Methodology

3. METHODOLOGY

The present study was formulated to evaluate the effect of the leaves of *Zea mays* on the cellular and molecular events triggered by strong oxidative agents. It also focuses on the anticancer effect of *Zea mays* leaves. These effects were evaluated using various *in vitro* models.

This project was conducted in four different phases. In phase I, the leaves of *Zea mays* at different periods of growth were analyzed for their antioxidant content. The period of growth exhibiting the maximum antioxidant content was selected for further phases. In phase II, the antioxidant effects of the different extracts of *Zea mays* leaves were assessed against a battery of *in vitro* experimental systems. Phase III involved the evaluation of the anti-apoptotic and anticancer effect of *Zea mays* leaves. The fourth phase involved the identification of the major active principle present in the leaves of *Zea mays*.

PHASE I

Zea mays seeds were obtained from a local market in Coimbatore district, Tamil nadu. They were grown within the university campus in pots. The plant was taken at different periods of growth namely 5, 10, 15, 20, 25 and 30 days after sowing. The plantlets were uprooted and washed thoroughly with running tap water. Then the leaves were blotted dry between folds of filter paper to remove water droplets. The leaves were then analyzed for enzymic and non-enzymic antioxidants using various methods as given below.

ENZYMIC ANTIOXIDANTS

The enzymic antioxidants analyzed were superoxide dismutase, catalase, peroxidase, glutathione S-transferase and glutathione reductase.

ASSAY OF SUPEROXIDE DISMUTASE

Superoxide dismutase activity was assayed spectrophotometrically following the method of Misra and Fridovich (1972).

PRINCIPLE

SOD uses the photochemical reduction of riboflavin as oxygen-generating system and catalyses the inhibition of NBT reduction. The SOD activity is based on the measurement of increase in absorbance at 560 nm due to the reduction of NBT to $NBTH_2$.

REAGENTS

1. Potassium phosphate buffer (500 mM, pH 7.8)
2. Methionine (450 μM)
3. Riboflavin (53 mM)
4. Nitro Blue Tetrazolium (NBT) (840 μM)
5. Potassium cyanide (200 μM)

PROCEDURE

Exactly 0.5g of fresh *Zea mays* leaves were homogenized with 3.0ml of potassium phosphate buffer, centrifuged at 2000rpm for 10 minutes and the supernatant was used for the assay.

The assay medium contained 50mM potassium phosphate buffer (pH 7.8), 45μM methionine, 5.3mM riboflavin, 84μM NBT, 20μM potassium cyanide and the enzyme (leaf) extract in a final volume of 3 ml. The amount of enzyme extract added to the medium was kept below one unit of enzyme to ensure accuracy. The tubes were kept in an aluminum foil-lined box maintained at 25°C. They were then exposed to 15W fluorescent light for 10 minutes. The reduced NBT was measured spectrophotometrically at 600nm. In the absence of the enzyme, the maximum reduction was observed.

One unit of enzyme activity was defined as the amount of enzyme giving a 50% inhibition of the reduction of NBT. The values were expressed as units/mg protein.

ASSAY OF CATALASE

The enzyme-catalyzed decomposition of H_2O_2 was measured by the method of Luck (1974) in the leaves of *Zea mays*.

PRINCIPLE

The rate of decomposition of H_2O_2 was measured spectrophotometrically from changes in absorbance at 240nm, since H_2O_2 absorbs light at this wavelength.

REAGENTS

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

PROCEDURE

Zea mays leaves (0.5g) at six different periods of growth (5, 10, 15, 20, 25 and 30 DAS) were homogenized in 2.5ml of phosphate buffer (pH 7.0) at 4°C and centrifuged at 5000rpm. The supernatant containing 20% homogenate of the leaves was used for the assay. H_2O_2 in phosphate buffer (3ml) was taken in an experimental cuvette, followed by the addition of 40µl of enzyme extract and mixed thoroughly. The time interval required for a decrease in absorbance by 0.05 units was recorded at 240nm. 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

The peroxidase activity was assayed by the method of Reddy *et al.* (1985).

PRINCIPLE

Peroxidase converts H_2O_2 to H_2O and O_2 in the presence of a hydrogen donor pyrogallol. The oxidation of pyrogallol to a coloured product called purpurogalli can be followed spectrophotometrically at 430nm. The formation of the product is proportional to the enzyme activity.

REAGENTS

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

PROCEDURE

Zea mays leaves (0.5g) on their 5,10,15,20,25 and 30 days after sowing were ground separately with 2.5ml of 0.1M phosphate buffer pH 6.5. The supernatant of 20% leaf homogenate was utilized for the assay of peroxidase.

The assay mixture containing 3.0ml of pyrogallol solution and 0.1ml of enzyme extract was taken in a 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 S-TRANSFERASE

The method proposed by Habig *et al.* (1974) was adopted for assaying the activity of GST.

PRINCIPLE

GST activity can be determined spectrophotometrically by monitoring the thioester formation at 340nm using 1-chloro-2,4-dinitrobenzene (CDNB) as the substrate.

REAGENTS

1. CDNB (1mM in ethanol)
2. Reduced glutathione (1mM)
3. Phosphate buffer (0.1M, pH 6.5)

PROCEDURE

Zea mays leaves (0.5g) at different periods of growth were macerated in 5.0ml of phosphate buffer. The homogenate was centrifuged at 5000rpm for 10 minutes and the supernatant was utilized for the assay.

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 adding 0.1ml of enzyme extract and the readings were recorded against distilled water blank for a minimum of three minutes. The whole assay mixture without the enzyme served as the control. The

enzyme activity was determined by monitoring the change in absorbance at 340nm in a spectrophotometer.

One unit of GST activity is defined as the nmoles of CDNB conjugated per minute.

ASSAY OF GLUTATHIONE REDUCTASE

Glutathione reductase activity was followed as per the method of David and Richard (1983).

PRINCIPLE

Glutathione reductase catalyses the conversion of oxidized glutathione to reduced glutathione employing NADPH as a substrate. The amount of NADPH utilized is a direct measure of enzyme activity, which is assayed by measuring the decrease in absorbance at 340nm.

REAGENTS

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

PROCEDURE

Zea mays leaves (0.5g) at different periods of growth were extracted by homogenization in to 5.0ml of phosphate buffer. The homogenate was spun at 5000g for 10 minutes and the supernatant was used for the assay.

In this method, 1.0ml of potassium phosphate buffer, 0.1ml of EDTA, 0.1ml of sodium azide and 0.1ml of oxidized glutathione were added and the volume was made up to 2.0ml with water. In the same tube, 0.1ml of the leaf extract was also added. The assay mixture was kept at room temperature for 3 minutes followed by the addition of 0.1ml of NADPH. The absorbance at 340nm was recorded at an interval of 15 seconds for 3 minutes. For each series of measurement, controls were done that contained water instead of oxidized glutathione.

One unit of GR is expressed as the μmoles of NADPH oxidized per minute.

NON-ENZYMIC ANTIOXIDANTS

The non-enzymic antioxidants ascorbic acid, tocopherol, total carotenoids, lycopene, total phenols, flavonoids, reduced glutathione and chlorophyll were analyzed in the leaves at different periods of growth.

ESTIMATION OF ASCORBIC ACID

The ascorbic acid levels were estimated in the leaves of *Zea mays* by the method of Roe and Keuther (1943).

PRINCIPLE

When treated with activated charcoal, ascorbate is converted to dehydroascorbate. It, in turn, reacts with 2,4-dinitrophenyl hydrazine to form osazones, which dissolve in sulphuric acid to produce an orange coloured solution. The absorbance can be measured spectrophotometrically at 540nm.

REAGENTS

1. Trichloroacetic acid (4%)
2. Sulphuric acid (9N)
3. 2,4-dinitrophenylhydrazine reagent (2% in 9N sulphuric acid)
4. Thiourea solution (10%)
5. Sulphuric acid (85%)
6. Standard ascorbate solution: 10mg ascorbate in 100ml of 4% TCA.

PROCEDURE

Zea mays leaves (1g) were ground in 4% TCA to extract ascorbate and the volume was made up to 10ml with the same. The debris was clarified by centrifugation at 2000rpm for 10 minutes and the supernatant obtained was treated with a pinch of activated charcoal and shaken well. The tubes were allowed to stand for 10 minutes at room temperature. The charcoal particles were then removed by centrifugation. Aliquots of 0.5ml and 1.0ml of the supernatant and working standard solution ranging between 20-100μg of ascorbate were taken and the volume was made upto 2.0ml with 4% TCA.

To each tube, DNPH reagent (0.5ml) was added followed by 2 drops of 10% thiourea solution. The tubes were incubated at 37°C for 3 hours. The osazone crystals formed were dissolved in 2.5ml of 85% H_2SO_4 in cold to avoid local heating. The tubes were then cooled in ice and incubated at room temperature for 30 minutes. The absorbance was recorded at 540nm.

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

ESTIMATION OF TOCOPHEROL

The tocopherol content was estimated in the *Zea mays* leaves using Emmerie-Engel reaction as explained by Rosenberg (1992).

PRINCIPLE

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

REAGENTS

1. Absolute alcohol
2. Xylene
3. 2,2'-dipyridyl (1.2g in 1 litre of n-propanol)
4. Ferric chloride (1.2g in 1 litre of ethanol, stored in brown bottle).
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

Zea mays leaves (2.5g) were homogenized with a small volume of 0.1N H_2SO_4 and the volume was made up to 50ml with 0.1N H_2SO_4 by adding slowly, without shaking. The assay mixture was allowed to stand overnight. The contents were then shaken vigorously and filtered through Whatman No.1 filter paper. Aliquots of the filtrate were used for the estimation.

The leaf extracts (1.5ml), standard (1.5ml) and water (1.5ml) were pippetted into three stoppered centrifuge tubes. Ethanol (1.5ml) and 1.5ml of xylene were added to all the tubes, mixed well and centrifuged. From the xylene layer, 1.0ml was transferred into another stoppered tube and 1.0ml of 2,2'-dipyridyl reagent was added and mixed well. This mixture was taken in the spectrophotometer cuvette and the extinctions of the test and the standard were read against the blank at 460nm. Then, in turn, beginning with the blank, 0.33ml of ferric chloride solution was added and mixed well. Exactly after 15 minutes, the absorbance of the red colour produced was read against blank at 520nm. The concentrations of tocopherol in the sample were calculated using the formula,

$$\text{Tocopherol}(\mu g) = \frac{\text{Reading at 520nm - Reading at 450nm}}{\text{Reading of standard at 520nm}} \times 0.29 \times 15$$

The results are expressed as µg tocopherol / g sample.

ESTIMATION OF TOTAL CAROTENOIDS AND LYCOPENE

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

PRINCIPLE

Petroleum ether was used to extract total carotenoids and lycopene and was estimated spectrophotometrically at 450nm and 503nm. At 503nm lycopene has a maximum absorbance while carotenes have only negligible absorbance.

REAGENTS

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

PROCEDURE

To avoid photolysis of carotenoids, all the steps subsequent to saponification were carried out in the dark. *Zea mays* leaves (0.5g) were homogenized and saponified with 2.5ml of 12% alcoholic KOH in a water bath at 60°C for 30 minutes. The saponified extract was then transferred into a separating funnel (plugged with glass wool along with

calcium carbonate) containing 10-15ml of petroleum ether. The extract was mixed gently, the lower aqueous phase was transferred to another separating funnel and the upper petroleum ether containing the carotenoid pigment was collected. The extraction was repeated until the aqueous phase became colourless.

A small quantity of anhydrous sodium sulphate was added to the petroleum ether extract to remove excess moisture. Whenever needed, the final volume of the petroleum ether was noted and diluted by a known dilution factor.

The yellow colour was read at 405nm and 503nm in a spectrophotometer using petroleum ether as a blank. The amount of total carotenoids and lycopene was calculated using the formula

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

$$\text{Amount of lycopene} = \frac{3.12 \times A_{503} \times \text{Volume of sample} \times \text{dilution} \times 100}{\text{Weight of the sample} \times 1000}$$

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

ESTIMATION OF TOTAL PHENOLS

The levels of total phenols were assayed using the method of Mallick and Singh (1980).

PRINCIPLE

Phenols react with phosphomolybdic acid in Folin-Ciocalteau reagent in alkaline medium to produce a blue coloured complex (molybdenum blue), which can be quantified 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

Ethanol (80%) of about 10X volume was used to prepare homogenate of 0.5g of *Zea mays* leaves. The homogenate was centrifuged at 10,000g for 20 minutes. The residue was re-extracted with 80% ethanol. The pooled supernatants were evaporated to dryness.

The residues were then dissolved in a known volume of distilled water. Aliquots ranging from (0.2ml to 2.0ml) were pipetted out into test tubes. The volume in each tube was made upto 3.0ml with water. 0.5ml of Folin-Ciocalteau reagent was added to all the tubes and mixed well. After 3 minutes, 2.0ml of 20% sodium carbonate solution was added and mixed thoroughly. The tubes were then kept in a boiling water bath for exactly one minute and allowed to cool at room temperature. The absorbance was measured against a reagent blank at 650nm in a spectrophotometer.

Standard catechol solutions corresponding to 2-10μg concentrations were also treated as above. A standard curve was constructed using an electronic calculator on the linear regression mode, using which the concentrations of phenols in the *Zea mays* samples were calculated. The values are expressed as mg phenols /g leaf.

ESTIMATION OF FLAVONOIDS

The flavonoid content in the leaves of *Zea mays* was quantified by the method of Cameron *et al.* (1943).

PRINCIPLE

Flavonoid is estimated by its reaction with vanillin to produce a coloured product, which can be read spectrophotometrically.

REAGENTS

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

EXTRACTION OF FLAVONOIDS

The leaves (0.5g) were extracted first with methanol : water mixture (2:1) followed by the same mixture in the ratio 1:1. The extracts were shaken well and allowed

to stand overnight. The pooled supernatants were then 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 th bulk of the flavonoids was then concentrated.

PROCEDURE

An aliquot of *Zea mays* leaf extract was pippetted out and evaporated to dryness. Four milliters of vanillin reagent was added and the tubes were heated for 15 minutes in a boiling water bath. The standard of varying concentrations was also treated in the same manner. The absorbance was measured at 340nm and the values were expressed as mg flavonoids / g leaf.

ESTIMATION OF REDUCED GLUTATHIONE

Moron *et al.* (1979) have proposed a method for the determination of reduced glutathione, which was adopted for this study.

PRINCIPLE

The amount of reduced glutathione is measured by its reaction with DTNB (5, 5'-dithiobis-2-nitro benzoic acid) (Ellmans reaction) to give a yellow colored product that absorbs at 412 nm.

REAGENTS

1. Phosphate buffer (0.2M, pH 8.0)
2. DTNB (0.6mM in 0.2M phosphate buffer)
3. TCA (5% and 25%)
4. Standard GSH (10 nmoles / ml in 5% TCA)

PROCEDURE

A 20% homogenate was prepared by homogenizing 0.5g of *Zea mays* leaves in 2.5ml of 5% TCA. The protein was precipitated by adding 125μl of 25% TCA to 0.5ml of tissue homogenate. The precipitated protein was centrifuged at 1000rpm for 10 minutes and cooled on ice. The supernatant (0.1ml) was used for the estimation of GSH.

The supernatant (0.1ml) was made up to 1ml with 0.2M sodium phosphate buffer (pH 8.0). Freshly prepared DTNB solution (2ml) was added to the tubes and the intensity of the yellow colour was read at 412nm in a spectrophotometer after 10 minutes.

Using the linear regression mode in the electronic calculator, a standard curve of GSH was plotted using the concentrations ranging from 2-10 nmoles of GSH and the values of the samples were read off it. The values are expressed as nmoles of GSH/g leaf.

ESTIMATION OF CHLOROPHYLL

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

PRINCIPLE

Chlorophyll is extracted in 80% acetone and its estimation is based on the absorption coefficient at 663nm and 645nm.

REAGENT

Acetone (80%, prechilled)

PROCEDURE

Zea mays leaves (1g) were extracted with 20ml of 80% acetone. The tubes were centrifuged for 5 minutes at 5000rpm and the supernatant was transferred to a volumetric flask. The extraction was repeated until the residue was colourless. After washing thoroughly the mortar and pestle with 80% acetone, the clear washings were then collected in the volumetric flask. The supernatant was made up to 100ml with 80% acetone. The absorbance of the green coloured solution was read at 645nm and 663nm against 80% acetone (blank). The total chlorophyll in the leaf was calculated using the formula,

$$\text{Total chlorophyll} = 202(A_{645}) + 8.02(A_{663}) \times \frac{V}{1000 \times W}$$

where

A = Absorbance at specific wavelength

V = Final volume of chlorophyll extract in 80% acetone

W = Fresh weight of leaves taken for extraction

The values are expressed as mg chlorophyll /g leaf.

PHASE II

The results obtained from phase I, which are presented in the next chapter, revealed that the 10th day plant showed maximum antioxidant content compared to the other time periods studied. Therefore, the 10th day plant was selected for further studies. In order to determine the chemical nature of the bioactive component, the leaf extracts were prepared in solvents of different polarity namely water, methanol and chloroform.

Phase II was designed to assess the antioxidant potential of *Zea mays* leaves with a battery of radicals and oxidants as targets. The molecular mechanism operating behind the antioxidant activity was studied by performing reducing and chelating assays. The biomolecular protective effects of *Zea mays* leaf extracts under oxidatively stressed conditions on purified molecules, isolated cells and *in vitro* models that simulated the *in vivo* environment were also assessed.

PREPARATION OF PLANT EXTRACTS

Fresh *Zea mays* leaves (1g) of 10th day plant were collected. The leaves were homogenized in 1ml of appropriate solvents (methanol or chloroform). The tubes were centrifuged at 2000rpm for 5 minutes and the supernatant was 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. Aqueous extracts were prepared fresh for each experiment.

EVALUATION OF RADICAL SCAVENGING EFFECTS OF *Zea mays* LEAF EXTRACTS

Zea mays leaf extracts were analyzed for their ability to nullify the effect of a sequence of oxidant moieties.

DPPH SCAVENGING EFFECT

The antioxidant activities of the plant extracts were assessed on the basis of the radical scavenging effect of the stable free radical DPPH as per the method of Mensor *et al.* (2001). The DPPH method permits to evaluate not only the electron or hydrogen atom-donating properties of antioxidants, but also the rate of their reaction towards the free radicals.

PRINCIPLE

The spectrophotometric technique employs the 2,2-diphenyl-2- picrylhydrazyl free radical ($DPPH^{\bullet}$), which shows a characteristic spectrum with a maximum absorbance close to 515nm in methanol. The addition of an antioxidant compound results in a decrease of absorbance proportional to the concentration and antioxidant activity of the compound.

REAGENTS

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

PROCEDURE

Zea mays leaf extracts (20µl corresponding to 10mg) were added with 0.5ml of methanolic solution of DPPH and 0.48ml of methanol. The reaction mixture was allowed to stand at room temperature for 30 minutes. Methanol served as blank and DPPH in methanol without the leaf extracts served as positive control. After incubation, the discolouration of the purple colour was read at 518nm in a spectrophotometer. The per cent extent of radical scavenging was calculated using the formula,

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

ABTS SCAVENGING EFFECT

Shirwaikar *et al.* (2006) have proposed a method for assessing ABTS (2,2'-azino-bis-3-ethyl benzthiazoline-6-sulphonic acid) radical cation de-colourization assay.

PRINCIPLE

The improved technique for the generation of $ABTS^{\bullet+}$ involves the direct production of the blue/green $ABTS^{\bullet+}$ chromophore through the reaction between ABTS and ammonium persulfate. This has absorption maxima at 745nm. Addition of antioxidants to the pre-formed radical cation reduces ABTS on a time-scale depending on the antioxidant activity, the concentration of the antioxidant and the duration of the

reaction. The method is applicable to the study of both water-soluble and lipid-soluble antioxidants, pure compounds, and food extracts.

REAGENT

ABTS solution (7mM with 2.45mM ammonium persulfate)

PROCEDURE

When ABTS solution (7mM) reacts with 2.45mM ammonium persulphate, ABTS radical cations ($ABTS^{+}$) were produced. The reaction mixture was allowed to react in the dark at room temperature for 12-16 hours before use. *Zea mays* leaf extracts (each 0.5ml) were added to 0.3ml of ABTS solution and the final volume was made upto 1ml with ethanol. The absorbance was read at 745 nm and the per cent inhibition of ABTS cation was calculated as follows:

$$\text{Inhibition (\%)} = \frac{(\text{Control} - \text{Test})}{\text{Control}} \text{x} 100$$

HYDROGEN PEROXIDE SCAVENGING EFFECT

The ability of *Zea mays* 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. Phosphate buffer (0.1M, pH 7.4)
2. H_2O_2 in phosphate buffer (40mM)

PROCEDURE

Hydrogen peroxide (40mM) solution was prepared in phosphate buffer (pH 7.4). *Zea mays* leaf extracts at the concentration of 10mg/10µl were added to H_2O_2 solution (0.6ml, 40mM) and the final volume was made up to 3 ml. The absorbance at 230nm was

determined against a blank containing phosphate buffer without H_2O_2. The per cent H_2O_2 scavenging ability of the plant extract was calculated using the formula,

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

HYDROXYL RADICAL SCAVENGING EFFECT

The scavenging capacity for hydroxyl radical was measured according to the method of Elizabeth and Rao (1990). The hydroxyl radical scavenging activity was measured by studying the competition between deoxyribose and the extracts for hydroxyl radicals generated with Fe_3^+/ascorbate/EDTA/H_2O_2 system. The hydroxyl radicals attack deoxyribose, which eventually results in TBARS formation, which can be quantified spectrophotometrically.

PRINCIPLE

The formation of $^{\bullet}$OH radicals was measured using 2'-deoxyribose oxidative degradation. The principle of the assay is the quantification of the major 2'-deoxyribose degradation product, malonaldehyde (MDA), by its condensation with TBA.

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 (200mM, pH 7.4)
7. Thiobarbituric acid (10%)
8. HCl (25%)

PROCEDURE

The final volume of 1ml of reaction mixture contained 2.8mM deoxyribose, 0.1mM $FeCl_3$, 0.1 mM EDTA, 1mM H_2O_2, 0.1mM ascorbate, 20mM phosphate buffer and 20µl of leaf extract. The reaction mixture was incubated at 37°C for 1 hour. At the end of the incubation period, 0.5ml of TBA and HCl were added and heated at 95°C for 20 minutes to develop the colour. After cooling, the amount of TBARS formed was

measured spectrophotometrically at 532nm against an appropriate blank. The hydroxyl radical scavenging activity was determined by fixing the TBARS production for positive control (H_2O_2) as 100% and the relative per cent TBARS were calculated for the extract treated groups.

SUPEROXIDE SCAVENGING EFFECT

The extent of inhibition of superoxide generation *in vitro* was quantified by the method of Winterbourn *et al.* (1975).

PRINCIPLE

The assay is based on the inhibition of the production of nitro blue tetrazolium formazon of the superoxide ion by the leaf extracts and is measured spectrophotometrically at 560nm.

REAGENTS

1. EDTA (0.1 M containing 1.5mg of NaCN)
2. Nitroblue tetrazolium (NBT - 1.5mM)
3. Riboflavin (0.12mM)
4. Phosphate buffer (0.067M, pH 7.6)
5. DMSO

PROCEDURE

Superoxide anions were generated in samples containing 3.0ml each of 0.02ml of the plant 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 plant extracts. The tubes containing assay mixture were then vortexed and the initial optical density was observed at 560nm. The tubes were illuminated uniformly by using a fluorescent lamp for 30 minutes. Again the absorbance was taken at 560nm. The difference in absorbance before and after illumination was indicative of superoxide anion scavenging activity.

The per cent inhibition of superoxide anion generation was calculated using the formula,

$$\% \text{ superoxide scavenged} = \frac{(A_{Control} - A_{Test})}{A_{Control}} \times 100$$

where,

$A_{Control}$ is the absorbance of the control reaction

A_{Test} is the absorbance in the presence of the extract

NITRIC OXIDE SCAVENGING EFFECT

The efficiency of the leaf extracts of *Zea mays* in inhibiting the *in vitro* generation of nitric oxide was studied 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 can be estimated spectrophotometrically with Griess reagent at 546nm. Scavengers of nitric oxide compete with oxygen leading to reduced production of nitric oxide.

REAGENTS

1. Sodium nitroprusside (100mM)
2. Phosphate buffered saline (pH 7.2)
3. Griess reagent (1% sulphanilamide, 2% H_3PO_4 and 0.1% naphthylethylene diamine dihydrochloride)

PROCEDURE

The assay mixture (3.0ml) containing 2.0ml of sodium nitroprusside, 0.5ml of PBS and 0.5ml of the *Zea mays* leaf extract (50mg) was incubated for 150 minutes at 25°C. At the end of the incubation period, 0.5ml of Griess reagent was added and allowed to stand for 30 minutes. Control tubes were measured without the leaf extracts. The pink coloured chromogen formed was then read at 546nm against a reagent blank.

$$(\%) \text{ Nitric oxide scavenged} = \frac{(A_{Control} - A_{Test})}{A_{Control}} \times 100$$

where,

$A_{Control}$ is the absorbance of the control reaction
A_{Test} is the absorbance in the presence of the sample of the extracts

ASSAY OF REDUCING PROPERTY

The reducing property was determined according to the modified method of Oyaizu (1986).

PRINCIPLE

Potassium ferricyanide and ferric chloride, when subjected to reduction by an antioxidant, give a coloured complex, which can be read spectrophotometrically at 700nm.

REAGENTS

1. Phosphate buffer (20mM, pH 6.6)
2. Potassium ferricyanide (1%)
3. Trichloroacetic acid (10 %)
4. Ferric chloride (0.1%)

PROCEDURE

Zea mays leaf extracts (20mg) were taken with 500µl of phosphate buffer (20mM, pH 6.6) and 500µl of 1% potassium ferricyanide was added and incubated at 50°C for 20 minutes. TCA was added to the mixture to a final concentration of 0.5% and centrifuged at 2000rpm for 15 minutes. The supernatants were mixed with 500µl of distilled water and ferric chloride (0.1%). The colour obtained was measured at 700nm.

ASSAY OF CHELATING PROPERTIES

The chelating property of *Zea mays* leaf extracts was determined based on the spectral changes following by the method of Brown *et al.* (1998).

PRINCIPLE

Ferrous chloride ($FeCl_2$) complexes with potassium hexacyanoferrate (potassium ferricyanide) and gives the coloured complex ferrous ferricyanide, called Turnbull's blue, with the maximum absorption at 700nm. The addition of antioxidant to the reaction mixture under the same conditions, decreases the absorption at 700nm. It shows that the antioxidant binds iron and makes it unavailable to $K_3[Fe(CN_6)]$ to form a complex and decreases the colour in a concentration-dependent manner.

REAGENTS

1. $FeCl_2$ (200μM)
2. Potassium ferricyanide (400μM)
3. EDTA-10μmoles in 1ml
4. TBS (20mM Tris HCl, 150mM NaCl, pH 7.4)

PROCEDURE

The reaction mixture containing ferrous chloride (200μM) and potassium ferricyanide (400μM) with or without *Zea mays* leaf extracts or EDTA (10μmoles) were added in a final volume of 1ml of water and mixed. The reaction mixture was incubated at 20°C for 10 minutes. The formation of the potassium hexacyanoferrate complex was measured at 700nm in spectrophotometer. The assay was carried out at 20°C to prevent Fe^{2+} oxidation.

Lower absorbance indicated higher iron chelating capacity. The control was without any chelating compound or test sample. The percent ferrous ion chelating effect was calculated from the equation,

$$(\%)\text{ iron chelating effect} = \frac{(\text{Control - Test})}{\text{Control}} \times 100$$

EFFECT OF *Zea mays* LEAF EXTRACTS ON OXIDATIVE DAMAGE TO BIOMOLECULES

Oxidative stress is characterized by an increase in reactive oxygen species (ROS) as a result of insufficient antioxidant defense. Overproduction of ROS is harmful to the body because the oxidation induced by ROS can result in cell membrane disintegration, membrane protein damage and DNA mutation, which can further initiate or propagate the development of many diseases (Geetha *et al.*, 2005).

Lipids are essential components of the membranes that surround the cells as well as other cellular structures, such as the nucleus and mitochondria, and constitute the immediate targets of ROS. In addition to damaging cells by destroying membranes, lipid peroxidation can result in the formation of reactive products that themselves can react with and damage DNA, which is considered to be the ultimate target of ROS (Canakci *et al.*, 2009).

EVALUATION OF THE EFFECTS OF *Zea mays* LEAF EXTRACTS ON MEMBRANE LIPIDS

All cellular membranes are especially vulnerable to oxidation due to their high concentrations of unsaturated fatty acids. The major damage to lipids is manifested as lipid peroxidation.

Lipid peroxidation in cells leads to direct damage of cell membranes with indirect damages of other cell constituents, caused by the reactivity of secondary products of this reaction. The damage caused by LPO is highly detrimental to the functioning of the cell and its survival (Balaban *et al.*, 2005).

Three different membrane model systems were selected in the present study to analyze the extent of LPO and protection rendered by the leaf extracts. 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 membrane and intact (live) cells. RBCs 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 prepared using Tris HCl buffer (40mM, pH 7.0) served as the source of internal membranes.

PRINCIPLE

The quantitative measurement of lipid peroxidation can be done by the thiobarbituric acid (TBA) test. The extent of formation of thiobarbituric acid reactive substances (TBARS) from the damaged lipids was quantitated by the reaction with TBA and used as an index of lipid peroxidation.

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 from the slaughter-house in a sterile container. The blood was defibrinated immediately using sterile acid-washed stones. After transferring the defibrinated blood to another container, it was diluted 1:1 with sterile isotonic KCl. The RBCs were pelleted by centrifugation at 3000xg for 10 minutes at 4°C. The supernatant was aspirated completely and the pellet was washed thrice with isotonic (1.15%) KCl. The washed pellet was treated with hypotonic KCl (0.5%) and allowed to lyse at 37°C for one hour. The lysate was then centrifuged at 5000Xg 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 then suspended in 1.5ml of TBS and 50µl aliquots were used for the assay as per the protocol of Dodge *et al.* (1963).

PROCEDURE

The assay mixture contained 50µl of RBC ghosts, 50µl of *Zea mays* leaf extracts, 50µl of $FeSO_4$ and 100µl of ascorbate, made up to 500µl with TBS. 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. To represent 100% oxidation, an assay medium containing all the other constituents except the plant extract was also prepared. The experimental medium corresponding to auto-oxidation contained only RBC ghosts. All the tubes were incubated for one hour at 37°C, followed by the addition of 0.5ml of 70% alcohol to all the tubes to arrest the reaction. TBA (1.0ml) was then added to all the tubes and heated in a boiling water bath for 20 minutes. After cooling to room temperature, the tubes were centrifuged to clear the solution and to the supernatant 0.5ml of acetone was added. The pink colour developed was measured at 535nm in a spectrophotometer.

ESTIMATION OF LPO IN GOAT LIVER HOMOGENATE

Goat liver was procured fresh from a slaughterhouse and washed thoroughly using Tris HCl buffer (40mM, pH 7.0). Using a motorized Teflon homogenizer, a 20% homogenate of the liver was prepared in the same buffer.

The homogenate was clarified by low speed centrifugation to remove the debris and this was used as the membrane source for the LPO assay performed according to the method of 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 containing 0.1ml of liver homogenate, 0.1ml of KCl, 0.1ml of $FeSO_4$ and 0.1ml of ascorbate was incubated for one hour at 37°C both in the presence and the absence of *Zea mays* leaf extracts (0.1ml corresponding to 50mg). TBA and acetic acid (1.5ml each) were added to 0.4ml of the assay mixture and mixed well. 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 colored chromophore obtained after centrifugation was measured at 532nm.

ESTIMATION OF LPO IN GOAT LIVER SLICES

The extent of inhibition of LPO in goat liver slices was estimated by the method proposed 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)

PROCEDURE

PREPARATION OF GOAT LIVER SLICES

The goat liver was collected fresh from a slaughterhouse, plunged in cold sterile PBS and maintained at 4°C till use. Using a sterile scalpel, thin slices of 1mm thickness were obtained.

PROCEDURE

Thin slices of goat liver (1.0g) were taken in 4.0ml of sterile PBS in flat-bottomed flasks, followed by the addition of oxidizing agent (550μM H_2O_2) and /or the extracts of *Zea mays* leaves. 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. To 1.0ml of homogenate, 2ml of TBA-TCA-HCl reagent was added to terminate the reaction. The mixture was heated in a boiling water bath for 15 minutes and the pink colour developed was read at 535nm against a reagent blank.

EFFECT OF THE EXTRACTS OF *Zea mays* LEAVES ON OXIDANT INDUCED DNA DAMAGE

ROS-induced DNA damage involves single- or double-stranded DNA breaks, purine, pyrimidine or deoxyribose modifications and DNA cross-links. DNA damage can result in either arrest or induction of transcription, induction of signal transduction pathways, replication errors and genomic instability, all of which are associated with carcinogenesis. To date, more than 100 oxidised DNA products have been identified (Valko *et al*., 2007). In the present investigation, the protective effect of *Zea mays* leaf extracts, in counteracting oxidant mediated DNA damage was assessed.

The DNA damage was studied *in vitro* in commercially available preparations of DNA and in intact cells. The DNA from different hierarchies of evolutionary development were selected for these assays. The commercially available preparations included viral DNA (λ DNA), herring sperm DNA and calf thymus DNA. The DNA from intact cells was from human peripheral blood cells.

ESTIMATION OF DAMAGE IN λ DNA

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

REAGENTS

1. λ DNA (2μg)
2. Tris buffer (30mM, pH 7.4)
3. H_2O_2 (30%)

4. $FeCl_3$ (500μM)
5. Agarose (1%) in 1X TAE buffer
6. EtBr (10mg/ml)
7. Gel loading dye (0.25% bromophenol blue, 0.25% xylene cyanol, 50% glycerol)
8. 50X TAE buffer (Tris base 24.2g, EDTA 18.612g, glacial acetic acid 5.71ml in a total volume of 100ml, pH 8.0)

PROCEDURE

The reaction was carried out in tris buffer (pH 7.4) at 37°C. Hydroxyl radicals were generated by allowing $FeCl_3$ and H_2O_2 to react. The reaction mixture contained 5μl of tris buffer or λ DNA (2μg) and 5μl of tris buffer or leaf extracts followed by the addition of 10μl H_2O_2 and 5μl of $FeCl_3$. λ DNA was then incubated at 37°C for 30 minutes. To the above mixture, 6μl of loading dye was added and electrophoresed in 1% agarose gel containing 3μg/ml EtBr, at 100V for 15 minutes. DNA bands were viewed under transilluminating UV light and photographed using an Alpha Digidoc digital gel documentation system (Alpha Innotech, USA). The intensity of the bands were quantified using the software of the system.

ESTIMATION OF DAMAGE IN HERRING SPERM AND CALF THYMUS DNA

The extent of DNA damage caused by hydrogen peroxide and the effect of *Zea mays* leaves on it was studied according to the method proposed by Aeschlach *et al.* (1994).

REAGENTS

1. Herring sperm DNA or calf thymus DNA (0.5mg/ml in 500mM tris buffer)
2. H_2O_2 (30%)
3. $MgCl_2$ (5mM)
4. $FeCl_3$ (50μm)
5. EDTA (0.1M)
6. TBA (1% w/v)
7. HCl (25%)
8. Tris buffer (10mM, pH 7.4)

PROCEDURE

The assay mixture (0.5 ml) contained 0.05ml of herring sperm DNA, 0.167ml of H_2O_2, 0.05ml of $MgCl_2$, 0.05ml of $FeCl_3$ (50μM) and leaf extract (10μl containing 10mg of extract prepared in tris buffer [10mM, pH 7.4]). The mixture was incubated at 37°C

for 1 hour. 0.05ml of 0.1M EDTA was added to terminate the reaction. The colour was developed by adding 0.5 ml of thiobarbituric acid and HCl, followed by incubation at 37°C for 15 minutes. After centrifugation, the extent of DNA damage was measured by the increase in absorbance at 532nm.

EVALUATION OF THE EXTENT OF DNA DAMAGE IN INTACT CELLS

The comet assay was used to quantify the extent of DNA damage in intact cells. It was carried out under alkaline conditions, basically as described by Singh *et al.* (1988). The intact cells used were peripheral blood cells. Prior to the assay, the cells were resuspended in Hank's Balanced Salt Solution (HBSS).

PRINCIPLE

The alkaline (pH > 13) single cell gel (SCG) electrophoresis assay, commonly known as comet assay, combines the simplicity of biochemical techniques for detecting DNA single strand breaks, alkali labile sites and the single cell approach typical of cytogenetic assays. This assay is one of the most sensitive assays to detect DNA damage.

REAGENTS

1. H_2O_2 (30%)
2. Low melting point agarose (LMPA – 0.5%)
3. Normal melting point agarose (NMPA – 1%)
4. Lysis solution (1M tris, pH 8.0, EDTA (0.5M), NaCl (2.5M), DMSO (10%), Triton X-100 (1%)) – The DMSO and triton X-100 were added fresh, just before use.
5. Neutralizing buffer (1M tris, pH 7.5)
6. Alkaline electrophoresis buffer (10N NaOH, 0.2M EDTA, pH >13)
7. EtBr (5μg/ml)

PROCEDURE

Peripheral blood cells were treated with H_2O_2 in the presence and the absence of *Zea mays* leaf extracts (20mg) and incubated at 37°C for 1 hour. At the end of the incubation period, an aliquot (150μl) of the cell suspension was mixed with equal volumes of 0.5% molten LMPA and maintained at 37°C. This suspension (75μl) was layered carefully onto 1% NMPA coated glass slides and spread evenly using a coverslip (22 x 40 mm). The slides were then immediately placed on slide trays held on ice in order

to solidify the agarose. The coverslip was removed carefully and the cell spread was overlaid with a layer of LMPA without cells. After solidification, the 'mini gels' were placed in cold lysing solution taken in coplin jars and incubated overnight at 4°C. The lysed cells were denatured in the alkaline electrophoresis buffer for 20 minutes and electrophoresed in the same buffer at 25V, 300mA for 20 minutes. After the electrophoresis, the gels were neutralized in Tris buffer for 10 minutes (pH 7.5) and stained with ethidium bromide solution. The slides were washed to remove excess ethidium bromide and air-dried. They were then scored for the presence of comet 'tails' under oil immersion in a fluorescent microscope (Nikon, Japan). Totally 100 cells per slide were scored and the frequency of DNA damage, as the number of comet bearing cells, was noted.

ASSESSMENT OF DNA REPAIR

In the present investigation, significant DNA damage was induced by H_2O_2, as reflected by the increased number of cells with comets. The extent of DNA damage was significantly reduced by the co-administration of the cells with the leaf extracts. This reduction could be due to the prevention of DNA damage (i.e., scavenging of the DNA damaging agent by the components of leaf extracts) or to the effective repair of the damaged DNA.

In order to ascertain the exact mechanism operating behind the reduction in the extent of DNA damage, Unscheduled DNA Synthesis (UDS) was followed in the Hep2 cell line exposed to H_2O_2. UDS is an indirect method of testing DNA repair capacity as cellular incorporation activity, which can be measured by scintillation counting (Hellman *et al.*, 1985).

PRINCIPLE

The incorporation of ^{3}HTdR (tritium labeled thymidine) into DNA follows the same route as incorporation of non-radioactive thymidine into DNA. This incorporation thus becomes an accurate measure of the rate of DNA synthesis, which, when unscheduled, is considered to be a measure of DNA repair. The uptake can be determined by liquid scintillation counting (LSC) of DNA from cells.

REAGENTS

1. ^{3}HTdR (from BARC, Mumbai)- specific activity 18,500 mCi/mmole
2. HBSS (Hank's Balanced Salt Solution)
3. Perchloric acid (0.6N and 0.4N)
4. 0.2 N Perchloric acid
5. Ethanol
6. Ether
7. Chloroform
8. KOH (0.3N)
9. Scintillation Cocktail (PPO – 4.0g, POPOP – 0.2g, naphthalene – 60g, ethylene glycol – 20ml, methanol – 100ml, 1,4-dioxan – to make up the final volume to 1 litre).

PROCEDURE

All discards were collected as radioactive wastes and disposed according to the standard procedures of BARC. The Hep2 cell line was procured from the National Centre for Cell Sciences (NCCS), Pune, India. The cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS). The cells were grown to confluence and were harvested by trypsinization (0.25%). The harvested cells were collected in DMEM containing 10% FBS and were held on ice till the analysis. The cells were subjected to oxidant and respective plant extract treatment along with ^{3}HTdR (0.4μCi/10^6 cells) at 37°C for one hour with mild shaking. After the incubation period, the volume of the samples was adjusted to 5ml using HBSS. Ice-cold 0.6N perchloric acid (2.5ml) was added and kept at 0°C for 10 minutes. The samples were centrifuged at 2000g for five minutes and the residue was washed thrice in cold 0.4N perchloric acid. The residues were extracted with ethanol, ethanol-chloroform (3:1) and ether. The fat free residue was subjected to alkaline digestion with 4.0ml of 0.3N KOH for 1 hour at 37°C. At the end of the incubation period, the tubes were cooled on ice and the DNA and protein were precipitated by adding 6ml of 0.2N perchloric acid. The tubes were cooled for 10 minutes at 2°C and centrifuged. 4ml of hot 0.6N perchloric acid was added to extract the DNA from the precipitate and hydrolysed for 15 minutes at 70°C, followed by additional extraction with 3.0ml of hot 0.6N perchloric acid. The DNA extracts were pooled and an aliquot of 4.0ml was taken for measuring the radioactivity in a Beckmann LS-1801 liquid scintillation counter. Correction for quenching was made using an external standard. The values were converted to dpm, and expressed as per cent extent of DNA repair.

The last part of phase II was planned to further probe the antioxidant property of *Zea mays* leaves in an *in vitro* model (goat liver slices). The extracts of the leaves were administered to the oxidatively stressed goat liver slices and the antioxidant status was analysed in them.

Precision-cut liver slices have proved useful for several pharmacological and toxicological investigations. Liver was the organ of choice because it is the metabolic organ and is responsible for the metabolic clearance of many xenobiotics. Liver slice is a microcosm of the intact liver and therefore it is an *in vitro* technique that offers the advantages of *in vivo* situation and hence is a more suitable model for the experimental analysis of antioxidant protection studies (Onderwater *et al.*, 2004).

The tissue preparation and the treatment groups set up were the same as that of the LPO in goat liver slices. 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.

At the end of the incubation period, a homogenate was prepared from the slices using the same incubation solution (PBS). The homogenate was centrifuged at 1500rpm for 5 minutes to clarify the debris and the supernatant was used for the analyses of both enzymic and non-enzymic antioxidants.

ASSAY OF ENZYMIC ANTIOXIDANTS

The enzymic antioxidants that were analysed were superoxide dismutase, catalase, peroxidase, glutathione S-transferase and glutathione reductase. All these enzymes were assayed following the same protocols as in Phase I of the study. Liver homogenate was used as the enzyme source instead of the leaf tissue.

DETERMINATION OF NON-ENZYMIC ANTIOXIDANTS

The non-enzymic antioxidants estimated in the tissue homogenate were ascorbic acid, tocopherol, vitamin A and reduced glutathione. The procedures adopted for the estimation of ascorbic acid, tocopherol and reduced glutathione were similar to those adopted in Phase I of the study, where instead of the leaf tissue, an aliquot of the liver homogenate was used in all the assays.

ESTIMATION OF VITAMIN A

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

PRINCIPLE

The assay is based on the spectrophotometric estimation of the colour produced by vitamin A, its 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)

PROCEDURE

All the procedures were carried out in the dark to avoid the interference of light. Liver homogenate and saponification mixture of 1 ml each were taken. The mixture was refluxed for 20 minutes at 60°C in the dark, and then cooled. To this, 20ml of water was added and mixed well. Vitamin A was extracted twice with 10ml portions of petroleum ether (40-60°C). The extracts were pooled, washed thoroughly with water and 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 of 1.0ml was evaporated to dryness at 60°C. The dried residue was dissolved in 1.0ml of chloroform. Standards (vitamin A palmitate) of concentrations ranging from 0 - 7.5µg were pipetted out into a series of clean, dry test tubes. The volumes in all the tubes were made upto 1.0ml with chloroform. 2.0ml of TCA reagent was added rapidly, mixed well and the absorbance of the blue colour formed was measured immediately at 620nm in a spectrophotometer. The procedure was repeated for the sample tubes. Vitamin A was expressed as mg/g tissue.

PHASE III

The results of phase II (presented in the next chapter) were suggestive of the fact that the leaf extracts of *Zea mays* are capable of scavenging free radicals and possess

reducing and chelating activity. They also protected the biomolecules against oxidative damage under *in vitro* conditions.

The leaf extracts of *Zea mays* could also improve the antioxidant status in the goat liver slices exposed *in vitro* to oxidative stress. Since the model was carefully planned to simulate *in vivo* conditions, it is perceivable that the effects also occur in the intact system. The methanolic extract elicited a better response than the aqueous and chloroform extracts. Our next objective was to focus on the influence of *Zea mays* leaf extracts in the apoptotic events under oxidative stress induced conditions. In order to minimize the use of animals for research purpose, alternative models were used.

The study was planned in tune with one of the primary objectives of our research group, which is to standardize the use of alternative experimental systems for studying plant extracts and products, thereby minimizing the use of live animals in research. This approach is part of a global effort, wherein several alternative systems to replace live experimental animals are being characterized.

Free radicals are recognised to play a part in both the induction of apoptotic cell death and in necrosis (Singh, 2007). Apoptosis of unrequired cells during development is undoubtedly beneficial to the organism, whereas inappropriate apoptotic cell death in some inflammatory disorders is likely to be deleterious (Thatte *et al.*, 2000).

Of interest to the scientists, is the concerted effort to study pharmacological modulation of programmed cell death. The attempt to influence the natural phenomenon of programmed cell death stems from the fact that it is reduced (as in cancer) or increased (as in neurodegenerative diseases) in several clinical situations. Thus, chemicals that can modify programmed cell death are likely to be potentially useful drugs. Recently, a variety of plant extracts have been investigated for their ability to influence the apoptotic process. Hence, our next objective was to focus on the influence of *Zea mays* leaf extracts in the apoptotic events under oxidative stress-induced conditions.

The yeast is a potentially useful eukaryotic model for studies of the effect of antioxidants at the cellular level, an attractive alternative to mammalian cell lines. Such studies can not only verify the results of the test tube experiments on the protection of macromolecules from the action of oxidants, but also reveal possible side effects of

antioxidants and products of their metabolism. Experiments with yeast are easy, cheap and avoid ethical questions inherent to the use of animals.

In vitro assays such as mammalian and human cell cultures, may all contribute information towards a weight of evidence and characterization sufficient to render the rodent bioassay unnecessary. It is also important to note that research using cell tissue culture or non-mammalian system is conducted not only as an alternative to using mammals but because a given alternative system best answers the question under study. *In vitro* studies also allow researchers to understand the discrete steps in specific sequences of events, which is difficult to do in whole animals.

Thus, with this background, the alternative models chosen for our study were chick embryo fibroblasts (which represent a primary cell culture system) and Hep2 (laryngeal carcinoma) cell line. They can be easily maintained and a very small quantity of sample is needed for testing.

Histologically, apoptosis is associated with cell shrinkage, membrane blebbing, chromatin condensation and the formation of fragmented chromatin into apoptotic bodies. All these cells were exposed to H_2O_2 and the influence of *Zea mays* leaf extracts was studied by membrane morphological and nuclear staining techniques. The experimental groups were the same for the yeast cells, primary chick embryo fibroblasts and cancer cell lines.

The results of the studies with H_2O_2 as the apoptosis-inducing oxidant revealed a differential response of the leaf extract treatment in non-cancerous (yeast and primary culture) and cancerous (Hep2) cells (details in the next chapter). This observation raised the query of what the effect of the extract would be in the presence of a standard chemotherapeutic agent that induces apoptosis.

The standard chemotherapeutic agent chosen for the present study was etoposide, which is known to cause apoptotic death in cells by inducing oxidative stress (Sermeus *et al.*, 2008). The effect of the *Zea mays* leaf extracts on the etoposide-induced effects was studied in primary cultured chick embryo fibroblasts and Hep2 cells, as representatives of non-cancerous and cancerous cells respectively.

Thus, the studies conducted in this phase of the study can be summarized as shown below.

CELL TYPE	**OXIDANT USED**
Saccharomyces cerevisiae Primary cultured chick embryo fibroblasts Hep2 cells	H_2O_2
Primary cultured chick embryo fibroblasts Hep2 cells	Etoposide

In all the cell types, the following treatment groups were set up:

- Untreated control cells
- H_2O_2 / etoposide treated (positive control) cells
- Aqueous extract of *Zea mays* leaves treated cells
- H_2O_2 / etoposide + Aqueous extract of *Zea mays* leaves treated cells
- Methanol extract of *Zea mays* leaves treated cells
- H_2O_2 / etoposide + Methanol extract of *Zea mays* leaves treated cells
- Chloroform extract of *Zea mays* leaves treated cells
- H_2O_2 / etoposide + Chloroform extract of *Zea mays* leaves treated cells

The extent of cell death was quantified in all the groups and morphological and nuclear staining methods were done to characterize the apoptosis-related events. The exact procedures for culturing and treating the different cells and the protocols adopted for analyzing various parameters are given below.

CULTURING OF YEAST CELLS

YPD medium (10g of yeast extract, 20g of peptone and 20g of dextrose in 1000ml of distilled water, pH 6.5) was prepared and sterilized by autoclaving after aliquoting. The cooled aliquots were stored at room temperature till use and checked regularly 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. After incubation, the medium was centrifuged at 1000g for 15 minutes to pellet out the

cells. The cells were then washed with saline and resuspended in a specific volume of assay medium for each assay.

CULTURING OF CHICK EMBRYO FIBROBLASTS

Fibroblast cells were isolated from chick embryo as explained below and were cultured using Dulbeccos modified Eagles medium (DMEM) (http://homepages.gac.edu/~cellab/chpts/chp12/ex12-10.html). The cells were seeded into 25cm^2 tissue culture flasks and incubated at 37°C in a CO_2 incubator (Napco, UK).

REAGENTS

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

PROCEDURE

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

Using a sterile metal hook or a bent glass rod, the embryo was gently lifted by holding the neck and placed in a 100mm petridish containing phosphate buffered saline (pH 7.4). It was washed repeatedly with sterile PBS to remove all yolk and / or blood. Then the embryo was transferred to a clean dish with PBS. The head, limbs and viscera were removed using sterile forceps and blades. Care was taken to remove the entire limb by pulling at the proximal end.

The remaining tissues were then placed in yet another sterile dish and washed with PBS. The washed tissue was finely minced with scissors and transferred to a flask containing PBS. With a sterile pipette, trypsin (2ml) was added and the suspension was stirred gently for 15-20 minutes, at 37°C.

DMEM with 10% FBS was used to resuspend the pellet. Cell count and viability was determined by trypan blue exclusion in a haemocytometer by taking 20µl from the suspended pellet. The cells were seeded in 25cm^2 plastic culture flasks containing DMEM

+ 10% FBS to a final concentration of 10^5 live cells / ml. Approximately 90-95% viability was observed.

CULTURING OF Hep2 CELL LINE

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

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

Hydrogen peroxide or etoposide at a concentration of 200μM was used as oxidants, as explained earlier. The concentration of plant extract used was 20mg. The cells were treated with the oxidant, both in the presence and the absence of the leaf extracts. The exposure of hydrogen peroxide and etoposide were given for one hour and 24 hours respectively at 37°C. The time points were arrived at by conducting a time-related response analysis of each cell type.

After treatment, the coverslips from the 6-well plates were removed and placed on a glass slide and sealed with vaseline. These slides were used for various staining techniques, whereas in 96-well plates, the medium was removed and replaced with fresh medium. These were used for checking the viability status.

ASSESSING THE EFFECT OF *Zea mays* LEAF EXTRACTS ON OXIDATIVE STRESS-INDUCED APOPTOSIS

The apoptotic consequences/events that occurred in the treated cells were followed by cytotoxicity assays (MTT and SRB) and various staining techniques (giemsa, EtBr, PI, DAPI and AO/EtBr staining).

CYTOTOXICITY ASSAY

The method of Igarashi and Miyazawa (2001) was employed for the assessment of cytotoxicity in the oxidant-induced cells both in the presence and the absence of the extract.

PRINCIPLE

3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) is converted into its formazan derivative by live cells. The amount of formazan produced is directly proportional to the number of surviving cells. After solubilization of the formazon with a suitable solvent, 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

After removing the serum and medium from the treated cells, 50µl of MTT was added and incubated at 37°C for 3 hours. To all the cells, 200µl of PBS was added and the liquid was then carefully aspirated. Acid propanol (200µl) was added and left overnight in the dark. The absorbance was read at 650nm in a microtiter 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 apoptotic index in the H_2O_2 treated chick embryo fibroblasts in the presence and the absence of the leaf extracts was studied by SRB as described 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 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. 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

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 TCA (40%) of 350µl was layered on top of the treated cells and incubated at 4°C for one hour. They were then washed 5 times with 200µl of cold PBS. SRB stain (350µl) was added to each well and left in contact with the cells for 30 minutes at room temperature. They were washed four times with 350µl portions of 1% acetic acid to remove the unbound dye. The protein bound dye was then solubilized by adding 10mM Tris (350µl) and were 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 microtitre plate reader (Anthos 2020, Austria) at 492nm. The cell viability was measured as the per cent absorbance compared to the control (untreated) cells.

MORPHOLOGICAL CHANGES IN THE CELLS

The morphological changes of the cells were observed in the presence and the absence of the leaf extract and / or H_2O_2. The coverslip with cells were stained with giemsa (diluted to 1:2 ratio) for 10 minutes and viewed under the phase contrast microscope (Nikon, Japan) as explained by Chih *et al.* (2001). The numbers of cells showing apoptotic morphological changes were counted in each experimental group per 100 cells in three different fields.

PRINCIPLE

Apoptosis is characterized by a series of well-documented membrane morphological changes, which can be observed by using liquid Giemsa staining technique.

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 cover slip containing cells placed on the glass slide and the stain was spread over the cells by placing another cover slip over it. The cells were observed for morphological changes under 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

The nuclear changes include condensation of nuclear contents 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 chick embryo fibroblasts in the presence and the absence of leaf extracts and / or H_2O_2 by PI, EtBr and AO/EtBr staining.

PROPIDIUM IODIDE STAINING

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

PRINCIPLE

PI is a fluorescent molecule that intercalates into nucleic acids and stains the nuclear changes in 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 the medium. The cells were permeabilized 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. Propidium iodide (10μl) was added and spread by placing a cover slip over it. The slides were then incubated at 37°C for 30 minutes in the dark.

The apoptotic cells with fragmented nuclei were then detected using the fluorescence microscope with G-2A filter (Nikon, Japan) at 400X magnification. The apoptotic ratio was then calculated by the formula mentioned earlier.

ETHIDIUM BROMIDE STAINING

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

PRINCIPLE

EtBr intercalates into nucleic acids, which helps to visualize the nuclear changes in apoptotic cells.

REAGENTS

1. PBS (Phosphate buffered saline)
2. Ethidium bromide - 50μg / ml in PBS.

PROCEDURE

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

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

DAPI STAINING

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

PRINCIPLE

DAPI is known to form 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. 3% paraformaldehyde in PBS
3. 0.2% Triton X-100 in PBS
4. DAPI (4'-6'-diamidino-2-phenyl indole) –1µg/ml in PBS.

PROCEDURE

The coverslip with cells were washed with PBS to remove medium with serum and fixed with 3% paraformaldehyde (50µl) for 10 minutes at room temperature. The fixed cells were then permeabilised with 0.2% Triton X-100 (50µl) for 10 minutes at room temperature and were incubated for 3 minutes with 10µl of 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.

ACRIDINE ORANGE / ETHIDIUM BROMIDE STAINING

AO/EtBr staining technique is used to differentiate between quiescent and actively proliferating cells. It is also used to measure apoptosis. Apoptotic cells were identified by AO/EtBr staining by the method proposed by Parks (1979).

PRINCIPLE

The combination of AO/EtBr staining technique is used to differentiate apoptotic and normal cells. Apoptotic cells, which have a larger fraction of DNA in the denatured form, display an intense red fluorescence and a reduced green emission when compared to non-apoptotic cells.

REAGENTS

1. PBS (Phosphate buffered saline)
2. Stock staining solution (100x): EtBr (50mg) and AO (15mg) were dissolved in 1 ml of 95% ethanol. 49ml of distilled water was added and mixed well. The solution was divided into 1ml aliquots and frozen.
3. Working solution: 1ml of aliquot of the 100X stock solution was thawed and diluted 100 times in phosphate buffered saline. The solution was mixed well and stored in an amber bottle at 4°C for up to one month.

PROCEDURE

To the treated cells, 10µl of AO/EtBr was added and spread by placing a coverslip over it. The stained slides were then incubated at room temperature for 5 minutes. The apoptotic cells with condensed chromatin and fragmented nuclei were identified by their red fluorescence and the normal cells were visualized by their green fluorescence, which were counted by using an upright fluorescent microscope (Nikon, Japan) using B-2A filter at 400X magnification.

The apoptotic ratio was calculated as

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

DROSOPHILA AS AN ALTERNATIVE MODEL

The alternative models used so far in the study represent the condition outside the biological system. However, in the *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 its efficacy. Thus, in order to draw concrete conclusion, we selected *Drosophila melanogaster*, an intact animal as a model system.

Drosophila melanogaster is a popular experimental animal because it is easily cultured in mass, has a short generation time, and mutant animals are readily obtainable (Ashburner *et al.*, 2005).

COLLECTION OF THE DROSOPHILA STOCK

The Drosophila stock (wild type) was collected from the Drosophila Stock Center, Mansagangotri, Mysore University.

PREPARATION OF MEDIUM

To 1 litre of boiled water, 100g of jaggery was added and cooked well. Then 100g of sooji was added, mixed and boiled well. Following this, 10g of agar and 7.5ml of propionic acid were added and cooked well. The mixture was then allowed to cool and transferred to sterile bottles. A few broken yeast granules were layered on the top of the medium and the bottles were plugged with autoclaved cotton. Additional yeast suspension was provided if necessary for the healthy growth of flies.

CULTURING OF DROSOPHILA

Drosophila was cultured in the prepared media at 25°C under 12 hours cycle of light and darkness. These cultures were maintained in the Drosophila colony laboratory in the university campus.

PREPARATION OF THE HOMOGENATE

The sex of the Drosophila was identified and the males and females were separated as soon as they hatched. The virgin male and female animals were then maintained in the above-mentioned diet and divided randomly for the various treatments.

TREATMENT GROUPS

As the results in the earlier phases of the study showed that the methanolic extract of the leaves was more effective than the aqueous and chloroform extracts, only the methanolic extract was used for testing in Drosophila. The following treatments were set up for each parameter for male and female animals separately.

1. Untreated group
2. *Zea mays* leaf extract treated group
3. H_2O_2 low dose (20mM)
4. H_2O_2 low dose (20mM) + *Zea mays* leaf extract treated group
5. H_2O_2 high dose (30mM)
6. H_2O_2 high dose (30mM) + *Zea mays* leaf extract treated group
7. CCl_4 low dose (125mM)
8. CCl_4 low dose (125mM) + *Zea mays* leaf extract treated group
9. CCl_4 high dose (195mM)
10. CCl_4 high dose (195mM) + *Zea mays* leaf extract treated group

The doses of the oxidants were selected based on the results of a pilot study, wherein varying doses of CCl_4 (50 to 200mM) and H_2O_2 (5 to 30mM) were studied. Two oxidants were chosen, in order to check if there existed a difference in the stress induced by a direct-acting oxidant (H_2O_2) and the one that requires metabolic activation (CCl_4).

The oxidants (H_2O_2 or CCl_4) and the plant extracts were mixed with the diet. The exposure was given for seven days to the virgin flies. After treatment, the flies were anaesthetized using diethyl ether and their wings were clipped off under a magnifying glass. They were then weighed, crushed using a micropestle and homogenized in ice-cold phosphate buffer (30mg of flies/300µl buffer) using an ultrasonic cell disruptor (Sonics, USA).

The homogenate was centrifuged and the supernatant was aliquoted. The samples were frozen at –85°C (Ilshin Ultra deep freezer, Korea), till analysis. All the analyses were performed in as short a duration as possible.

PARAMETERS ANALYSED

As the volume of the homogenate obtained was very less compared to the volume obtained in other traditional animal models, all the assays were performed using a nanospectrophotometer Optizen 3220 UV Bio (Korea), wherein the assay volume required ranges from 0.7 μl to 4μl only.

DETERMINATION OF ENZYMIC ANTIOXIDANTS

The enzymic antioxidants analyzed in the tissue homogenate were superoxide dismutase, catalase and peroxidase. The procedures adopted for the determination of the activities of enzymic antioxidants were the same as those for the leaf analysis (Phase I). An aliquot (20μl) of the homogenate obtained from the treated Drosophila was used instead of plant extract.

DETERMINATION OF NON-ENZYMIC ANTIOXIDANTS

The non-enzymic antioxidants determined in the homogenate namely, ascorbic acid, vitamin E and reduced glutathione, were assayed by the methods as elaborated in phase I of this chapter. Instead of the plant extract, the homogenate prepared after treatment was used for the estimations.

PHASE IV

The outcome of the first three phases of this research work clearly indicated that the methanolic extract of *Zea mays* leaves exhibited strong antioxidant and anticancer potential against oxidative stress induced under *in vivo*-simulated *in vitro* conditions.

There is a growing interest in correlating phytochemical constituents of a plant with its pharmacological activity. Thus, in the next phase, phytochemical analysis of the leaf extract was carried out to identify the active principle operating behind the antioxidant activity of the leaf extract against oxidative stress.

PRELIMINARY PHYTOCHEMICAL SCREENING

The methanolic extract of *Zea mays* leaves were tested for the presence of various phytochemicals as described by Khandelwal (2002).

DETECTION OF ALKALOIDS

MAYER'S TEST

An aliquot 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 were observed for the formation of cream coloured precipitate.

DRAGENDROFF'S TEST

A fraction of the extract was treated with Dragendroff's reagent and observed for the formation of reddish orange coloured precipitate.

WAGNER'S TEST

A small fraction of the extract was treated with Wagner's reagent (1.27g of iodine and 2g of KI in 100ml of distilled water) and observed for the formation of reddish brown coloured precipitate.

DETECTION OF PHENOLICS

FERRIC CHLORIDE TEST

An aliquot of the extract was treated with 5% $FeCl_3$ reagent and observed for the formation of deep blue-black 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 SODIUM HYDROXIDE TEST

A fraction of the extract was treated with 1N aqueous sodium hydroxide and observed for the formation of yellow-orange colouration.

SULPHURIC ACID TEST

A fraction of the extract was treated with concentrated sulphuric acid 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 HCl, heated slightly and observed for the formation of dark pink colour.

The results of the qualitative analysis indicated the presence of phenolics and flavonoids. Following this, the leaves were subjected to various spectral analyses to identify the active biomolecule.

ABSORPTION SPECTRUM

A spectral analysis was done by a survey scan of the *Zea mays* leaf extracts in a nanospectrophotometer (Optizen, Korea) in the UV range (190nm to 300nm).

HPLC ANALYSIS

The residue of the methanolic extract of *Zea mays* leaves was dissolved in an appropriate volume of HPLC grade acetonitrile and injected into the 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 1000 psi and the mobile phase used was acetonitrile and water in the ratio of 15:85 containing 1% acetic acid with a run time of 30 minutes of 1ml/minute flow rate.

HPTLC ANALYSIS

The HPTLC (CAMAG, Switzerland) analysis of the methanolic extract of *Zea mays* leaves was performed for the detection of alkaloids, flavonoids and phenolics. The conditions used are given below.

ALKALOIDS

The plate was fixed in a scanner stage and scanned at 500nm. The mobile phase used was ethylacetate : methanol : water in the ratio (10:1.35:1). Dragondoff's reagent was sprayed followed by 10% ethanolic sulfuric acid reagent over the plate and heated at 120°C for 5 minutes in a hot air oven. The presence of alkaloids was confirmed by the presence of a bright orange coloured zone at daylight mode from the chromatogram.

PHENOLICS

The plate was scanned at 500nm. The mobile phase used was toluene : chloroform : acetone in the ratio (4:25:3.5). The plate was sprayed with 25% aqueous Folin Ciocalteau reagent and heated at 120°C for 5 minutes in a hot air oven. The presence of phenolics was confirmed by the appearance of blue or blue-grey coloured zone at daylight.

FLAVONOIDS

The mobile phase used was ethylacetate : butanone : formic acid in the ratio (5:3:1). The plate was scanned at 366nm. The plate was sprayed with 1% ethanolic aluminium chloride reagent and heated at 120°C for 5 minutes in a hot air oven. The presence of flavonoids was confirmed by the appearance of yellow and yellow green fluorescence at UV 366nm.

IR SPECTRAL ANALYSIS

The IR spectral analysis was carried out in the methanolic extract of *Zea mays* leaves, using a scanning Michelson inferometer and Fourier Transformation (Shimadzu, Japan). A residue of the methanolic extract of *Zea mays* leaves 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 was analysed using a Shimadzu gas chromatography apparatus (Model qp 5000 GC-MS) using a DB-S capillary column (30m) equipped with QP MS detector (EI, 70 ev) with helium as a carrier gas at a flow rate of 1ml/minute. The compounds were identified using the database available in the software provided.

STATISTICAL ANALYSIS

The biochemical parameters studied were subjected to statistical analysis using SigmaStat (Version 3.1) and Agress (Version 3.01) statistical software. Statistical significance was determined by three-way analysis of variance for Drosophila studies and two-way analysis of variance with $P<0.01$ considered significant was adopted to all the other parameters under study to test the level of statistical significance.

The results obtained for the various parameters analyzed in the four major phases and the salient findings of the study are presented in the next chapter.