Methodology

3. METHODOLOGY

The aim of the present study was to investigate the antioxidant properties of *Artemisia vulgaris* leaves. The plant sample was collected from Coimbatore and was grown within the university campus as pot culture. The plant was identified and certified by the Botanical Survey of India, Tamil Nadu Agricultural University, Coimbatore. The voucher specimen was collected and maintained. Fresh leaves were collected and cleaned to remove adhering dust particles, washed under running tap water and gently blotted dry between folds of tissue paper. The extracts were prepared using appropriate buffers or solvents as described for each assay.

This study was conducted in four different phases. In phase I, the leaves of *Artemisia vulgaris* were analyzed for their antioxidant contents and the leaf extracts were tested for their radical quenching effects. In phase II, the biomolecule protective effects of *Artemisia vulgaris* leaf extracts were determined and the antioxidant effects evoked by the leaf extracts in an *in vivo*-simulated *in vitro* system subjected to oxidative stress was analyzed. In Phase III, oxidative stress-induced apoptotic events in selected *in vitro* systems and anticancer activities of *Artemisia vulgaris* leaf extracts in a cancer cell line were studied. In the final phase, a phytochemical analysis was done to identify the active principle rendering the antioxidant activity in *Artemisia vulgaris* leaf extracts.

PHASE I

The first phase of the study was conducted in two sections. In the first section of the phase, the leaves of *Artemisia vulgaris* were analyzed fresh for

their antioxidant contents and in the second section, the leaf extracts were tested for their radical quenching activities. The methodology adopted for analyzing these parameters are given below.

ANALYSIS OF ANTIOXIDANTS IN *Artemisia vulgaris* LEAVES

The leaves of *Artemisia vulgaris* were tested for the activities of enzymic (superoxide dismutase, catalase, peroxidase, glutathione reductase, glutathione S-transferase and polyphenol oxidase) and non-enzymic (ascorbic acid, tocopherol, reduced glutathione, total carotenoids, lycopene, total phenols, flavonoids and chlorophyll) antioxidants. The procedures used for the assays are given below.

ENZYMIC ANTIOXIDANTS

ASSAY OF SUPEROXIDE DISMUTASE

Superoxide dismutase activity was determined by the method proposed by Kakkar *et al.* (1984).

PRINCIPLE

The assay of SOD is based on the inhibition of formation of NADH-phenazine methosulphate-nitroblue tetrazolium formazon, the extent of which can be assayed spectrophotometrically at 560nm.

REAGENTS

1. Sodium pyrophosphate buffer (0.025M, pH 8.3)
2. Phenazine methosulphate (PMS) (186μM)
3. Nitroblue tetrazolium (NBT)) (300μM)
4. NADH (700μM)

5. Glacial acetic acid
6. n-butanol

PROCEDURE

PREPARATION OF ENZYME EXTRACT

Artemisia vulgaris leaves (0.5g) were ground with 3.0ml of sodium pyrophosphate buffer, centrifuged at 2000g for 10 minutes and the supernatant was used for the assay.

ASSAY

The assay mixture contained in a total volume of 3.0ml, 1.2ml of sodium pyrophosphate buffer, 0.1ml of PMS, 0.3ml of NBT, 0.2ml of enzyme preparations and 1.0ml of water. NADH (0.2ml) was added to start the reaction. The assay mixture was incubated at 30°C for 90 seconds and the reaction was stopped by the addition of 1.0ml of glacial acetic acid. n-butanol (4ml) was added to the above mixture, allowed to stand for 10 minutes and then centrifuged at 2000g for 5 minutes. The intensity of the chromogen in the butanol layer was measured at 560nm against butanol as blank. The system devoid of enzyme served as control. One unit of enzyme activity is defined as the amount of enzyme causing a 50% reduction in NBT oxidation/minute.

ASSAY OF CATALASE

Catalase activity was assayed spectrophotometrically by the method proposed by Luck (1974).

PRINCIPLE

The UV light absorption of hydrogen peroxide can be easily measured between 230 and 250nm. On decomposition by catalase, the absorption of H_2O_2 decreases with time and is proportional to the activity of the enzyme. The enzyme activity can be arrived at from this decrease.

REAGENTS

1. Phosphate buffer (0.067M, pH 7.0)
2. Hydrogen peroxide (30% in phosphate buffer)

PROCEDURE

PREPARATION OF ENZYME EXTRACT

Artemisia vulgaris leaves (0.5g) were homogenized in 2.5ml of phosphate buffer at 4°C and the homogenate was centrifuged at 2000g for 10 minutes. The supernatant obtained was used for the assay.

ASSAY

H_2O_2-phosphate buffer (2.9ml) was pipetted out into a quartz cuvette. The enzyme extract (0.1ml) was rapidly added and mixed thoroughly. The time required for a decrease in absorbance by 0.05 units was recorded. The H_2O_2-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

Peroxidase activity was assayed by the method of Reddy *et al.* (1995) in the fresh leaves of the plant.

PRINCIPLE

In the presence of the hydrogen donor pyrogallol, peroxidase converts H_2O_2 to water and oxygen. The oxidation of pyrogallol to the coloured product purpurogalli can be quantified spectrophotometrically at 430nm. The formation of the product is proportional to the activity of the enzyme and can be used as a measure of the same.

REAGENTS

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

PROCEDURE

PREPARATION OF ENZYME EXTRACT

A 20% homogenate was prepared in 0.1M phosphate buffer (pH 6.5) from the leaves. The homogenate was then centrifuged at 3000g for 15 minutes. The supernatant was used as the enzyme source.

ASSAY

Pyrogallol solution (2.4ml) and enzyme extract (0.1ml) were pipetted out into an experimental cuvette. The spectrophotometer was adjusted to read zero at 430nm. To the experimental cuvette, 0.5ml of 1% H_2O_2 was added and the change in absorbance was recorded for every 30 seconds up to 3 minutes. One unit of peroxidase activity is defined as the change in absorbance per minute at 430nm.

ASSAY OF GLUTATHIONE REDUCTASE

Glutathione reductase activity was determined by the method of David and Richard (1983).

PRINCIPLE

The enzyme glutathione reductase involves the conversion of oxidized glutathione to its reduced form by using NADPH as a 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

Artemisia vulgaris leaves (0.5g) were crushed and extracted into 5ml of phosphate buffer. The debris was removed by centrifugation at 5000g for 10 minutes. The supernatant was used for the assay.

ASSAY

The reaction mixture contained in a final volume of 3.0ml, EDTA (0.1ml), sodium azide (0.1ml), oxidized glutathione (0.1ml), enzyme source (0.1ml) and water. The reaction mixture was incubated for 3 minutes, after which NADPH (0.1ml) was added to the reaction mixture. The absorbance was

recorded at an interval of 15 seconds for 3 minutes at 340nm. The assay mixture free from oxidized glutathione served as control. One unit of glutathione reductase is defined as the μmoles of NADPH oxidized/minute.

ASSAY OF GLUTATHIONE S-TRANSFERASE

The method proposed by Habig *et al.* (1974) was employed for the assessment of glutathione S-transferase.

PRINCIPLE

GST conjugates with GSH and CDNB and the extent of conjugation causes a proportionate change in the absorption at 340nm, which can be followed spectrophotometrically.

REAGENTS

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

PROCEDURE

PREPARATION OF ENZYME EXTRACT

A 20% homogenate was prepared in 0.1M phosphate buffer from the leaf tissue of the plant. The supernatant was used for the assay after centrifugation of the homogenate at 5000g for 10 minutes.

ASSAY

The substrates for GST (GSH and CDNB, 0.1ml each) were taken in a test cuvette along with 0.1M phosphate buffer to make a volume of 2.9ml. The reaction was started by the addition of 0.1ml of the enzyme source to this mixture. The readings were recorded against distilled water blank for a minimum of 3 minutes. The complete assay mixture without the enzyme source served as the control. The enzyme activity was determined by recording the changes in absorbance at 340nm. One unit of GST activity is defined as the nmoles of CDNB conjugated/minute.

ASSAY OF POLYPHENOL OXIDASE

The method proposed by Esterbauer *et al.* (1977) was used to simultaneously assay catechol oxidase and laccase spectrophotometrically, which was used to assay polyphenol oxidase in the leaf of the selected plant.

PRINCIPLE

Phenol oxidases are copper proteins, which catalyze the aerobic oxidation of certain phenolic substrates to quinines, which are auto-oxidized to dark brown pigments generally known as melanins, which can be estimated spectrophotometrically at 495nm.

REAGENTS

1. Reaction medium - Tris-HCl (50mM, pH 7.2), sorbitol (0.4M), NaCl (10mM)
2. Catechol (0.01M)
3. Phosphate buffer (0.1M, pH 6.5)

PROCEDURE

PREPARATION OF ENZYME EXTRACT

The enzyme extract was prepared by macerating 5g of leaf tissue in 20ml reaction medium containing tris-HCl. The homogenate was centrifuged at 2000g for 10 minutes at 4°C, the supernatant was used for the assay.

ASSAY

Both phosphate buffer (2.5ml) and catechol solution (0.3ml) was pipetted out into the experimental cuvette and the spectrophotometer was set at 495nm. The sample (0.2ml) was added to the same cuvette and the changes in absorbance were monitored for every 30 seconds up to 5 minutes. One unit of either catechol oxidase/laccase is defined as the amount of enzyme that transforms one µmole of dihydrophenol to one µmole of quinine/minute. The activity of PPO can be calculated using the formula,

Enzyme unit = K X (ΔA/minute)

where,

K for catechol oxidase = 0.272

K for laccase = 0.242

NON-ENZYMIC ANTIOXIDANTS

ESTIMATION OF ASCORBIC ACID

The levels of ascorbic acid in *Artemisia vulgaris* leaves were quantified spectrophotometrically by the method of Roe and Keuther (1943).

PRINCIPLE

Activated charcoal treatment converts ascorbate to dehydroascorbate, which reacts with 2,4-dinitrophenyl hydrazine to form osazone. Osazone

dissolves in H_2SO_4 to give an orange coloured solution, whose absorbance can be measured spectrophotometrically at 540nm.

REAGENTS

1. Trichloro acetic acid (TCA) (4%)
2. Sulphuric acid (H_2SO_4) (9N)
3. 2,4-dinitrophenyl hydrazine (DNPH) (2% in 9N H_2SO_4)
4. Thiourea (10%)
5. H_2SO_4 (85%)
6. Standard ascorbate solution: 10mg ascorbic acid in 100ml of 4% TCA.

PROCEDURE

EXTRACTION OF ASCORBIC ACID

The leaves of the candidate plant (1g) was macerated in a mortar and pestle in 4% TCA and the homogenate was made up to 10ml, centrifuged at 2000rpm for 10 minutes. The resulting supernatant was treated with a pinch of activated charcoal. The centrifugation was repeated to remove the charcoal residue. Aliquots (0.5ml) of the supernatants were used for the estimation.

ESTIMATION

Aliquots (0.2 to 1.0ml) of the working ascorbate solution were made up to 2.0ml with 4% TCA. DNPH reagent (0.5ml) was added to each tube, followed by two drops of thiourea solution and incubated at 37°C for 3 hours. The osazone crystals formed were dissolved by the addition of 85% H_2SO_4 (2.5ml) on ice to avoid local heat generation. The plant sample was treated in the way similar to that of ascorbate standard.

To the blank alone, DNPH reagent and thiourea were added after the addition of H_2SO_4. The absorbance was read at 540nm. The concentration of ascorbic acid in the samples was calculated from the standard curve constructed on an electronic calculator set to the linear regression mode and expressed as mg ascorbate/g leaf.

ESTIMATION OF TOCOPHEROL

The spectrophotometric method proposed by Rosenberg (1992) was adopted to estimate the level of tocopherol in *Artemisia vulgaris* leaves.

PRINCIPLE

The Emmerie-Engel reaction can be employed to estimate tocopherol. 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. Tocopherol and carotenes are first extracted with xylene and the extraction read at 460nm to measure carotenes. A correlation is made for this 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 (1.2g/L in ethanol)
5. Standard solution of D,L-α tocopherol: 10mg/L in absolute alcohol (91mg of α-tocopherol is equivalent to 100mg of tocopherol acetate).
6. Sulphuric acid (0.1N)

PROCEDURE

PREPARATION OF PLANT EXTRACT

The leaves were crushed in a small volume of 0.1N sulphuric acid, transferred to a standard flask and the total volume of the homogenate was made up to 50ml with 0.1N sulphuric acid. The content of the flask was allowed to stand overnight. The next day, the homogenate was shaken vigorously and filtered through Whatman No.1 filter paper. Estimation of tocopherol was done using aliquots of the filtrate.

ESTIMATION

The plant extract (1.5ml), standard (1.5ml) and water (1.5ml) were pipetted out into three centrifuge tubes namely test, standard and blank respectively. To all the tubes, xylene (1.5ml) was added, stoppered, mixed well and centrifuged. The xylene layer (1.0ml) was taken and transferred to another set of stoppered tubes, 1.0ml of 2,2'-dipyridyl was added to each and mixed. The reaction mixture (1.5ml) was taken in a spectrophotometric cuvette and the extinction of test and standard were read against the blank at 460nm. Ferric chloride solution (0.33ml) was added and after exactly 15 minutes, the absorbance of the red colour was read against blank at 520nm. The amount of tocopherol in the sample was calculated using the formula,

$$\text{Tocopherol } (\mu g) = \frac{\text{Reading at 520nm - Reading at 450nm}}{\text{Reading of standard at 520nm}} \times 0.29 \times 0.15$$

The results are expressed as µg tocopherol/g sample.

ESTIMATION OF REDUCED GLUTATHIONE

The amount of reduced glutathione present in the leaf sample was estimated by the method proposed by Moron *et al.* (1979).

PRINCIPLE

Reduced glutathione (GSH) was measured by its reaction with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) (Ellman's reaction) to give a yellow coloured compound that absorbs at 412nm.

REAGENTS

1. TCA (5%)
2. TCA (25%)
3. Sodium phosphate buffer (0.2M, pH 8.0)
4. DTNB (0.6M in 0.2M sodium phosphate buffer)

PROCEDURE

PREPARATION OF PLANT EXTRACT

The leaves of *Artemisia vulgaris* (0.5g) were ground with 2.5ml of 5% TCA. The precipitated protein was centrifuged at 1000rpm for 10 minutes. The homogenate was cooled on ice and the supernatant (0.1ml) was taken for the estimation of GSH.

ESTIMATION

The volume of different aliquots (0.2 to 1.0ml) was made up to 1ml with phosphate buffer. Freshly prepared DTNB (2ml) was added to the tubes and the intensity of the yellow colour was read at 412nm in a spectrophotometer after

10 minutes. A standard curve of GSH was prepared using concentrations ranging from 2 to 10nmoles of GSH. The concentration of GSH in the samples was calculated from this and the result were expressed as nmoles GSH/g leaf.

ESTIMATION OF TOTAL CATORENOIDS AND LYCOPENE

The method proposed by Zakaria *et al.* (1979) was used for the estimation of total carotenoids and lycopene.

PRINCIPLE

The total carotenoids and lycopene in the sample were extracted in petroleum ether. The total carotenoids were estimated in UV-visible spectrophotometer at 450nm. After measuring the total carotenoids at 450nm, the same extract can be used for estimating lycopene at 503nm. Lycopene has a maximum absorbance at 503nm, where the other carotenoids have only negligible absorbance.

REAGENTS

1. Petroleum ether (40 to 60°C)
2. Anhydrous sodium sulphate
3. Calcium carbonate
4. Alcoholic potassium hydroxide (KOH) (12%)

PROCEDURE

All the steps subsequent to the saponification were carried out in the dark to avoid photolysis of carotenoids. The leaves (5.0g) were ground and saponified with 2.5ml of 12% ethanolic KOH in a water bath at 60°C for 30 minutes. The saponified extract was transferred into a separating funnel

(packed with glass wool and calcium carbonate) containing 10-15ml of petroleum ether, mixed and allowed to separate. The petroleum ether layer containing the carotenoid pigments was transferred into another separating funnel. The extraction was repeated until the aqueous phase was colourless. A small quantity of anhydrous sodium sulphate was added to the petroleum ether extract to remove excess moisture, if any. The final volume of the petroleum ether layer was noted and diluted (if needed) by a known dilution factor. The absorbance of the yellow colour was read at 450nm and 503nm using petroleum ether as blank. The amount of total carotenoids and lycopene was calculated using the formula,

$$\text{Amount of total carotenoids} = \frac{P \times 4 \times V \times 100}{W} \text{ mg}$$

where,

P = Optical density of the sample

V = Volume of the sample

W = Weight of the sample

$$\text{Lycopene} = \frac{3.12 \times \text{O.D}_{\text{sample}} \times \text{Volume of sample} \times \text{dilution} \times 100}{1 \times \text{weight of the sample} \times 1000}$$

The total carotenoids and lycopene are expressed as mg/g leaf.

ESTIMATION OF TOTAL PHENOLS

The method described by Mallick and Singh (1980) was adopted in the present study to estimate total phenols.

PRINCIPLE

Phenols react with phosphomolybdic acid in Folin-Ciocalteau reagent in alkaline medium and produce blue coloured complex (molybdenum blue), which can be read spectrophotometrically at 650nm.

REAGENTS

1. Ethanol (80%)
2. Folin-Ciocalteau reagent (1N)
3. Sodium carbonate (20%)
4. Standard solution: 10mg catechol in 100ml of distilled water

PROCEDURE

PREPARATION OF PLANT EXTRACT

Pre-weighed leaf sample (0.5g) was ground in 5ml of 80% ethanol. The homogenate was centrifuged at 10,000rpm for 20 minutes. The supernatant was collected and the residue was re-extracted with 2.5ml of 80% ethanol. After repeated centrifugation, the supernatants were collected and pooled. The ethanol was evaporated and the residue was dissolved in a known volume of distilled water and used for the estimation of phenol.

ESTIMATION

Aliquots (0.2 to 2.0ml) of the standard catechol solution were made up to 3ml with distilled water. Folin-Ciocalteau (0.5ml) reagent was added to each test tube. After 3 minutes, 2.0ml of 20% sodium carbonate was added to each tubes. After mixing the tubes thoroughly, all the tubes were heated in a boiling water bath for exactly one minute and allowed to cool at room temperature. The

blue colour was recorded at 650nm against a reagent blank. The concentration of phenols in the sample was calculated from the standard curve constructed on an electronic calculator set to the linear regression mode and expressed as mg phenols/g leaf.

ESTIMATION OF FLAVONOIDS

The levels of flavonoids were assayed by the method explained by Cameron *et al.* (1943).

PRINCIPLE

Flavonoids react with vanillin to produce a coloured product that can be measured spectrophotometrically at 360nm.

REAGENTS

1. Vanillin reagent (1% vanillin in 70% sulphuric acid)
2. Sulphuric acid (70%)
3. Methanol : water (2:1)
4. Methanol : water (1:1)
5. Hexane
6. Catechin standard (110µg/ 1ml)

PROCEDURE

PREPARATION OF PLANT EXTRACT

The leaves were weighed (0.5g) and divided equally into two parts. One part was extracted with methanol : water (2:1) and other part was extracted with methanol : water in the ratio of 1:1. These homogenates were allowed to stand over night. The next day, the two extracts were combined and evaporated to

1/3rd the original volume. The resultant aqueous extract was cleared of low polarity contaminants by extracting with hexane. The solvent-extracted aqueous layer containing the bulk of the flavonoids was then concentrated.

ESTIMATION

An aliquot of the leaf extract was pipetted out and evaporated to dryness. Aliquots of the standard (0.2 to 1.0ml) were taken in test tubes and made up to 1.0ml with distilled water. Vanillin reagent (4ml) was added to all the test tubes and heated in a boiling water bath for 15 minutes. The absorbance was measured at 340nm after cooling. The values are expressed as mg flavonoids/g leaf.

ESTIMATION OF CHLOROPHYLL

The chlorophyll content of the leaves was estimated by the procedure explained by Witham *et al.* (1971).

PRINCIPLE

Chlorophyll is extracted in 80% acetone and the estimation of chlorophyll is based on the absorbance coefficient at 663nm and 645nm.

REAGENT

Acetone (80%, prechilled)

PROCEDURE

The leaves were weighed (1g) and extracted with 20ml of 80% acetone. The extract was centrifuged at 5000rpm for 5 minutes. The resulting supernatant was transferred to a 100ml volumetric flask. The residue was

re-extracted with 20ml of 80% acetone. The centrifugation was repeated and the supernatant was transferred to the same volumetric flask. This procedure was repeated until the residue was colourless. The total volume of the supernatant in the volumetric flask was made up to 100ml with 80% acetone. The absorbance of the green colour was read at 645nm and 663nm against 80% acetone blank. The total chlorophyll in the leaf was calculated using the formula,

$$\text{Total chlorophyll} = 20.2\,(\text{A645}) + 8.02\,(\text{A663}) \times \frac{V}{1000 \times W}$$

where,

A = absorbance of specific wave length

V= final volume of the extract

W= fresh weight of the leaves

The values are expressed as mg chlorophyll/g leaf.

RADICAL SCAVENGING EFFECTS OF *Artemisia vulgaris*

The results of the previous section, which are presented in the next chapter, revealed the leaves of *Artemisia vulgaris* to be a good source of both enzymic and non-enzymic antioxidants. The second section of phase I of the study was performed to test the radical scavenging activities of the leaf extracts of *Artemisia vulgaris*. The radical quenching potential of the leaf extracts were tested *in vitro* against a battery of radicals namely DPPH (a stable radical), ABTS (cation radical), superoxide, nitric oxide, non-radical oxidant H_2O_2 and hydroxyl radicals.

SOLVENT EXTRACTION

In order to understand the active principle rendering the antioxidant activities, the leaves were serially extracted into solvents of increasing polarity

using a Soxhlet apparatus. The solvents used were petroleum ether, benzene, chloroform, ethyl acetate and methanol. For this, fresh leaves of *Artemisia vulgaris* (5g) were collected, washed, dried and packed in a thimble. This was subjected to Soxhlet extraction using the solvents mentioned above. After extraction, the solvents were evaporated to dryness and the yields of the extracts were calculated. The residues were re-dissolved in a known volume of the same solvents in which they were extracted.

Apart from the solvent extracts, a fresh aqueous extract was also prepared. A homogenate of the leaves (1g/1ml) was prepared in double distilled water, centrifuged at 2000rpm for 5 minutes and the supernatant was used as the fresh aqueous extract. The different solvent extracts and the aqueous extract were tested for their DPPH, ABTS, $SO^{\bullet}$ and NO radical scavenging effects. The procedures for each are explained below.

DPPH SCAVENGING EFFECTS

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

PRINCIPLE

Antioxidants react with DPPH (1,1'-diphenyl-2-picryl hydrazyl) and converted into α,α'-diphenyl-β-picryl hydrazine by donating its OH group. This can be identified by the conversion of purple colour to yellow colour.

DOT-BLOT RAPID SCREENING 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 (0.3mM in methanol)
3. Methanol

PROCEDURE

All the six different extracts (3µl each) were spotted on a TLC plate and allowed to air dry. This was placed upside down in DPPH solution for 10 seconds. The spots exhibiting radical scavenging antioxidant activity were observed.

DPPH SPECTROPHOTOMETRIC ASSAY

The method proposed by Mensor *et al.* (2001) was adopted to test the DPPH scavenging ability of different solvent extracts of the leaves of the candidate plant.

REAGENTS

1. DPPH (0.3mM in methanol)
2. Methanol

PROCEDURE

The different solvent extracts and crude aqueous extract (5µl) was added with 0.5ml of methanolic solution of DPPH and 0.495ml of methanol. The mixture was then allowed to stand at room temperature for 30 minutes. DPPH-methanol solution was used as positive control and methanol alone acted as blank. After incubation, the conversion of purple colour to yellow colour was

read at 518nm in a spectrophotometer. The per cent inhibition was calculated using the following formula;

$$\text{Scavenging activity (\%)} = 100 - \frac{\text{A(Control)} - \text{A(Sample)}}{\text{A(Control)}} \times 100$$

ABTS SCAVENGING EFFECTS

ABTS (2,2'-azino-bis-3-ethyl benzthiazoline-6-sulphonic acid) radical cation de-colourisation assay proposed by Shirwaikar *et al.* (2006) was employed to assess the radical-scavenging effect of the leaf extracts of the candidate plant.

PRINCIPLE

ABTS is a chromogen, which changes into a coloured mono-cation radical form ($ABTS^+$) in the presence of oxidative agent and the $ABTS^+$ has an absorption peak at 750nm. Antioxidants will reduce $ABTS^+$ into its colourless form and the extent of decolourisation corresponds to the per cent reduction of $ABTS^+$.

REAGENTS

1. Ethanol
2. ABTS solution (7mM ABTS with 2.45mM ammonium persulfate). The solution was incubated at room temperature for 12-16 hours before use.

PROCEDURE

The six different extracts (100µl each) were added to ABTS solution (300µl) and the final volume of each was made up to 1ml with ethanol. The

absorbance was read at 745nm and the percentage inhibition was calculated using the formula,

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

SUPEROXIDE SCAVENGING ACTIVITY

The efficiency of the leaf extracts to inhibit the *in vitro* generation of $SO^{\bullet}$ was studied by the method of Winterbourn *et al.* (1975).

PRINCIPLE

The extent of superoxide generation was studied on the basis of 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 sodium cyanide/10 ml)
2. Nitroblue tetrazolium (NBT) (1.5mM)
3. Riboflavin (0.12mM)
4. Phosphate buffer (0.067M, pH 7.6)
5. Dimethyl sulfoxide (DMSO)

PROCEDURE

The assay mixture contained 0.02ml of plant (solvent and crude aqueous) extracts with 0.2ml of EDTA, 0.1ml of NBT, 0.05ml of riboflavin and 2.63ml of phosphate buffer. DMSO, instead of plant extract, was considered as control. All tubes were vortexed and the initial absorbance was read at 560nm. The tubes were illuminated uniformly using a fluorescent lamp for 30 minutes. The

absorbance was read again at 560nm. The difference in optical density before and after illumination is the measure of superoxide generation and the percentage inhibition was calculated using the formula,

$$\% \text{ Superoxide Scavenged} = \frac{\text{A(Control)} - \text{A(Sample)}}{\text{A(Control)}} \times 100$$

NITRIC OXIDE SCAVENGING ACTIVITY

The method developed by Green *et al.* (1982) was employed to test the inhibition of *in vitro* generated nitric oxide by the leaf extracts.

PRINCIPLE

An aqueous solution of sodium nitroprusside spontaneously generates nitric oxide at physiological pH, which interacts with oxygen to produce nitrite ions, which is measured at 546nm.

REAGENTS

1. Sodium nitroprusside (100mM)
2. Phosphate buffered saline (PBS) (pH 7.4)
3. Griess reagent (1% sulphanilamide, 2% phosphoric acid and 0.1% naphthyl ethylene diamine dihydrochloride)

PROCEDURE

Sodium nitroprusside (2.0ml), phosphate buffered saline (0.5ml) and each of the six different plant extracts (0.5ml) were mixed and incubated at 25°C for 30 minutes. Griess reagent (0.5ml) was added and allowed to stand for 30 minutes. The control tube was prepared without leaf extracts. The

absorbance of the pink coloured chromogen was read at 546nm against a reagent blank.

In the assays described above (DPPH, ABTS, $SO^{\bullet}$ and NO scavenging), out of the six different extracts (petroleum ether, benzene, chloroform, ethylacetate, methanol and aqueous) examined, the methanolic extract exhibited maximum activity. However, there was considerable activity in the other extracts also. Therefore, in further studies, aqueous, methanol and chloroform extracts were used.

PREPARATION OF PLANT EXTRACTS

Artemisia vulgaris leaves (1g) were collected, washed and homogenized thoroughly in 10ml each of methanol and chloroform. The homogenates were centrifuged at 2000rpm for 5 minutes and the organic extracts were dried at 60°C protected from light. The residue was weighed and dissolved in DMSO to obtain a final concentration of 20mg in 5µl of DMSO. An aqueous extract was also prepared fresh for each experiment. The fresh leaves of *Artemisia vulgaris* (1g) was ground with 1ml double distilled water, centrifuged and the supernatant was used for the assay.

HYDROXYL RADICAL SCAVENGING EFFECTS

The effect of *Artemisia vulgaris* leaf extracts on oxidant-induced damage to deoxyribose *in vitro* was quantified as the amount of thiobarbituric acid reactive substances (TBARS) formed, according to the procedure explained by Elizabeth and Rao (1990).

PRINCIPLE

Hydroxyl radicals are generated from a Fe^{2+}/ascorbate/EDTA/H_2O_2 system, which attacks deoxyribose and eventually produces TBARS. The ability of the plant extracts to inhibit TBARS formation is measured spectrophotometrically at 532nm.

REAGENTS

1. Deoxyribose (28mM)
2. $FeCl_3$ (1mM)
3. EDTA (1mM)
4. H_2O_2 (10mM)
5. Ascorbate (1mM)
6. KH_2PO_4-KOH buffer (20mM, pH 7.4)
7. Thiobarbituric acid (1%)
8. HCl (25%)

PROCEDURE

The reaction mixture contained deoxyribose (0.1ml), $FeCl_3$ (0.1ml), H_2O_2 (0.1ml), ascorbate (0.1ml), buffer (0.1ml) and 20µl of leaf extracts which corresponded to 10mg concentration. The total volume was made up to 1ml with water. The tubes were capped tightly and incubated in a water bath at 37°C for one hour. The reaction was terminated by the addition of TBA (0.5ml) and HCl (0.5ml). The tubes were heated in a boiling water bath for 20 minutes for colour development. The intensity of the pink colour formed, as the indication of TBARS formation, was measured at 532nm. The per cent TBARS produced

for positive control (H_2O_2) was fixed as 100% and the relative per cent TBARS was calculated for the extract treated groups.

HYDROGEN PEROXIDE SCAVENGING EFFECTS

The effect of the leaf extracts to scavenge H_2O_2 was determined by the method proposed by Ruch *et al.* (1989).

PRINCIPLE

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

REAGENTS

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

PROCEDURE

The plant extract was diluted to a concentration of 10mg in 10µl. This extract (10µl corresponding to 10mg) was added to 0.6ml of H_2O_2 solution and the final volume was made up to 3ml with the same buffer. After 10 minutes, the absorbance values at 230nm of the reaction mixtures were recorded against a blank containing phosphate buffer without H_2O_2 for each sample. The per cent inhibition was calculated using the formula,

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

PHASE II

The aim of the second phase of the study was to determine the biomolecule protective effects of *Artemisia vulgaris* leaves and to analyze the antioxidant effects evoked by the leaf extracts in *in vivo*-simulated *in vitro* systems subjected to oxidative stress. The biomolecule-protecting effects of the leaf extracts were studied on lipids and DNA.

EFFECTS OF *Artemisia vulgaris* LEAF EXTRACTS ON MEMBRANE LIPIDS

Lipid peroxidation (LPO) is regarded as one of the basic mechanisms of tissue damage caused by free radicals. LPO initiated in the presence of hydroxyl radical, resulting in the production of malondialdehyde (MDA), directly produces oxidative stress (Muller *et al.*, 2007). Lipids are the most susceptible molecules to free radical attack, followed by DNA. Therefore, the biomolecule protective effects of *Artemisia vulgaris* leaf extracts were studied on lipids first.

Three different membrane preparations were used in the present study to analyze the extent of LPO and the protection mediated by the leaf extracts of *Artemisia vulgaris*. These different models were employed in order to ascertain whether the lipid composition and the nature of the membrane influenced the effect of the leaf extracts on the extent of LPO. The membrane models used were plasma membrane, internal membranes and intact cells. RBC ghosts prepared from goat blood were used as a source of plasma membranes and fresh liver slices of 1mm thickness were used as intact live cells; goat liver homogenate served as the source of internal membranes.

PRINCIPLE

The oxidation of polyunsaturated fatty acids by oxidizing agents (ferrous ions, ascorbate or H_2O_2) leads to the production of malondialdehyde (MDA), which reacts with thiobarbituric acid (TBA), yielding a pinkish red chromogen, which can be measured at 535nm.

INHIBITION OF *in vitro* LIPID PEROXIDATION IN GOAT RBC GHOSTS

The method explained by Dodge *et al.* (1963) was employed to prepare goat 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 RBC GHOSTS

Fresh goat blood (50ml) was collected in a clean sterile container containing acid-washed stones for defibrination. The defibrinated blood was transferred into a fresh sterile container and diluted with sterile isotonic KCl (1:1). The mixture was centrifuged at 3000g for 10 minutes at 4°C. The

supernatant was discarded and the pellet was washed thrice with isotonic KCl. The resulting pellet was suspended in hypotonic KCl and incubated at 37°C for one hour for complete lysis. The lysate was centrifuged at 5000g for 15 minutes at 4°C. The centrifugation was repeated until a pale pink pellet containing erythrocyte ghosts was obtained. The ghost preparation was suspended in 1.5ml of TBS and used as a source of membrane lipids.

PROCEDURE

The assay mixture contained RBC ghosts (0.1ml), leaf extracts (0.1ml), $FeSO_4$ (0.1ml), ascorbate (0.1ml) and TBS (0.1ml). A blank was also prepared by eliminating plant extract and lipid source but only $FeSO_4$, ascorbate and TBS to a final volume of 0.5ml. An assay medium corresponding to 100% oxidation was prepared by adding all other constituents except the plant extracts. The experimental medium corresponding to auto-oxidation contained only the RBC ghosts. All the tubes were heated in a water bath at 37°C for one hour. The reaction was stopped by the addition of 70% alcohol (0.5ml). TBA (0.5ml) was added to all the tubes and heated in a boiling water bath for 20 minutes to allow colour development. After cooling, acetone (0.5ml) was added to all the tubes. The intensity of the pink colour, as the indication of TBARS produced, was measured at 535nm.

INHIBITION OF *in vitro* LIPID PEROXIDATION IN LIVER HOMOGENATE

Goat liver was collected fresh from the slaughterhouse and washed with TBS to remove blood. A 20% goat liver homogenate was prepared in ice-cold TBS using Teflon homogenizer. The homogenate was centrifuged to remove

the debris and the supernatant was used as a membrane source for the LPO assay (Okhawa *et al.*, 1979).

REAGENTS

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

PROCEDURE

The reaction mixture contained 0.1ml of liver homogenate, 0.1ml of KCl, 0.1ml of $FeSO_4$ and 0.1ml of ascorbate. The mixture was incubated for one hour at 37°C both in the presence and the absence of *Artemisia vulgaris* leaf extracts (0.1ml corresponding to 50mg). An aliquot (0.4ml) of the assay mixture was mixed with 1.5ml each of TBA and acetic acid. The mixture was heated in a boiling water bath for 20 minutes and allowed to cool at room temperature, followed by the addition of 1.0ml of distilled water and 5.0ml of a mixture of n-propanol and pyridine. The pink coloured chromophore obtained after centrifugation was measured at 532nm.

INHIBITION OF *in vitro* LIPID PEROXIDATION IN LIVER SLICES

The extent of inhibition of LPO in goat liver slices was followed by the method of Dodge *et al.* (1963)

REAGENTS

1. Phosphate buffered saline (PBS)
2. H_2O_2 (30%)

3. Alcohol (70%)
4. TBA (1%)
5. Acetone

PREPARATION OF GOAT LIVER SLICES

The goat liver was collected fresh from the slaughterhouse, plunged in ice-cold sterile PBS and maintained at 4°C till use. Very thin slices (1mm) of the liver were cut using a sterile blade.

PROCEDURE

Thin slices of goat liver (250mg) were taken in 1.0ml of sterile PBS in flat-bottomed flasks. The slices were incubated in the presence and/or the absence of the leaf extracts of *Artemisia vulgaris* (corresponding to an extract concentration of 20mg) and oxidant (5µl of 30% H_2O_2). Appropriate controls were also set up. The assay mixture was incubated at 37°C with mild shaking for one hour. At the end of the incubation period, the goat liver slices were homogenized using a Teflon homogenizer and the homogenate was used for the assay.

An aliquot of the homogenate (0.5ml) was mixed with 0.5ml of 70% alcohol to terminate the lipid peroxidation reaction. TBA (1ml) was added to all the tubes and heated in a boiling water bath for 20 minutes. After cooling at room temperature, 500µl of acetone was added and the pink colour developed was measured spectrophotometrically at 535nm as an indication of TBARS formed.

EFFECTS OF *Artemisia vulgaris* LEAF EXTRACTS ON DNA DAMAGE INDUCED BY THE OXIDANT

Free radical-induced DNA damage *in vivo* can result in deleterious biological effects such as initiation and promotion of cancer. Chemical characterization and quantitation of such DNA damage is essential for an understanding of its biological consequences and cellular repair (Genestra, 2007). Hence, the *Artemisia vulgaris* leaf extracts were tested for their DNA protective effects *in vitro*.

The protective effect of the leaf extracts on oxidant-induced DNA damage *in vitro* was studied using purified and commercially available DNA. The pure DNA used were of different hierarchical levels of evolution. They were λ DNA (linear phage DNA), pUC 18 DNA (circular plasmid DNA) and herring sperm DNA (genomic, haploid DNA from higher eukaryote).

ESTIMATION OF DNA DAMAGE IN λ DNA AND pUC 18 DNA

The method proposed by Chang *et al.* (2002) was used to determine DNA damage.

REAGENTS

1. λ DNA/pUC 18 DNA (2μg/5μl)
2. Tris buffer (50mM, pH 7.4)
3. H_2O_2 (30%)
4. $FeCl_3$ (500μM)
5. Agarose (1%) in 1X TAE buffer
6. TAE buffer (50X) (tris base 24.2g, glacial acetic acid 5.71ml and EDTA 18.61g, in a total volume of 100ml, pH 8.0).

7. EtBr (10mg/ml)
8. Gel loading dye (1.28g bromophenol blue, 0.25g xylene caynol and 50ml glycerol in a total volume of 100ml).

PROCEDURE

The reaction mixture (30µl) contained 5µl of tris buffer or pUC 18 / λ DNA and 5µl of tris buffer or plant extract, 10µl of 30% H_2O_2 and 5µl of $FeCl_3$ and incubated at 37°C for 15 minutes for pUC 18 DNA and 30 minutes for λ DNA. The assay mixture (10µl) was mixed with 5µl of gel loading dye and loaded onto 1% agarose gel with 5µg/ml EtBr. Electrophoresis was carried out at 100V for 15 minutes in a submerged gel electrophoretic apparatus. The DNA was visualized in a UV transilluminator and photographed using Alpha Digidoc digital gel documentation system (Alpha Innotech, UK).

ESTIMATION OF DNA DAMAGE USING HERRING SPERM DNA

The H_2O_2-induced DNA damage in herring sperm DNA was studied by the method described by Aeschlach *et al.* (1994).

REAGENTS

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

PROCEDURE

The assay mixture contained herring sperm DNA (50µl), H_2O_2 (0.167ml), $FeCl_3$ (0.05ml) and leaf extract (5µl of 10mg/ml). The total volume was made up to 0.5ml with tris buffer. The mixture was incubated at 37°C for one hour. EDTA (10µl) was added to terminate the reaction. TBA (0.5ml) and HCl (0.5ml) were added and incubated at 37°C for 15 minutes for colour development. After centrifugation, the supernatant was taken and the extent of DNA damage was measured by the increase in absorption at 532nm spectrophotometrically.

EVALUATION OF THE EFFECTS OF *Artemisia vulgaris* LEAF EXTRACTS ON THE ANTIOXIDANT STATUS OF TISSUE *in vitro*

Liver slice technology is a relatively new addition to the battery of *in vitro* assays of xenobiotic metabolism and toxicity evaluation (Thohan and Rosen, 2008). Precision-cut liver slices can provide a system where all liver cell-types are present in their natural environment (Bovenkamp *et al.*, 2005), thereby preserving the cell–cell and cell–extracellular matrix interactions. Precision-cut liver slices can contribute to the reduction of animal experiments. In the present study, precision-cut liver slices were employed as an *in vitro* system to assay the antioxidant potential of *Artemisia vulgaris* leaf extracts, as earlier studies in our laboratory have proved this technique to be a reliable alternative to live animal studies (Varier, 2002; Saraswathi, 2006; Sumathi, 2007; Vidya, 2007; Kiruthika, 2010).

PREPARATION OF GOAT LIVER SLICES

The goat liver was obtained fresh from a slaughterhouse and transported to the laboratory on ice. The liver was plunged into ice-cold PBS and maintained at 4°C till use. The precision-cut slices of 1mm thickness of the liver were cut using sterile scalpel.

INDUCTION OF OXIDATIVE STRESS

Precision-cut liver slices (0.25g) were taken in 1ml of PBS in a flat bottomed flask. Hydrogen peroxide (H_2O_2) was used to induce oxidative stress at a final concentration of 500μM. The liver slices were treated in the presence or the absence of leaf extracts (20μl) and H_2O_2 and incubated at 37°C for 1 hour with mild shaking. After incubation, the mixture was homogenized using a Teflon homogenizer. The homogenate was centrifuged at 1500rpm for two minutes and the supernatant was used for the analysis of various enzymic and non-enzymic antioxidants.

The treatment groups set up for each assay were

1. Untreated control
2. Treated with H_2O_2 (standard oxidant)
3. Treated with aqueous extract of *Artemisia vulgaris* leaves
4. Treated with methanol extract of *Artemisia vulgaris* leaves
5. Treated with chloroform extract of *Artemisia vulgaris* leaves
6. Treated with aqueous extract of *Artemisia vulgaris* leaves + H_2O_2
7. Treated with methanol extract of *Artemisia vulgaris* + H_2O_2
8. Treated with chloroform extract of *Artemisia vulgaris* + H_2O_2

ANALYSIS OF ENZYMIC ANTIOXIDANTS

The enzymic antioxidants, namely superoxide dismutase, catalase, peroxidase, glutathione reductase and glutathione S-transferase, were assayed in the goat liver slices to evaluate the enzymic antioxidant status of the liver slices. The protocols adopted for the determination of the activities of enzymic antioxidants were the same as those for the leaf analyses (phase I). An aliquot of the liver slice homogenate was used as the enzyme source instead of the leaf extracts.

DETERMINATION OF NON-ENZYMIC ANTIOXIDANTS

The non-enzymic antioxidants analysed in the liver homogenate were ascorbic acid, tocopherol, vitamin A and reduced glutathione. Vitamin C, tocopherol and reduced glutathione were analysed by the methods explained in phase I of this chapter. The plant extract was replaced by liver slice homogenate, which acted as the non-enzymic antioxidant source.

ESTIMATION OF VITAMIN A

The estimation of vitamin A in goat liver homogenate in the presence and the absence of leaf extracts were done by the method proposed by Bayfield and Cole (1980).

PRINCIPLE

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

REAGENTS

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

PROCEDURE

Liver homogenate (1.0ml) was mixed with 1.0ml of saponification mixture and reflexed for 20 minutes at 60°C in the dark. All the steps subsequent to saponification were carried out in the dark. Vitamin A was extracted twice with 10ml of petroleum ether (40-60°C). The extracts were pooled, washed thoroughly with water and the layers were separated using a separating funnel. When the petroleum ether fraction was clear, a pinch of sodium sulphate (anhydrous) was added to remove the excess moisture. The volume of the extract was noted and a fraction (1.0ml) was evaporated to dryness at 60°C. The residue was dissolved in 1.0ml of chloroform. Aliquots of the standard (vitamin A palmitate) were pipetted out into a series of clean, dry test tubes in the concentration ranging from 0 to 7.5µg. The volume in all the tubes was made up to 1.0ml with chloroform. TCA reagent (2.0ml) was added rapidly, mixed well and the absorbance of blue colour was read immediately at 620nm in a spectrophotometer. The vitamin A level was expressed as µg/g tissue.

PHASE III

The results of the first two phases (presented in the next chapter) showed that the *Artemisia vulgaris* leaves are a good source of antioxidants. The leaf extracts are potent scavengers of free radicals and also possess biomolecule protective effects against oxidative stress under *in vitro* conditions. The leaf extracts of *Artemisia vulgaris* could also improve the status of antioxidants in goat liver slices exposed to oxidative stress.

The next objective of the study was to understand the role of *Artemisia vulgaris* leaf extracts in the apoptotic events in cells subjected to oxidative stress. Apoptosis plays an essential role in the development of tissues and their homeostasis throughout life. Apoptosis is a well-recognized form of cell death with some typical hallmarks (Merino and Bouillet, 2009; Son *et al.*, 2009).

One of the prime focuses of our research group is to standardize the use of alternative experimental system for studying the antioxidant potential of plant extracts. Using *in vitro* systems will help minimize animal suffering. Yeast cells can act as a potentially useful eukaryotic model system to study the effects of antioxidants at cellular level. Yeast cells are easily handled, cheap and avoid ethical questions inherent to the use of animals. Yeast is a standard object of genetic manipulation and expression of protein, including antioxidant proteins (Zyracka *et al.*, 2005). Primary chick embryo fibroblasts are another group of cells that possess a very high proliferative potential and high rate of metabolism (Sanders *et al.*, 2001).

With this knowledge, the third phase was formulated to study the effect of the plant extracts on events associated with apoptotic death induced by oxidative stress and to test the anticancer activity of *Artemisia vulgaris* leaf

extracts in a cancer cell line. Two different untransformed cell types (yeast and primary chick embryo fibroblasts) were used to understand the oxidative stress-induced apoptosis, and the effect of *Artemisia vulgaris* leaf extracts on the process. Oxidative stress was induced by a standard oxidant H_2O_2 at a final concentration of 200μM, while the concentration of plant extract used was 20mg.

PARAMETERS ANALYSED

The extent of cell death was quantified (SRB and MTT assays) in all the groups and the characteristic features of apoptosis were studied by performing various parameters like morphological changes (giemsa staining), apoptotic index (propidium iodide staining) and nuclear events (EtBr and DAPI staining). In order to perform the parameters, the following treatment groups were set up.

The treatment groups set up for both the cell types were:

- Untreated (negative) control cells
- H_2O_2 treated (positive control) cells
- Aqueous extract of *Artemisia vulgaris* leaves treated cells
- Methanol extract of *Artemisia vulgaris* leaves treated cells
- Chloroform extract of *Artemisia vulgaris* leaves treated cells
- Aqueous extract of *Artemisia vulgaris* leaves treated cells + H_2O_2
- Methanol extract of *Artemisia vulgaris* leaves treated cells + H_2O_2
- Chloroform extract of *Artemisia vulgaris* leaves treated cells + H_2O_2

CULTURING OF YEAST CELLS

YPD medium was prepared by mixing 10g of yeast extract, 20g of peptone and 20g of dextrose (pH 6.5) and the total volume of the medium was

made up to 1000ml with water. The YPD medium was sterilized by autoclaving after aliquoting. The aliquots were cooled and stored at room temperature till use. They were regularly checked for contamination.

Yeast cells were inoculated into the medium on the penultimate day of each assay and the flask was incubated in a temperature-controlled orbital shaker at 30°C overnight. Overnight grown cultures of *Saccharomyces cerevisiae* cells were spun down and the cell pellet was 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 and plant extracts. 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

The fibroblasts were prepared from chick embryo and cultured in Dulbeccos Modified Eagles Medium (DMEM) as per the procedure described in http://homepages.gac.edu/~cellab/chpts/chp12/ex12-10.html. The cells were cultured in 25cm^2 tissue culture flasks and incubated at 37°C in a CO_2 incubator (Napco, UK).

REAGENTS

1. Complete medium [DMEM, 10% FBS and antibiotics (1X from 100X penicillin-streptomycin)]
2. Phosphate buffered saline (PBS)
3. Trypsin-EDTA (0.25% trypsin in 1mM EDTA)
4. Alcohol (70%)

PROCEDURE

The live embryo was seen as a shadow when holding an egg in the bright light source. An egg containing a live 8^{th} or 9^{th} day-old chick embryo was taken in a beaker with the blunt end up and swabbed with 70% alcohol. The blunt end of the egg was carefully punctured with the point of a sterile scissors and a circle of shell was cut away carefully to expose the underlying membrane called chorioallantois. The chorioallantoic membrane was carefully cut with a second pair of sterile scissors and removed to expose the embryo.

The embryo was gently lifted by the neck using a sterile hook or a bent glass rod, and placed in a 100mm petridish containing PBS. It was then washed several times with PBS by transferring the embryo to fresh petriplates. After removal of all yolk and/or blood, the embryo was transferred to a clean dish with PBS. The head, limbs and viscera were removed using two sterile forceps. Care was taken to remove the entire limb by pulling at the proximal end.

The remaining tissues of the embryo were taken to yet another sterile dish and washed with PBS. Then the embryo was finely minced with scissors and transferred to a flask containing PBS. After allowing the tissue pieces to settle, the PBS was removed with a sterile pipette. Trypsin (2ml) was added and the solution was stirred gently for 15 to 20 minutes.

The pellet was resuspended in fresh DMEM with 10% FBS. From this, 20µl of the culture was taken to determine cell count and viability by trypan blue exclusion in a haemocytometer. Then the cells were seeded in $25cm^2$ sterile tissue culture flask containing complete medium to a final concentration of 10^5 live cells/ml. The tissue culture flasks were incubated in a CO_2 incubator in a 5% CO_2 and 95% humidity atmosphere.

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

EVALUATING THE EFFECT OF *Artemisia vulgaris* LEAF EXTRACTS ON OXIDATIVE STRESS-INDUCED APOPTOSIS

The apoptotic events that occurred in the treated cells were followed by cytotoxic assays and various staining techniques.

MTT DYE REDUCTION ASSAY

The MTT [3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide] reduction assay described by Igarashi and Miyazawa (2001) was employed to elucidate the cytotoxicity of the sample.

PRINCIPLE

Living cells convert MTT into its formazon derivative. The number of surviving cells can be determined by the amount of MTT formazon produced, which is measured in a microtitre plate reader after solubilization with a suitable solvent.

REAGENTS

1. PBS
2. MTT (3mg/ml in PBS)
3. Acid-propanol (isopropanol in 0.04N HCl)
4. HCl (0.04N)

PROCEDURE

After the incubation period, the medium was removed. The treated cells (100µl) were incubated with 50µl of MTT at 37°C for 3 hours with mild shaking. At the end of the incubation period, 200µl of PBS was added to all the samples and the liquid was carefully aspirated. Acid-propanol (200µl) was added and left overnight in dark. The absorbance was read at 650nm in a microtitre plate reader (Anthos 2020, Austria). The optical density of the oxidant-induced cells was fixed as 100% viability and the per cent viability of the cells in the other treatment groups were calculated relative to this.

SULPHORHODAMINE B (SRB) ASSAY

The SRB assay explained by Skehan *et al.* (1990) was employed to determine the cell viability in the presence and the absence of leaf extracts in the oxidant-treated cells.

PRINCIPLE

Sulphorhodamine B (SRB) is a pink coloured aminoxanthane dye with two sulphonic groups. Under midly acidic conditions, SRB binds to basic amino acids in the proteins in TCA fixed cells to provide a sensitive index of cellular protein content, which is directly proportional to cell viability.

REAGENTS

1. TCA (40%)
2. TCA (1%)
3. SRB (0.4% in 1% TCA)
4. Acetic acid (1%)

5. Tris (10mM, pH 10.5)
6. PBS

PROCEDURE

After the treatment, the medium was completely removed from each well and washed with 200µl PBS to remove the traces of medium and serum. Ice-cold 40% TCA (350µl) was layered on top of the cells and incubated at 4°C for one hour, after which the pellet was collected and washed 5 times with cold PBS (200µl). SRB stain (350µl) was added to each well and left in contact with the cells for 30 minutes at room temperature, after which they were washed 4 times with 350µl of 1% acetic acid to remove any unbound dye. Then, 350µl of 10mM tris was added to solubilize the protein-bound dye and was shaken gently for 20 minutes on a gyratory shaker. The tris layer in each well was transferred to a new 96-well plate and the absorbance was read in a microtitre plate reader (Anthos 2020, Austria) at 496nm. The optical density of the oxidant-induced cells was fixed as 100% viability and the per cent viability of the cells in the other treatment groups were calculated relative to this.

MORPHOLOGICAL CHANGES OF APOPTOSIS

The morphological changes of the cells were followed in the presence and the absence of leaf extracts and/or oxidant. The treated cells were stained with giemsa stain and the morphological changes were viewed under phase contrast microscope (Nikon, Japan) as explained by Chih *et al.* (2001).

PRINCIPLE

During apoptosis, the cells undergo a series of well-documented morphological changes, which can be observed after staining with giemsa stain.

REAGENTS

1. Phosphate buffer saline (PBS), pH 7.4
2. Liquid giemsa stain (1:2 dilution in PBS)

PROCEDURE

The diluted giemsa stain (10µl) was added to the treated cells and the stain was spread by placing a coverslip over it. The cells were observed under 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 OF APOPTOSIS

The nuclear changes of apoptosis include condensation of nuclear content into clumps of heterochromatin at the nuclear periphery, nuclear fragmentation and final packaging of the nuclear fragments into membrane enclosed apoptotic bodies. The nuclear changes during apoptosis were studied in the presence and the absence of leaf extracts and / or oxidant by PI, EtBr and DAPI staining.

PROPIDIUM IODIDE STAINING

The method proposed by Sarker *et al.* (2000) was employed.

PRINCIPLE

Apoptotic cells, among other typical features, are characterized by DNA fragmentation and consequently, loss of nuclear DNA content which can be analysed using a fluorochrome such as propidium iodide that intercalates into nucleic acid and stains it.

REAGENTS

1. PBS
2. Acetone : methanol (1:1)
3. Propidium iodide (5µg/ml in PBS)

PROCEDURE

After treatment, the cells were permeabilised with 50µl of acetone : methanol (1:1) mixture at -20°C for 10 minutes. The slides were taken out and kept at room temperature for drying. PI (10µl) stain was added to each slide, spread with a coverslip and incubated at 37°C for 30 minutes in the dark. The apoptotic cells were detected using G-2A filter of the fluorescence microscope (Nikon, Japan) at 400X magnification. At least 100 cells were scored for each slide. The apoptotic ratio was calculated by the formula mentioned earlier.

ETHIDIUM BROMIDE STAINING

The method explained by Mercille and Massie (1994) was adopted to study the nuclear changes of apoptotic cells.

PRINCIPLE

Ethidium bromide is a molecule that intercalates into nucleic acid and can be used to visualize the nuclear changes in apoptotic cells.

REAGENTS

1. PBS
2. Ethidium bromide (EtBr) (50µg/ml in PBS)

PROCEDURE

To the treated cells, 10µl of EtBr was added and spread by placing a coverslip over it. The stained cells were incubated at room temperature for 5 minutes. The apoptotic cells were scored by counting the cells with condensed chromatin and fragmented nuclei under fluorescent microscope (Nikon, Japan) using G-2A filter at 400X magnification. The apoptotic ratio was calculated by the formula mentioned earlier.

DAPI STAINING

The oxidant-treated cells with or without the leaf extracts and untreated control cells were scored for DAPI staining using an inverted fluorescent microscope (Motic, Hong Kong) as explained by Rashmi *et al.* (2003).

PRINCIPLE

DAPI (4'-6-diamidino-2-phenylindole) forms a fluorescent complex with double stranded DNA. Because of this property, DAPI is a useful tool to distinguish apoptotic nuclei (intensely stained, fragmented nuclei and

condensed chromatin) from normal nuclei. The presence of nuclear apoptotic bodies and chromatin migration can also be observed after DAPI staining.

REAGENTS

1. PBS
2. Paraformaldehyde (3%)
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 permeabilised with 0.2% triton X-100 (50μl) for 10 minutes at room temperature and incubated for 3 minutes with 10μl of DAPI by placing a coverslip over the cells to enable uniform spreading of the stain. The cells were scored 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.

The results showed that the viability of untransformed cells did not decrease with the leaf extracts of *Artemisia vulgaris*. This observation led to the query of what the effect of leaf extracts would be in the presence of a standard chemotherapeutic agent that induces apoptosis. According to the available literature, etoposide is a commonly used anticancer drug, which is known to exert its action by inducing apoptosis by oxidative stress (Lee *et al.*, 2007b). Hence, etoposide was used as an oxidative stress-inducing factor on Hep2 (laryngeal carcinoma) cells at a concentration of 200μM.

Another set of experiments were conducted in parallel using chick embryo fibtoblasts, wherein the stress was induced using etoposide instead of H_2O_2. This was done to compare the response of etoposide and the leaf extracts on cancerous (Hep2) and non-cancerous (chick embryo fibroblasts) cells. The experimental groups and parameters analysed were similar to those of untransformed cells, as explained earlier.

CULTURING OF Hep2 CELL LINE

The Hep2 (laryngeal carcinoma) cell line was procured from National Centre for Cell Science (NCCS), Pune, India. The cell count was done and the cell viability was tested by trypan blue using haemocytometer. The cells were incubated in a CO_2 incubator in a 5% CO_2 and 95% humidity atmosphere.

Once the cells attained confluent growth, the cells were trypsinized using Trypsin-EDTA (PAA) and the required number of cells like 10^5 and 10^3 cells/ml was seeded into 6-well and 96-well plates respectively for carrying out various assays. In each well of the 6-well plates, a clean, dry, sterile coverslip was placed before the cells were seeded, followed by the incubation in a CO_2 incubator in a 5% CO_2 and 95% humidity atmosphere (Innova CO-170, United States).

The cells were treated in the presence and/or the absence of leaf extracts (20mg) and etoposide (200μM) and incubated for 24 hours in a 5% CO_2 and 95% humidity atmosphere. After treatment, the coverslip was removed and placed on a glass slide, sealed with vaseline and used for various staining techniques. In 96-well plates, the medium was replaced with fresh medium and used for checking the viability of the cells by MTT and SRB assays.

PHASE IV

The results of the first three phases revealed that the *Artemisia vulgaris* leaf extracts exhibited antioxidant, radical quenching, biomolecule protective and apoptosis-modulating effects against oxidative stress induced under *in vivo*-simulated *in vitro* conditions. The final phase of the study was formulated to identify the active principle(s) rendering the antioxidant responses evoked by leaf extracts against oxidative stress.

PRELIMINARY PHYTOCHEMICAL SCREENING

The methanolic extract of *Artemisia vulgaris* was screened for the presence of phytochemicals, according to the method proposed by Khandelwal (2002).

DETECTION OF ALKALOIDS

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 coloured precipitate.

Dragendroff's test

An aliquot of the extract was tested with Dragendroff's reagent and observed for the formation of reddish orange precipitate.

Wagner's test

A fraction of the extract was treated with Wagner's reagent (1.2g of iodine and 2g of potassium iodide in 100ml distilled water) and observed for the formation of reddish brown coloured precipitate.

DETECTION OF PHENOLICS

Ferric chloride test

To a fraction of the extract, 5% $FeCl_3$ solution was added and observed for the formation of deep blue colour.

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

Aqueous NaOH test

To a fraction of the extract, a drop of 1N aqueous NaOH solution was added and observed for the formation of yellow orange colouration.

Sulphuric acid (H_2SO_4) test

A fraction of the extract was treated with concentrated H_2SO_4 and observed for the formation of orange colour.

Schinodo's test

A fraction of the extract was treated with a piece of magnesium turnings followed by a few drops of concentrated hydrochloric acid, heated slightly and observed for the formation of dark pink colour.

UV ABSORPTION SPECTRAL ANALYSIS

The UV absorption spectra of the components present in the methanolic extracts of *Artemisia vulgaris* was evaluated by a survey scan in a

nanospectrophotometer (Optizen 3220bio, Korea). The instrument was set to the scan mode and the absorption spectrum was obtained in the range of 190nm-350nm.

HPTLC ANALYSIS

The methanolic residue (100mg) of *Artemisia vulgaris* leaves was dissolved in methanol (1ml) and centrifuged at 3000rpm for 5 minutes. The supernatant was collected and used as a sample for HPTLC analysis. The test sample (3µl) was loaded as an 8mm band in the 5 X 10 Silica gel 60 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 and developed up to 90mm.

The developed plates were dried in hot air to evaporate the solvents from the plates. The plates were kept in 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 and sesquipterpenoids, and at UV 366nm for flavonoids. Finally, the plates were fixed in the scanner stage and scanned at 500nm for alkaloid, phenolics and sesquiterpenoids, and 366nm for flavonoids. The peak table, peak display and peak densitogram of alkaloids, phenolics, flavonoids and sesquiterpenoids were noted.

HPTLC PROFILE OF ALKALOIDS

The mobile phase used was ethylacetate : methanol : water (10:1.35:1). The developed plates were sprayed with Dragandroff's reagent followed by ethanolic sulphuric acid reagent. Then the plates were heated at 120°C for 5 minutes in a hot air oven. Nicotin 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-Ciocalteu 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 in the ratio of 5:3:1. The plate was sprayed with 1% ethanolic 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 366nm.

HPTLC PROFILE OF SESQUITERPENOIDS

The mobile phase used was n-hexane : ethyl acetate (7.2:2.9). After development, the plate was sprayed with 5% ethanolic molybdo-phosphoric

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

HPLC ANALYSIS

The residue of the methanolic extract of the *Artemisia vulgaris* leaves was dissolved in HPLC grade acetonitrile and 20µl of the sample was injected in to the HPLC apparatus (Shimadzu, Japan equipped with UV detector and a reverse phase C18 column). The sample analysis was performed at room temperature, in the wavelength range of 200-320nm at 1000psi. Acetonitrile and water in the ratio of 15:85 containing 1% acetic acid was used as the mobile phase, with a run time of 30 minutes at a flow rate of 1ml/minute.

IR SPECTRAL ANALYSIS

IR spectral analysis was carried out in the methanolic extract of *Artemisia vulgaris* leaves using a scanning Michelson inferometer and Fourier Transformation (Shimadzu, Japan). A residue of the methanolic 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.

GC-MS ANALYSIS

The methanolic extract of *Artemisia vulgaris* leaves was analyzed using a Shimadzu gas chromatography apparatus (Model qp 5000 GC-MS) 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 using the WILEY database available in the software provided.

STATISTICAL ANALYSIS

The parameters analysed in all the phases of the study were subjected to statistical analysis using SigmaStat (Version 3.1) statistical software. Statistical significance was determined by one-way analysis of variance with $P<0.05$ considered significant and, one-way ANOVA, followed by post-hoc DMRT analysis.

The results obtained for the various parameters analysed in all the four phases and the salient observations made during the study are presented in the next chapter.