract of *B. monnieri* leaves was analyzed using a Shimadzu gas chromatography apparatus (Model: GC8000 series and MS was MD800) using a DB-S capillary column (30m) equipped with QP MS detector (EI, 70 ev) with helium as a carrier gas at a flow rate of 1ml/minute. The compounds were identified by comparison with the standards, and also matched with the in-built libraries.

IR SPECTRAL ANALYSIS

IR spectral analysis was carried out in the methanol extract of *B. monnieri* leaves using a scanning Michelson inferometer and Fourier Transformation (Shimadzu, Japan). A residue of the methanol extract was placed in the beam. The intensities that are reduced in the inferogram were subjected to Fourier Transform. This Fourier Transform is the infrared absorption spectrum of the sample.

STATISTICAL ANALYSIS

All the parameters studied were analysed statistically using SigmaStat statistical package (Version 3.1). One way ANOVA with $P<0.05$ was considered significant and, one way ANOVA followed by post-hoc Fischer analysis was done to test the levels of statistical significance.

The results obtained for the various parameters analyzed during the different phases of the study are presented in the next chapter.

PLATE 1

Bacopa monnieri