Methodology

3. METHODOLOGY

The aim of the study was to explore and identify the antioxidant potential of a novel and under-exploited natural source of antioxidants namely *Rhinacanthus nasutus*. The plant was collected from Coimbatore district, Tamil Nadu. Fresh leaves were collected and cleaned to remove adhering dust particles, washed under running tap water and gently blotted dry between folds of filter paper. The extracts were prepared fresh using appropriate buffers or solvents as indicated for each assay.

PHASE I

In Phase I, both enzymic and non-enzymic antioxidant components were analysed in the leaves of *Rhinacanthus nasutus*. The methodology adopted for analyzing these parameters is given below.

ENZYMIC ANTIOXIDANTS

The enzymic antioxidants analysed in the leaves were superoxide dismutase, catalase, peroxidase, glutathione S-transferase and polyphenol oxidase.

ASSAY OF SUPEROXIDE DISMUTASE (SOD)

Superoxide dismutase activity was determined by the method of Misra and Fridovich (1972).

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

REAGENTS

1. 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

Rhinacanthus nasutus leaves (0.5g) were ground with 3.0 ml of potassium phosphate buffer, centrifuged at 2000 rpm for 10 minutes and the supernatant was used for the assay. The incubation medium contained, in a final volume of 3.0 ml, 50 mM potassium phosphate buffer (pH 7.8), 45 μM methionine, 5.3 mM riboflavin, 84 μM NBT and 20 μM potassium cyanide. The amount of homogenate added to this medium was kept below one unit of enzyme to ensure sufficient accuracy.

The tubes were placed in an aluminium foil-lined box maintained at 25°C and equipped with 15W fluorescent lamps. Reduced NBT was measured spectrophotometrically at 600 nm after exposure to light for 10 minutes. The maximum reduction was evaluated in the absence of the enzyme. One unit of enzyme activity was defined as the amount of enzyme giving a 50% inhibition of the reduction of NBT. The values were calculated as units/mg protein.

ASSAY OF CATALASE

Catalase activity was assayed spectrophotometrically following the method of Luck (1974) in the fresh leaves of the plant.

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

REAGENTS

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

PROCEDURE

A 20% homogenate of the leaves was prepared in phosphate buffer (0.067M, pH 7.0) and the homogenate was employed for the assay. The samples were read against a control without homogenate, but containing the H_2O_2-phosphate buffer.

To the experimental cuvette, 3 ml of H_2O_2-phosphate buffer was added, followed by the rapid 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 method proposed by Reddy *et al.* (1995) was adopted for assaying the activity of peroxidase.

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

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

A 20% homogenate was prepared in 0.1M phosphate buffer (pH 6.5) from the leaves of the plant and employed for the assay. Pyrogallol solution (3.0 ml) and enzyme extract (0.1 ml) were pipetted out into a cuvette. The spectrophotometer was adjusted to read zero at 430nm. To the test cuvette 0.5 ml of 1% H_2O_2 was added and mixed. 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 of Habig *et al.* (1974) was employed for the assessment of glutathione S-transferase in the leaves of the selected plant.

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

REAGENTS

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

PROCEDURE

Rhinacanthus nasutus leaves (0.5g) were homogenized with 5.0 ml of phosphate buffer. The homogenate was centrifuged at 5000rpm for 10 minutes and the supernatant was used for the assay. The enzyme activity was determined by monitoring the change in absorbance at 340 nm in a spectrophotometer. The assay mixture contained 0.1ml of GSH, 0.1 ml of CDNB and phosphate buffer in a total volume of 2.9 ml. The reaction was started by the addition of 0.1ml of enzyme extract to this mixture and the readings were recorded against distilled water blank for a minimum of three minutes. The complete assay mixture without the enzyme served as the control to monitor non-specific binding of the substrates.

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

ASSAY OF POLYPHENOL OXIDASE (PPO)

Esterbauer *et al.* (1977) have reported a method for the spectrophotometric estimation of catechol oxidase and laccase simultaneously, which was used to assay PPO in the leaf extract of the candidate plant.

Phenol oxidases are copper proteins of wide occurrence in nature, which catalyse the aerobic oxidation of phenolic substrates to quinones, which are autooxidized to dark brown pigments generally known as melanins, which can be estimated spectrophotometrically at 495nm.

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

REAGENTS

1. Reaction medium - Tris HCl (50 mM, pH 7.2), Sorbitol (0.4 M), NaCl (10 mM)
2. Catechol solution (0.01 M, pH 6.5)

PROCEDURE

The enzyme extract was prepared by grinding 5g of *Rhinacanthus nasutus* leaves using mortor and pestle in about 20ml reaction medium containing 50mM Tris HCl, pH 7.2, 0.4M sorbitol and 10 mM NaCl. The homogenate was centrifuged at 20,000g for 10 minutes and the supernatant was used for the assay. The assay mixture contained 2.5ml of 0.1M phosphate buffer and 0.3ml of catechol solution (0.01M), which was added into the cuvette. The spectrophotometer was set at 495 nm. The enzyme extract (0.2ml) was added to the same cuvette and the change in absorbance was recorded for every 30 seconds up to 5 minutes.

The activity of PPO was calculated using the formula,

Enzyme unit = K x (Δ/min)

where,

K for catechol oxidase = 0.272

K for laccase = 0.242

NON-ENZYMIC ANTIOXIDANTS

The non-enzymic antioxidants analysed in *Rhinacanthus nasutus* leaves were ascorbic acid, tocopherol, total carotenoids, lycopene, reduced glutathione, total phenols and chlorophyll.

ESTIMATION OF ASCORBIC ACID

Ascorbic acid content in the leaves was estimated by the method of Roe and Keuther (1943). Ascorbate is converted to dehydroascorbate by treatment with activated charcoal or bromine. Dehydro ascorbic acid then reacts with 2,4-dinitrophenyl hydrazine to form osazones, which dissolves in sulpuric acid to give an orange coloured solution, whose 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

Ascorbate was extracted into 4% TCA by homogenizing 1g of *Rhinacanthus nasutus* leaves in it and the volume was made up to 10ml with 4% TCA. The supernatant obtained after centrifugation at 2000 rpm for 10 minutes was treated with a pinch of activated charcoal, shaken well and kept for 10 minutes. Centrifugation was repeated once again to remove the charcoal residue. The volumes of the clear supernatants obtained were noted.

Two different aliquots of the supernatant were taken for the assay (0.5ml and 1.0 ml). The assay volumes were made up to 2.0 ml with 4% TCA. Aliquots of the working standard solution containing 20-100µg of ascorbate were pipetted

into clean dry test tubes, the volumes of which were also made up to 2.0 ml with 4% TCA.

DNPH reagent (0.5ml) was added to all the tubes, followed by two drops of 10% thiourea solution. The osazones formed after incubation at 37°C for 3 hours, were dissolved in 2.5ml of 85% H_2SO_4, in cold, with no appreciable rise in temperature. To the blank alone, DNPH reagent and thiourea were added after the addition of H_2SO_4. After incubation for 30 minutes at room temperature, the samples were read at 540 nm and the levels of ascorbic acid in the samples were determined using the standard graph constructed on an electronic calculator set to the linear regression mode and expressed as mg ascorbate /g leaf.

DETERMINATION OF TOCOPHEROL

The levels of tocopherol in the leaves were estimated spectrophotometrically by the method of Rosenberg (1992).

Tocopherols can be estimated using Emmerie-Engel reaction, which is based on the reduction of ferric to ferrous ions by tocopherols, which forms a red colour with 2,2'-dipyridyl. Tocopherols and carotenes were 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 one 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

A small volume of 0.1N sulphuric acid was used for homogenizing 2.5g of *Rhinacanthus nasutus* leaves and the volume was finally made up to 50 ml by adding 0.1N sulphuric acid slowly, without shaking and allowed to stand overnight. The contents of the flask were shaken vigorously on the next day and filtered through Whatman No.1 filter paper. Aliquots of the filtrate were used for the estimation. Into 3 stoppered centrifuge tubes (test, standard and blank), 1.5ml of plant extract, standard and water respectively were pipetted out. To all the tubes, 1.5ml each of ethanol and xylene were added, stoppered, mixed well and centrifuged.

After centrifugation, the xylene layer was transferred into another stoppered tube, taking care not to include any ethanol or protein. To 1.0 ml of xylene layer, 1.0ml of 2,2'-dipyridyl reagent was added to each tube, stoppered and mixed. This mixture was taken in the colorimetric cuvettes 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, mixed well and after exactly 15 minutes, the test and the standard were read against the blank at 520nm.

The levels of tocopherol in the leaf sample was calculated using the formula

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

ESTIMATION OF TOTAL CAROTENOIDS AND LYCOPENE

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

The total carotenoids and lycopene in the sample are extracted in petroleum ether. The total carotenoids are 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. 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

All the steps subsequent to the saponification were carried out in the dark to avoid photolysis of carotenoids. Saponification was done with 5g of leaves using 2.5ml of 12% ethanolic potassium hydroxide in a water bath at 60°C for 30 minutes. The saponified extract was then transferred into a separating funnel (packed with glass wool and calcium carbonate) containing 10-15ml of petroleum ether and 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. To the petroleum ether extract, a small quantity of anhydrous Na_2SO_4 was added to remove excess moisture, if any. The final volume of the petroleum ether extract was noted and diluted if needed by a known dilution factor.

The absorbance of the yellow colour was read at 450nm and 503nm in a spectrophotometer using petroleum ether as a blank. The amount of total carotenoids and lycopene was calculated using the formula,

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

where,

P = optical density of the sample

V = Volume of the sample

W = Weight of the sample

$$\text{Lycopene} = \frac{3.12 \times OD_{sample} \times \text{Vol of sample} \times \text{dilution} \times 100}{1 \times \text{weight of the sample} \times 1000}$$

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

ESTIMATION OF REDUCED GLUTATHIONE

The method proposed by Moron *et al.* (1979) was used for the estimation of reduced glutathione.

Reduced glutathione (GSH) is measured by its reaction with DTNB (5,5'-dithiobis-2-nitrobenzoic acid) (Ellman's 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 obtained by homogenizing 0.5g of leaf tissue in 2.5 ml of 5% TCA. To precipitate the protein, 125 µl of 25% TCA was added to 0.5 ml of tissue homogenate. The precipitated protein was centrifuged at 1000rpm for 10 minutes. The homogenate was cooled on ice and 0.1ml of the supernatant was taken for the estimation. The supernatant was made up to 1 ml with 0.2M

sodium phosphate buffer (pH 8.0). Two ml of freshly prepared DTNB solution was added to the tubes and the intensity of the yellow colour formed was read at 412 nm in a spectrophotometer after 10 minutes.

A standard curve of GSH was prepared using concentrations ranging from 2-10 nanomoles of GSH in an electronic calculator set to the linear regression mode and the values of the samples were read off it. The values are expressed as nmoles of GSH /g leaf.

DETERMINATION OF TOTAL PHENOLS

Total phenols were assayed by the method proposed by Mallick and Singh (1980) in the leaves of the candidate plant.

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

REAGENTS

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

PROCEDURE

The homogenate was prepared with 0.5g of *Rhinacanthus nasutus* leaves and 10X volumes of 80% ethanol. The homogenate was centrifuged at 10,000 rpm for 20 minutes. The residue was re-extracted with 80% ethanol. The supernatants were pooled and evaporated to dryness. The residue was then dissolved in a known volume of distilled water. Different aliquots (0.2 to 2.0ml) were pipetted

out into test tubes. The volume in each tube was made up to 3.0ml with water. To all the tubes, 0.5 ml of Folin-Ciocalteau reagent was added and mixed. After 3 minutes, 2.0ml of 20% sodium carbonate solution was added to each tube. After mixing the tubes thoroughly, all the tubes were kept in a boiling water bath for exactly 1 minute, and allowed to cool. The absorbance was measured at 650 nm against a reagent blank.

ESTIMATION OF CHLOROPHYLL

The chlorophyll content of the leaves was determined using the procedure described by Witham *et al.* (1971).

Chlorophyll is extracted in 80% acetone and the absorption at 663nm and 645nm are read in a spectrophotometer. Using the absorption coefficient, the amount of chlorophyll is calculated.

REAGENTS

Acetone (80%, prechilled).

PROCEDURE

Chlorophyll was extracted from 1g of *Rhinacanthus nasutus* leaves using 20 ml of 80% acetone. The supernatant was transferred to a volumetric flask after centrifugation at 5000 rpm for 5 minutes. The extraction was repeated until the residue was colourless. The mortar and pestle was washed thoroughly with 80% acetone and the clear washings were collected in the volumetric flask. The volume in the flask was made up to 100ml with 80% acetone. The absorbance of the green coloured solution was read at 645 and 663nm against the solvent (80% acetone) blank. The total chlorophyll in the leaf was calculated using the formula,

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

where

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 outcome of the first phase, which is described in the next chapter, revealed that the leaves of *Rhinacanthus nasutus* were found to be a rich source of both enzymic and non-enzymic antioxidants. In order to determine the bioactive component responsible for the antioxidant potential of the leaves of the selected plant, extracts were prepared in three different solvents differing in polarity namely water, methanol and chloroform and all the three extracts were taken for further analyses of the study.

Phase II was formulated to study the radical scavenging ability of the candidate plant *Rhinacanthus nasutus*. The leaves were extracted with solvents of different polarity and the solvents used were chloroform and methanol. Apart from the solvent extracts, a crude aqueous extract of the leaves was also prepared.

PREPARATION OF PLANT EXTRACTS

Fresh leaves of *Rhinacanthus nasutus* (Plate 1) were collected and 1g of them was homogenized thoroughly in 10ml of appropriate solvent. The organic extracts were dried at 60°C protected from light. The residue was weighed and dissolved in dimethyl sulfoxide (DMSO) to obtain a final concentration of 20mg in 5µl of DMSO. Aqueous extracts were prepared fresh when experiments were performed.

In all the scavenging assays, the oxidant used to induce oxidative damage was hydrogen peroxide (200μM final concentration). The treatment groups set up to study the antioxidant effect of *Rhinacanthus nasutus* were as follows:

1. Untreated (negative) control
2. H_2O_2 (positive) control
3. H_2O_2 + Aqueous extract of *Rhinacanthus nasutus* leaves
4. H_2O_2 + methanolic extract of *Rhinacanthus nasutus* leaves
5. H_2O_2 + chloroform extract of *Rhinacanthus nasutus* leaves
6. Aqueous extract of *Rhinacanthus nasutus* leaves
7. Methanolic extract of *Rhinacanthus nasutus* leaves
8. Chloroform extract of *Rhinacanthus nasutus* leaves

EVALUATION OF RADICAL-SCAVENGING EFFECTS OF *Rhinacanthus nasutus* LEAF EXTRACTS

The antioxidant effects of the leaf extracts were assessed by the ability to scavenge free radicals.

DPPH SCAVENGING EFFECTS

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

DPPH radical reacts with an antioxidant compound that can donate hydrogen, and gets reduced. DPPH, when acted upon by an antioxidant, is converted into diphenyl-picryl hydrazine. The degree of discolouration from purple to light yellow can be measured at 518nm, which is a measure of the scavenging effect of antioxidant extracts.

REAGENTS

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

PROCEDURE

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

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

ABTS SCAVENGING EFFECTS

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

REAGENTS

ABTS Solution (7mM with 2.45 mM ammonium persulfate).

PROCEDURE

ABTS radical cations ($ABTS^{+}$) were produced by reacting ABTS solution (7 mM) with 2.45 mM ammonium per sulphate. The mixture was allowed to stand in the dark at room temperature for 12-16 hours before use. All the three different extracts (each 0.5 ml) were added to 0.3 ml of ABTS solution and the final volume

was made up to 1ml with ethanol. The absorbance was read at 745 nm and the per cent inhibition was calculated using the formula

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

HYDROGEN PEROXIDE SCAVENGING EFFECTS

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

REAGENTS

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

PROCEDURE

A solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer (pH 7.4). Plant extracts at the concentration of 10mg/10µl was added to H_2O_2 solution (0.6ml, 40mM). The total volume was made up to 3ml. The absorbance of the reaction mixture was recorded at 230nm. The blank solution contained phosphate buffer without H_2O_2. The percentage of H_2O_2 scavenging of plant extracts was calculated as

$$\text{\% scavenged hydrogen peroxide} = \frac{(A_o - A_1) \times 100}{A_o}$$

A_o - Absorbance of control

A_1 - Absorbance in the presence of plant extract and standards

HYDROXYL RADICAL SCAVENGING EFFECTS

The DNA damage induced *in vitro* by hydrogen peroxide generated hydroxyl radicals in the presence and the absence of plant extracts was quantified as thiobarbituric acid reactive substances spectrophotometrically as per the procedure given by 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 result in TBARS formation, which can be quantified spectrophotometrically.

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

PROCEDURE

The reaction mixture contained in a final volume of 0.98ml, 2.8mM deoxy ribose, 0.1mM $FeCl_3$, 0.1mM EDTA, 1mM H_2O_2, 0.1mM ascorbate and 20mM buffer. 20µl of plant extract was added such that the final volume was 1ml. The reaction mixture was incubated for one hour at 37°C. Deoxyribose degradation was measured as TBARS by adding 0.5 ml of TBA and 0.5 ml of HCl and boiling

in a water bath for 20 minutes. It was then allowed to cool and the absorbance was measured at 532 nm.

ESTIMATION OF DNA DAMAGE USING HERRING SPERM DNA

The DNA damage caused by hydrogen peroxide and the influence of *Rhinacanthus nasutus* leaves on it was studied according to the method reported by Aeschlach *et al.* (1994).

REAGENTS

1. Herring sperm 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. The reaction was terminated by the addition of 0.05ml of 0.1M EDTA. The colour was developed by adding 0.5 ml of thiobarbituric acid and 0.5ml of 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.

INHIBITION OF *in vitro* LIPID PEROXIDATION IN LIVER HOMOGENATE

Goat liver homogenate was used for the induction of lipid peroxidation, mediated by $FeSO_4$ as a pro-oxidant and the efficiency of the leaf extracts of *Rhinacanthus nasutus* in inhibiting the *in vitro* lipid peroxidation was studied as per the method of Okhawa *et al.* (1979) by the measurement of thiobarbituric acid reactive substances spectrophoto-metrically at 535nm in the experimental mixture.

REAGENTS

1. Tris buffered saline (TBS) (10 mM Tris, 0.5 M NaCl, pH 7.4)
2. Ferrous sulphate (10 µM, prepared fresh in TBA)
3. Thiobarbituric acid (1% in TBS)
4. Alcohol (70%)
5. Acetone
6. Goat liver homogenate prepared in TBS (5%)

PROCEDURE

A 5% goat liver homogenate was prepared in cold TBS and 50 µl of it was used in the assay. Fresh plant tissue (0.5g) was weighed accurately and homogenized in 1 ml of cold TBS. Aliquots of 50µl of it were used in the assay. Ferrous sulphate at a final concentration of 10 µmoles was added to the assay medium to induce oxidation.

The final volumes in the test tubes were made up to 500 µl with cold TBS. Controls were prepared for each sample, containing the respective plant extract (50µl), liver homogenate (50µl) and TBS to make up the final volume to 500µl. Pro oxidant was not added to the control tubes.

A blank containing no plant extract, no liver homogenate but only $FeSO_4$ and TBS to make a final volume of 500 µl was also prepared. An assay medium corresponding to 100% oxidant was prepared by adding all the other constituents except the plant extract and the volume was made up to 500 µl with cold TBS. The experimental medium corresponding to auto oxidation contained only the liver homogenate and TBS to make up the final volume to 500 µl. All the tubes were incubated at 37°C for one hour. Following the incubation period, 500 µl of 70% alcohol was added to all the tubes to stop the reaction. One ml of 10% TBA was added to all the tubes, followed by boiling in a hot water bath for 20 minutes. After cooling to room temperature, the tubes were centrifuged. To the clear supernatants collected into tubes, 500 µl of acetone was added and the TBARS was measured at 535nm in a spectrophotometer.

PHASE III

Phase III was conceived with an approach of using alternatives to animals. Such alternative experimental systems serve the purpose of substituting live animals used in research. Earlier studies done in our laboratory using goat liver slices as an *in vitro* model that simulated *in vivo* conditions brought out successful results (Saraswathi, 2006; Sreelatha, 2006; Vidhya, 2007; Sumathi, 2007; Kalaivani, 2007). The alternative models chosen for the present study included goat liver slices, primary cell cultures, *Saccharomyces cerevisiae*, and cell lines.

PREPARATION OF GOAT LIVER SLICES

Liver plays a major role in coordinating the internal environment of the body. Hence the foremost *in vitro* model adapted in this phase was goat liver slices. The goat liver was collected fresh from a slaughter house, plunged into cold, sterile PBS and maintained at 4°C till the assay. Very thin (1mm) slices of the goat liver were cut accurately using sterile scalpel.

TREATMENT GROUPS

One gram of goat liver slice was taken in 4.0ml of sterile PBS. Hydrogen peroxide, at 0.2M concentration was used as an oxidant for the induction of oxidative stress in the liver slices. The leaves of the candidate plant were collected fresh, washed, cleaned and dried. The leaf homogenate was prepared using 1g of leaf sample in the appropriate solvent and the homogenate was centrifuged to clarify the debris. The solvent used for the preparation included methanol, chloroform and water. The supernatant was collected after centrifugation and dried at 60°C well protected from light. The residue obtained after drying the solvent was weighed and dissolved in a known amount of DMSO to yield a concentration of 20mg / 5µl. DMSO was maintained at a minimum level to avoid DMSO induced events, if any. The solvents used for aqueous and organic extracts of *Rhinacanthus nasutus* leaves were added and incubated at 37°C for one hour with mild shaking. The treatment groups designed for this phase were as follows:

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

After 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 various enzymic and non-enzymic antioxidants.

ASSAY OF ENZYMIC ANTIOXIDANTS

The enzymic antioxidant status in the liver slices was assessed by assaying the activities of SOD, catalase, peroxidase and glutathione S-transferase. All these enzymes were assayed following the same protocols used for the Phase I analysis. The leaf extracts of the plant, used as the enzymic sources in Phase I studies were replaced by an aliquot of the liver slice homogenate.

DETERMINATION OF NON-ENZYMIC ANTIOXIDANTS

The non-enzymic antioxidants estimated were ascorbic acid, tocopherol, vitamin A and reduced glutathione. The procedures adopted for the assay of ascorbic acid, tocopherol and reduced glutathione in the different treatment groups of liver slice homogenate were exactly the same as those followed for the leaf extracts analyses (Phase I). An aliquot of the tissue homogenate was used instead of leaf tissue in all the assays. Vitamin A was assayed by the method of Bayfield and Cole (1980).

ESTIMATION OF VITAMIN A

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

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

Liver homogenate and saponification mixture of 1 ml each was taken. The mixture was refluxed for 20 minutes at 60°C in the dark. It was cooled and to this 20ml of water was added. Vitamin A was extracted twice with 10ml portions of petroleum ether (40-60°C). The extracts were pooled, washed thoroughly with water, separating the layers using 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. Aliquots of the standard (vitamin A palmitate) were pipetted out into a series of clean, dry test tubes in the concentration range of 0-7.5µg. The volumes in all the tubes were made upto 1.0ml with chloroform. From a fast delivery pipette, 2.0ml of TCA reagent was added with a rapid mixing of the contents of the tube. 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.

Total antioxidant activity of the treated liver slice homogenates was done using DPPH assay as explained in Phase I and the extent of inhibition of *in vitro* lipid peroxidation was also evaluated, as explained earlier.

The results of the first part of the Phase III analysis (presented in the next chapter) revealed that the methanolic extract of *Rhinacanthus nasutus* leaves exhibited maximum antioxidant efficacy against the consequences of oxidant induced damage. Therefore, only the methanolic extract of the leaves of the candidate plant was taken for all the subsequent analyses in this study.

Further, chick embryo fibroblasts, *Saccharomyces cerevisiae* cells and cell lines were used as various alternative models to study the antioxidant potential and apoptosis related events.

CULTURING OF CHICK EMBRYO FIBROBLASTS

Dulbecco's modified Eagle's medium [DMEM] was used for culturing the chick embryo fibroblast cells. As an initial step, the fibroblasts were isolated from chick embryo as described below (http://homepages.gac.edu/~cell ab/chpts/chpt12/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. Complete medium (DMEM, 10% FBS and antibiotics)
2. PBS (phosphate buffered saline)
3. Trypsin-EDTA (0.25% trypsin in 1mM EDTA)

PROCEDURE

An egg containing a live 13 or 14-day-old chick embryo was taken in a beaker with the blunt end up and swabbed with ethanol. The top of the egg was carefully punctured with the point of a pair of sterile scissors and a circle of shell was cut away carefully to expose the underlying membrane (the chorioallantois). With a second pair of sterile scissors, the chorioallantoic membrane was carefully cut and removed, to expose the embryo.

The embryo was gently lifted by the neck, using a sterile metal hook or a bent glass rod, and the embryo was placed in a 100mm Petri dish containing sterile phosphate buffered saline. It was then washed several times with sterile PBS by transferring the embryo to fresh Petri plates.

After removal of all yolk and/or blood, the embryo was moved to a clean dish with PBS. Using two sterile forceps, the head, limbs and viscera were removed. Care was taken to remove the entire limb by pulling at the proximal end. The remaining tissues of the embryo were taken to yet another sterile dish and

washed with PBS. Then the embryo was finely minced with scissors. The minced tissue was transferred to a flask containing PBS. After allowing the tissue pieces to settle, the PBS was removed with a sterile pipette. Two ml of trypsin was added and the solution was stirred gently for 15-20 minutes, at 37°C.

The pellet was resuspended in fresh DMEM + 10% FBS. From this, 20µl was taken to determine cell count and viability by trypan blue exclusion in a haemocytometer. Then the cells were seeded in 25cm^2 plastic culture flasks containing DMEM + 10% FBS to a final concentration of 10^5 live cells / ml. The viability always ranged between 90-95%.

Then the primary chick embryo cells were cultured in the required volume (5ml/25cm^2 bottle) of DMEM (PAA) supplemented with 10 percent FBS (PAA). Penicillin and streptomycin (PAA) was also added to the medium to 1 X final concentration from a 100X stock.

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

Rhinacanthus nasutus leaves were homogenized in 1ml of solvent (methanol). The homogenate was centrifuged and the supernatant was taken and dried at 60°C. The residue obtained after evaporating the methanol was weighed and dissolved in a known volume of DMSO to yield a concentration of 20mg / 5µl.

TREATMENT GROUPS

The experiment was set up in 4 groups as follows

1. Primary cells alone (negative control)
2. Primary cells + H_2O_2 (positive control)
3. Primary cells + methanolic extract of *Rhinacanthus nasutus* leaves.
4. Primary cells + H_2O_2 + methanolic extract of *Rhinacanthus nasutus* leaves.

The cells were subjected to the treatment for one hour at 37°C. The concentration of H_2O_2 used for all the assays was 200µM and the plant extract concentration used was 20mg in 5µl of DMSO. After treatment, the cover slip alone was removed, placed over a glass slide and secured with vaseline. The cells were washed with PBS and then used for the analyses of various parameters.

PARAMETERS ANALYSED

The procedures adopted for the determination of the activities of enzymic antioxidants (SOD, catalase, peroxidase and glutathione reductase) in the primary cells of chick embryo fibroblasts were the same as those for the tissue slices. The non-enzymic antioxidants determined were ascorbic acid, tocopherol, vitamin A and reduced glutathione, which were assayed by the methods as elaborated in Phase II of the study for liver slices. Total antioxidant potential and lipid peroxidation were also assessed in the primary cells using the protocols as explained in Phase II of the study for liver slices.

Typsinised cells were used as antioxidant source in all the enzymic and non-enzymic antioxidant analyses performed. From the trypsinised cells, 10^4- 10^6 cells were taken for each assay. Based on the number of cells used for the assays, the total volume of the reaction mixture was reduced and the absorbance of the resultant chromogen was measured using a nanospectrophotometer (Optizen, Korea).

EFFECT OF *Rhinacanthus nasutus* LEAVES ON APOPTOSIS IN CHICK EMBRYO FIBROBLASTS

Severe oxidative stress can result in programmed cellular death (Skulachev, 2006). The programmed cell death process of apoptosis is characterized by tell-tale cellular, nuclear and molecular events, which can be quantified using various microscopic techniques. In the present study, the apoptotic events were also observed using various parameters in the chick embryo fibroblasts as presented below.

MORPHOLOGICAL CHANGES OF THE APOPTOTIC CELLS

During apoptosis, the cells undergo various morphological changes like cell shrinkage, membrane blebbing and formation of apoptotic bodies. These changes in the fibroblasts were observed under phase contrast microscopy. The morphological appearance of the fibroblasts were followed in the presence and the absence of the leaf extract and / or H_2O_2. The cells were stained with giemsa for 10 minutes and observed under the phase contrast microscope (Nikon, Japan) as described by Chih *et al.* (2001).

REAGENTS

1. Complete medium (DMEM, 10% serum and antibiotics)
2. PBS (phosphate buffered saline)
3. Liquid Giemsa stain (1:1 dilution in PBS)

PROCEDURE

The diluted giemsa stain (10µl) was added to the slide and spread by placing another cover slip over it. Then they were observed under phase contrast microscope (Nikon, Japan) at 400X magnification for morphological changes. 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 apoptotic nuclei can be identified by the condensed chromatin gathering at the periphery of the nuclear membrane or a total fragmented morphology of nuclear bodies. These nuclear changes were investigated in the chick embryo fibroblasts in the presence and the absence of leaf extracts and / or H_2O_2 by ethidium bromide staining as explained by Mercille and Massie (1994) with minor modifications and propidium iodide staining by Sarker *et al.* (2000).

ETHIDIUM BROMIDE STAINING

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

REAGENTS

1. Complete medium (DMEM, 10% serum and antibiotics)
2. PBS (phosphate buffered saline)
3. Ethidum bromide - 50µg / ml in PBS

PROCEDURE

The treated cells were incubated for 5 minutes with 10µl of ethidium bromide and spread by placing a cover slip over it. The apoptotic cells were scored by counting the cells with condensed chromatin and fragmented nuclei under inverted fluorescent microscope (Moticam, China) using DAPI filter at 400 X magnification. The apoptotic ratio was calculated as,

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

PROPIDIUM IODIDE STAINING

Propidium iodide is a fluorescent molecule that intercalates into nucleic acids and can stain the nuclear changes in apoptotic cells.

REAGENTS

1. Complete medium (DMEM, 10% serum and antibiotics)
2. PBS (phosphate buffered saline)
3. Acetone : Methanol (1:1)
4. Propidium iodide (PI) - 5μg / ml in PBS

PROCEDURE

After the treatment, the medium was removed and the cells were washed with PBS to remove the traces of medium and serum. The cells were permeabilized with 50μl of acetone : methanol (1:1) mixture at -20°C for 10 minutes. Then 10μl of propidium iodide was added, spread by placing a cover slip and incubated at 37°C for 30 minutes in the dark.

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

CYTOTOXICITY ASSAY

The 2-(4,5-dimethyl-2-tetrazoyl)-2,5-diphenyl-2,4 tetrazolium salt (MTT) dye reduction assay described by Igarashi and Miyazawa (2001) was applied to assess the extent of cytotoxicity in the oxidant induced cells in the presence and the absence of the extract.

Living cells convert MTT into its formazan derivative. The number of surviving cells can be determined by the amount of MTT formazan produced,

which is measured in a micro titer plate reader after solubilization with a suitable solvent.

REAGENTS

1. Complete medium : DMEM, 10% serum and antibiotics
2. PBS (phosphate buffered saline)
3. MTT – 3mg/ml in PBS
4. Isopropanol in 0.04N HCl (acid-propanol)

PROCEDURE

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

CELL VIABILITY ASSAY

The extent of apoptosis in the H_2O_2 treated chick embryo fibroblasts, in the presence and the absence of leaf extracts was studied by sulphorhodamine B assay as proposed by Skehan *et al.* (1990).

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. The SRB assay provides a sensitive measure of drug-induced cytotoxicity and is useful in quantitating clonogenicity and is well suited to high volume, automated drug screening.

REAGENTS

1. Complete medium (DMEM, 10% serum and antibiotics)
2. PBS (phosphate buffered saline)
3. 40% TCA
4. Sulphorhodamine B - 0.4% in 1% TCA
5. 1% acetic acid
6. 10mM Tris (pH 10.5)

PROCEDURE

After the various treatments, the medium was removed and washed with 200µl PBS to remove the traces of medium and serum. A portion of 350µl of ice cold 40% TCA was layered on top of the treated cells and incubated at 4°C for one hour, after which they were washed 5 times with 200µl of cold PBS. The buffer was removed, 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 portions of 1% acetic acid to remove the unbound dye. Then 10mM tris (350µl) was added to each well to solubilize the protein-bound dye. They were shaken gently for 20 minutes. Then the tris layer in each group was transferred to a new 96-well plate and the absorbance was read in a micro titer plate reader (Anthos 2020, Austria) at 492nm. The cell survival was measured as the per cent absorbance compared to the control (untreated) cells.

DETECTION OF DNA FRAGMENTATION

DNA fragmentation, which generally occurs at the late phase of apoptosis, was analysed using agarose gel electrophoresis as proposed by Yin *et al.* (1994).

DNA fragmentation that occurs during apoptosis breaks the chromosomal DNA to smaller fragments with 3' overhang. These fragments, when run on an agarose gel, form a typical laddering pattern after electrophoresis.

REAGENTS

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

PROCEDURE

The tubes were centrifuged after incubation and washed twice with cold PBS. The cell pellet was lysed in 1 ml lysis buffer. After 10 minutes on ice, the lysate was centrifuged at 13,000g for 10 minutes at 4°C. The nucleic acids in the supernatant were extracted with phenol-chloroform-isoamyl alcohol in the ratio of 25:24:1. For every 500 µl of aqueous phase, 37.5µl of 4M NaCl was added and the nucleic acids were precipitated with two volumes of ice cold ethanol at -20°C overnight. The pellet was then rinsed with ice cold 70% ethanol, air dried and dissolved in 20 µl of TE buffer.

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

documented using Alpha Digidoc gel documentation system (Alpha Innotech, UK).

YEAST CELLS AS AN *in vitro* SYSTEM

Yeast cells were chosen as they are considered to be an ideal model organism for eukaryotic cells and the entire genomic and proteomic characterization of these cells are being pursued as a prototype of all the higher eukaryotic systems.

CULTURING OF YEAST CELLS

YPD medium (10g of yeast extract, 20g of peptone and 20g of dextrose, pH 6.5 for 1000ml) was prepared and 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. The cells were pelleted by centrifugation at 1000g for 15 minutes. The pellet was then washed with saline and resuspended in a specific volume of assay medium for each experiment.

TREATMENT GROUPS

The following treatment groups were set up for each parameter, to study the effect of methanolic extract of the leaves of *Rhinacanthus nasutus* on oxidatively stressed *Saccharomyces cerevisiae* cells.

1. Untreated (negative) control
2. H_2O_2 treated (positive) control
3. Methanolic extract of *Rhinacanthus nasutus* leaves treated group
4. H_2O_2 + methanolic extract of *Rhinacanthus nasutus* leaves treated group

The concentration of hydrogen peroxide used for all the assays was 200μM. H_2O_2 was diluted fresh from a 30% solution just before addition. The concentration of plant extract was 20mg. The oxidant and plant extract exposure was given for one hour.

PARAMETERS ANALYSED

The apoptotic events that occurred after the oxidant treatment were analysed using various parameters (giemsa, EtBr, PI and DAPI staining) as described for the primary chick embryo fibroblasts.

DNA FRAGMENTATION

DNA fragmentation in yeast cells was quantified using diphenyl amine method as described by Boraschi and Maurizi (1998). The cells were collected by trypsinization using trypsin – EDTA (PAA). The collected cells were distributed equally to the tubes and the treatments were performed in the distributed fractions. The DNA from all the treated groups of cells was sedimented and used for the assay.

This method is based on the notation that extensively fragmented double-stranded DNA can be prepared from chromosomal DNA upon centrifugal sedimentation and colorimetrically quantified upon reaction with diphenylamine (DPA), which binds to deoxyribose.

REAGENTS

1. TTE solution (TE buffer - 10mM Tris and 1mM EDTA with 0.2% triton X-100, pH 7.4)
2. TCA (5% and 25%)
3. Acetaldehyde solution (16mg of acetaldehyde in 10ml of distilled water)

4. DPA solution (10ml glacial acetic acid was added to 150mg of DPA in a 50ml polypropylene tube and mixed thoroughly. To that, 50µl of acetaldehyde solution was added, mixed thoroughly and stored in a cool, dark place)
5. YPD medium

PROCEDURE

The cells were taken equally in tubes labeled B and treatments were performed in that according to the treatment groups. After that, the tubes were centrifuged at 200Xg at 4°C for 10 minutes. The supernatants were transferred to new tubes labeled S. To the pellets in tubes B 1.0ml of TTE solution was added and vortexed to release the fragmented chromatin from the nuclei.

To separate the fragmented DNA from intact chromatin, tubes B were centrifuged at 20,000Xg for 10 minutes at 4°C. From tubes B, supernatants were carefully transferred to new tubes labeled T. To the small pellets in tubes B 1.0 ml of TTE solution was added. Then 1.0 ml of 25%TCA was added to all tubes and vortexed. The precipitation was allowed to proceed overnight at 4°C.

The supernatants were then discarded by aspiration. Then the DNA was hydrolysed by adding 160µl of 5% TCA to each pellet and heating for 15 minutes at 90°C. A blank was also prepared having 160µl of 5% TCA alone.

Freshly prepared DPA solution (320µl) was added to all the tubes. The tubes were incubated for 4 hours at 37°C or overnight at room temperature for colour development. Aliquots of 200µl of the colored solution were transferred from each tube to wells of a 96-well micro titer plate.

The optical density was read at 600nm with a micro titer plate reader (Anthos 2020, Austria). The per cent fraction of fragmented DNA was calculated using the formula

$$\text{Per cent fragmentation} = \frac{T+S}{T+S+B} \times 100$$

where, S, T and B are the absorbances at 600nm of fragmented DNA in the S, T and B fractions respectively.

The fragmented DNA released by cells undergoing apoptosis and lysis is recovered in the fraction S. Since many substances present in the fraction could heavily interfere with the OD measurement, the following formula was also applied,

$$\text{Per cent fragmentation} = \frac{T}{T+B} \times 100$$

CELL LINES AS AN ALTERNATIVE MODEL

Use of intact cells as an alternative model to live animals has achieved by culturing human carcinoma cell line Hep2 (laryngeal carcinoma). The Hep2 cell line was procured from the National Centre for Cell Science (NCCS), Pune, India. The cells were maintained in DMEM supplemented with 10% FBS. The cells were grown to confluency and were harvested by trypsinization (0.25%).

Etoposide, which is a standard anti-cancer chemotherapeutic agent that is known to exert its action by inducing apoptosis (Lee *et al.*, 2007b), was used as the standard stress-inducing agent. Since etoposide is known to act via apoptosis, the cellular events related to the process of apoptosis were followed in the presence and the absence of *Rhinacanthus nasutus* leaf extracts. Etoposide induces oxidative stress, which leads to apoptosis (Custodio *et al.*, 2002). The standard oxidant used in this part of the study was etoposide. Since the *in vitro* system used was cancer cell lines, the extent of oxidative stress and the protection rendered by the leaf extracts could be better studied using etoposide.

When the cells reached the state of confluency, the cells were trypsinised and the required number of cells was seeded on to 6-well and 96-well plates for carrying out various assays. The well plates were incubated in a CO_2 incubator (Napko, UK) in a 5% CO_2 and 95% humidity atmosphere. Various treatment groups were set up for each parameter as given below.

TREATMENT GROUPS

1. Untreated (negative) control
2. Etoposide treated (positive) control
3. Methanolic extract of *Rhinacanthus nasutus* leaves treated group
4. Etoposide + methanolic extract of *Rhinacanthus nasutus* leaves treated group.

The concentration of etoposide used for all the assays was 200μM, while the concentration of plant extract used was 20mg in 5μl of DMSO.

PARAMETERS ANALYSED

All the apoptotic events analysed in the yeast cells were also followed in the cancer cell lines. In addition to ethidium bromide and propidum iodide staining methods, the nuclear changes in Hep2 cells were also observed by following DAPI (4'-6'-diamidino-2-phenyl indole) staining prodedure as given by Rashmi *et al.*(2003).

DAPI forms fluorescent complexes with double stranded DNA. Because of this property, DAPI is a useful tool to distinguish apoptotic cells from normal cells.

REAGENTS

1. Complete medium (DMEM, 10% FBS and antibiotics)

2. PBS (phosphate buffered saline)
3. 3 % paraformaldehyde in PBS
4. 0.2% Triton X-100 in PBS
5. DAPI (4'-6'-diamidino-2-phenyl indole) – 1μg / ml in PBS

PROCEDURE

After removing the medium and serum, the treated cells were fixed with 3% paraformaldehyde (50μl) for 10 minutes at room temperature, permeabilised with 0.2% triton X-100 (50μl) for 10 minutes at room temperature. Then they were incubated for 3 minutes with 10μl of DAPI after placing a coverslip over the cells to enable uniform spreading of DAPI.

The apoptotic cells were scored by counting the cells with condensed chromatin and fragmented nuclei under fluorescent microscope (Nikon, Japan) using UV2A filter at 400X magnification. The apoptotic index was calculated as given earlier.

PHASE IV

The results of the first three phases of the present study revealed that the methanolic extract of *Rhinacanthus nasutus* leaves exhibited strong antioxidant potential against oxidative assaults induced under *in vivo* simulated *in vitro* conditions. In the fourth phase, a preliminary phytochemical screening of the leaf extract was carried out to identify the principle component responsible for the antioxidant response evoked by the leaf extract against oxidative stress.

PRELIMINARY PHYTOCHEMICAL SCREENING

The methanolic extract prepared from *Rhinacanthus nasutus* leaves were tested for the presence of various known phytochemicals (Khandelwal *et al.*, 2002).

DETECTION OF ALKALOIDS

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

b) **Dragendroff's test:**

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

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

DETECTION OF PHENOLICS

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

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

DETECTION OF FLAVONOIDS

a) **Aqueous sodium hydroxide test**: A fraction of the extract was treated with 1N aqueous NaOH solution and observed for the formation of yellow-orange colouration.

b) **Sulphuric acid test**: A fraction of the extract was treated with concentrated sulphuric acid and observed for the formation of orange colour.

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

The results of the qualitative analysis showed the presence of alkaloids, phenolics and flavonoids in the leaves of *Rhinacanthus nasutus*. Further, the leaves were extracted using the extraction protocols specific for alkaloids, phenolics and flavonoids (Harborne, 1973; Vitale *et al.,* 1995) as given below.

EXTRACTION OF ALKALOID, PHENOLIC AND FLAVONOID FRACTIONS

TOTAL ALKALOID FRACTION

Fresh leaves of *Rhinacanthus nasutus* (5g) were extracted with 20ml of ethanol : 28% NH_4OH, (95:5) at room temperature overnight. The extract was filtered and concentrated under reduced pressure to a fumy residue, which was extracted twice with 1N HCl (10ml each) and filtered. Alkaloids were liberated at pH 9.8 by the addition of 0.7M Na_2CO_3. The solution was extracted with methylene chloride (3X5 ml). The organic extract was dried over anhydrous sodium sulphate to yield the total alkaloid fraction.

TOTAL PHENOL FRACTION

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

FLAVONOID FRACTION

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

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

TLC OF ALKALOIDS, PHENOLICS AND FLAVONOIDS

The extracted fractions were subjected to Thin Layer Chromatography on silica gel G60 F_{254} plates (EMerck). The alkaloid fraction was developed with CH_2Cl_2 : ethanol : 28% ammonium hydroxide (85:14:1) and sprayed with Dragendroff's reagent. Phenolics were separated with acetic acid : chloroform (45:5) and flavonoids with n-butanol : acetic acid : water (4:1:5) and both were detected with vanillin-H_2SO_4 (10% vanillin in ethanol : concentrated sulphuric acid in 2:1 ratio) spray reagent. The R_f values of the spots were calculated as the ratio of the distance traveled by the solute to that by the solvent front.

SPECTRAL ANALYSIS

A preliminary spectral analysis was done by a survey scan of the methanolic extract of *Rhinacanthus nasutus* in a nanospectrophotometer (Optizen, Korea) after which the samples were taken for HPLC analysis.

HPLC ANALYSIS

HPLC analysis was conducted with a Shimadzu chromatograph equipped with photodiode array detector and a 250mm reverse phase column. Shade dried *Rhinacanthus nasutus* leaves were powdered and dissolved in appropriate volume of HPLC grade methanol solvent and injected into the apparatus. The sample analysis of the powdered leaf sample was performed at room temperature, in the wavelength range of 200-400nm at 1000 psi and the mobile phase used was acetonitrile and water in the ratio of 60:40.

GC-MS ANALYSIS

The powdered plant material was analysed using an Agilant-5 gas chromatography-MS spectrometer using a HP-5 column equipped with SEM detector with helium as a carrier gas at a flow rate of 1.5psi. The compounds were identified using the database available in the light of the available literature in the journals and books.

IR SPECTRAL ANALYSIS

Prior to IR analysis, the methanolic extract of *Rhinacanthus nasutus* was subjected to thin layer chromatography using the solvent system ethyl acetate and ethanol in 1:1 ratio. It gave three spots, the middle spot being more distinct, indicative of a single compound. The third spot corresponds to chlorophyll and was not considered. The first and second spots were then separated using preparative TLC and eluted into methanol, and the IR spectral analysis were carried out with the eluted spots using IR spectrophotomer (Shimadzu).

STATISTICAL ANALYSIS

The analysis was performed using SigmaStat statistical package (version 3.1). 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 was adopted to the parameters under study to test the level of significance.

The results obtained for the various biochemical assays performed in all the four phases of the study and the salient findings made during the study are discussed in the next chapter.

LEAVES OF *Rhinacanthus nasutus*

PLATE 1

4. RESULTS

Free radicals and Reactive Oxygen Species are by-products arising from numerous physiological and biochemical processes. Research findings have shown that excessive reactive oxygen species may be harmful to biomolecules and promote aging, cancer and cardiovascular diseases. Consequently, protecting organisms from oxidative damage by the use of antioxidants is one approach to prevent these diseases (Gad *et al.,* 2007).

The antioxidant properties of plants can be correlated with oxidative stress defense and different human diseases. In this respect, antioxidants, especially flavonoids and other polyphenolic compounds, have received the greatest attention (Shrikumar and Ravi, 2007).

Natural products have served as a major source of drugs for centuries, and about half of the pharmaceuticals in use today are derived from natural products. The use of natural substances, particularly those derived from plants, to control diseases, is a centuries old practice that has led to the discovery of more than half of all modern pharmaceuticals. A growing worldwide interest in the use of phytopharmaceuticals, as complimentary or alternative medicine either to prevent or ameliorate many diseases has been noted in recent years (Krishna *et al*., 2008).

Ethnobotanical survey reveals that the roots of the *Rhinacanthus nasutus* possess medicinal value. Uprooting the plant for the experimental purpose can be avoided if the leaf part is chosen, which in turn minimize the damage caused to the plant system. Therefore, the main aim of the present study was to assess the antioxidant potential of the *Rhinacanthus nasutus* leaves.

PHASE I

As an initial step towards understanding the antioxidant potential of the leaves, the enzymic and non-enzymic antioxidants were analysed in the leaves of *Rhinacanthus nasutus*.

ENZYMIC ANTIOXIDANT ACTIVITIES IN THE LEAVES OF *Rhinacanthus nasutus*

The enzymic antioxidants analysed were SOD, CAT, POD, GST and PPO (catechol oxidase and laccase) and the activities obtained are presented in Table I.

TABLE I

ENZYMIC ANTIOXIDANT ACTIVITY OF *Rhinacanthus nasutus* LEAVES

ENZYMES	ACTIVITY
SOD (U^{*}/ mg of protein)	0.006±0.0007
CAT (U^{+}/ mg of protein)	33.55 ± 0.82
POD ($U^{\$}$/ mg of protein)	0.06 ±0.006
GST ($U^{@}$/ mg of protein)	3.20 ± 0.08
Catechol oxidase ($U^{\#}$/ mg of protein)	0.25 ± 0.02
Laccase ($U^{\#}$/ mg of protein)	0.23 ± 0.04

The values are means ± S.D. of triplicates

*1 Unit = Amount of enzyme that causes 50% reduction in NBT oxidation.

+1 Unit = Amount of enzyme required to decrease the absorbance at 240nm by 0.05 units.

$ 1Unit = change in absorbance at 430nm per minute

@1 Unit = nmoles of NADPH oxidized

Amount of catechol oxidase / laccase which transforms 1 unit of dihydrophenol to quinine / minute

The leaves of *Rhinacanthus nasutus* possessed considerable activities of all the enzymes analysed. It can be deduced from the tabulated values that the plant leaf is an excellent source of antioxidants.

NON-ENZYMIC ANTIOXIDANT LEVELS IN THE LEAVES OF *Rhinacanthus nasutus*

Table II depicts the non-enzymic antioxidant levels in *Rhinacanthus nasutus* leaves. The major representatives of the non-enzymic antioxidants, namely ascorbate, tocopherol, total carotenoids, reduced glutathione, total phenols and chlorophyll were estimated in the leaf extract of *Rhinacanthus nasutus*. It was observed that the leaves of *Rhinacanthus nasutus* were found to exhibit moderate levels of all the non-enzymic antioxidant assays performed.

TABLE II

NON-ENZYMIC ANTIOXIDANT LEVELS OF *Rhinacanthus nasutus* LEAVES

PARAMETER	LEVELS / g LEAF
Ascorbic acid (mg)	3.91 ± 0.04
Tocopherol (µg)	88.67 ± 0.55
Total carotenoids (mg)	74.15 ± 1.06
Lycopene (mg)	63.24 ± 1.51
GSH (nmoles)	292.6 ± 3.2
Total phenols (mg)	63.63 ± 7.0
Chlorophyll (mg)	1.39 ± 0.2

The values are means ± S.D. of triplicates

It is evident from the results of phase I that *Rhinacanthus nasutus* contained potent antioxidants and can be subjected to further analysis.

PHASE II

The second phase of this study involved the analysis of the free-radical scavenging and antioxidant potential of the leaves of *Rhinacanthus nasutus*. In order to understand the nature of the extract exhibiting the strongest antioxidant activity, the leaves were extracted into solvents of differing polarity (water,

methanol, chloroform). These extracts were then assessed for their radical scavenging effects against a sequence of oxidant moieties that included a stable radical DPPH, the unstable, non-radical oxidant (H_2O_2), ABTS cation radical and hydroxyl radicals. The effect of the extracts were also tested on DNA and lipids exposed to oxidative stress *in vitro*.

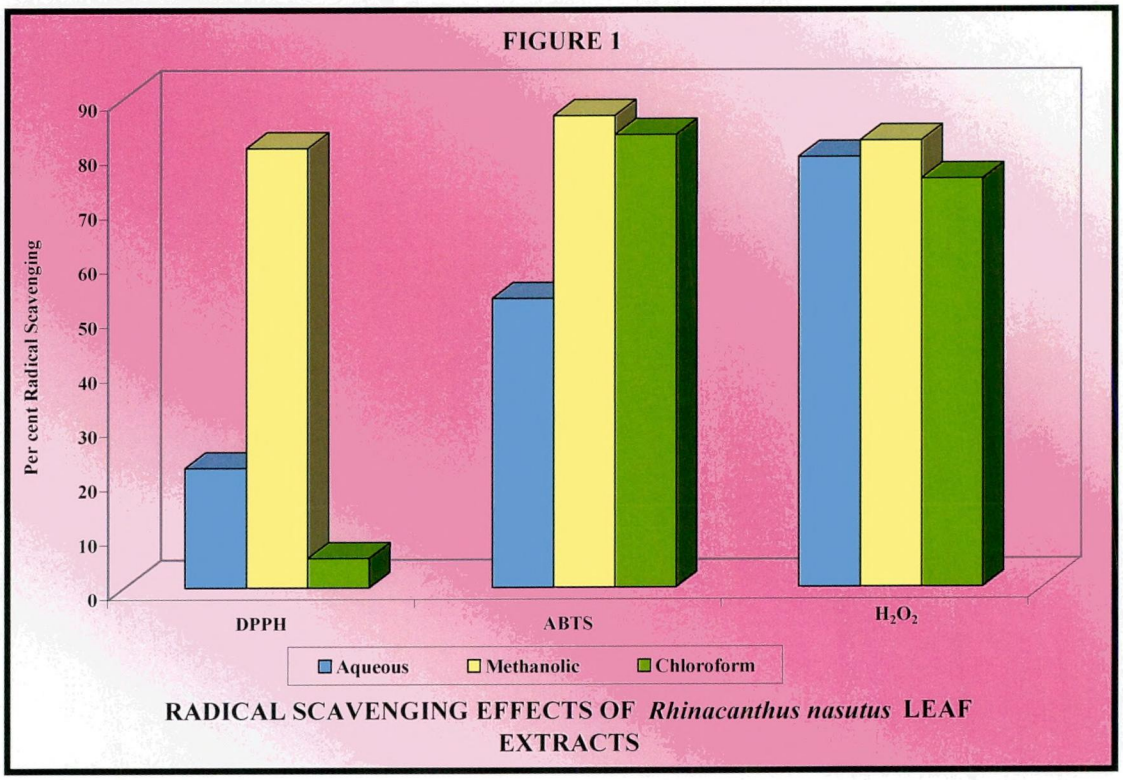
RADICAL SCAVENGING ABILITY OF *Rhinacanthus nasutus* LEAF EXTRACTS

The radical scavenging effects of the three extracts of *Rhinacanthus nasutus* leaves were studied on DPPH, H_2O_2 and ABTS. The results obtained are presented in Figure 1. Among the three extracts analysed, the methanolic extract of *Rhinacanthus nasutus* leaves showed a powerful DPPH scavenging ability followed by the aqueous extract and the least scavenging effect was obtained from the chloroform extract. All the three extracts exhibited strong H_2O_2 scavenging ability. While the activities were on par with each other for the aqueous and methanolic extracts, it was slightly lower in the chloroform extract. All the three extracts readily scavenged the ABTS radical cation. The maximum extent of ABTS scavenging was mediated by the methanolic extract, followed closely by the chloroform extract. The aqueous extract exhibited a lower activity in comparison.

PROTECTIVE EFFECTS OF *Rhinacanthus nasutus* LEAVES ON H_2O_2 INDUCED DAMAGE TO DEOXY RIBOSE

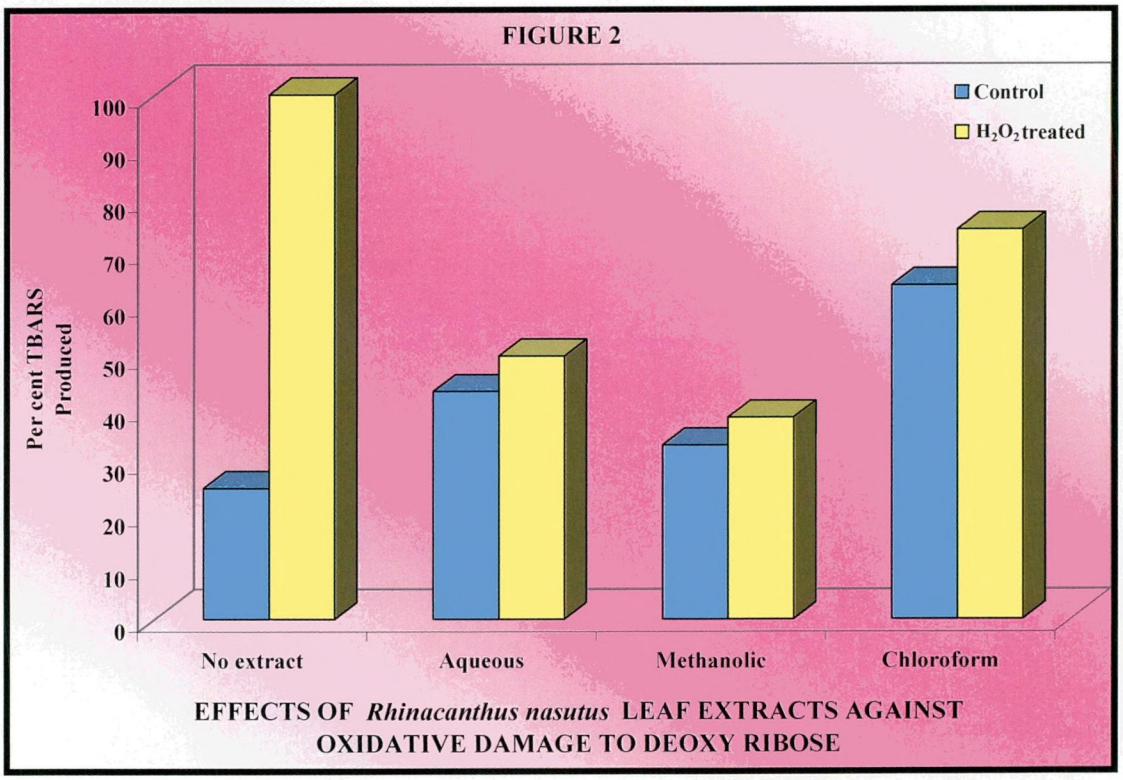
The protective effects of herbal extracts against oxidative damage induced to deoxy ribose is considered as an indicator of the hydroxyl radical scavenging ability of the extracts. Hydroxyl radicals have been implicated as highly damaging species in free radical pathology. This radical has the capacity to join nucleotides in DNA, cause strand breakage, which contributes to mutagenesis, carcinogenesis and cytotoxicity (Murthy *et al.*, 2006).

FIGURE 1



RADICAL SCAVENGING EFFECTS OF *Rhinacanthus nasutus* LEAF EXTRACTS

FIGURE 2



EFFECTS OF *Rhinacanthus nasutus* LEAF EXTRACTS AGAINST OXIDATIVE DAMAGE TO DEOXY RIBOSE

The effect of *Rhinacanthus nasutus* leaf extracts on H_2O_2-induced damage to deoxy ribose was quantified as the amount of TBARS formed, and the results obtained are represented in Figure 2. The values of H_2O_2 treated group was fixed at 100 and other groups was calculated relative to this.

H_2O_2 exposure resulted in a steep increase in the extent of damage, which was very effectively counteracted by the different extracts of *Rhinacanthus nasutus* leaves. The effect of methanolic extract was more pronounced than the other two extracts in scavenging hydroxyl radicals, closely followed by the aqueous extract.

PROTECTIVE EFFECTS OF *Rhinacanthus nasutus* LEAVES ON H_2O_2 INDUCED DAMAGE TO HERRING SPERM DNA

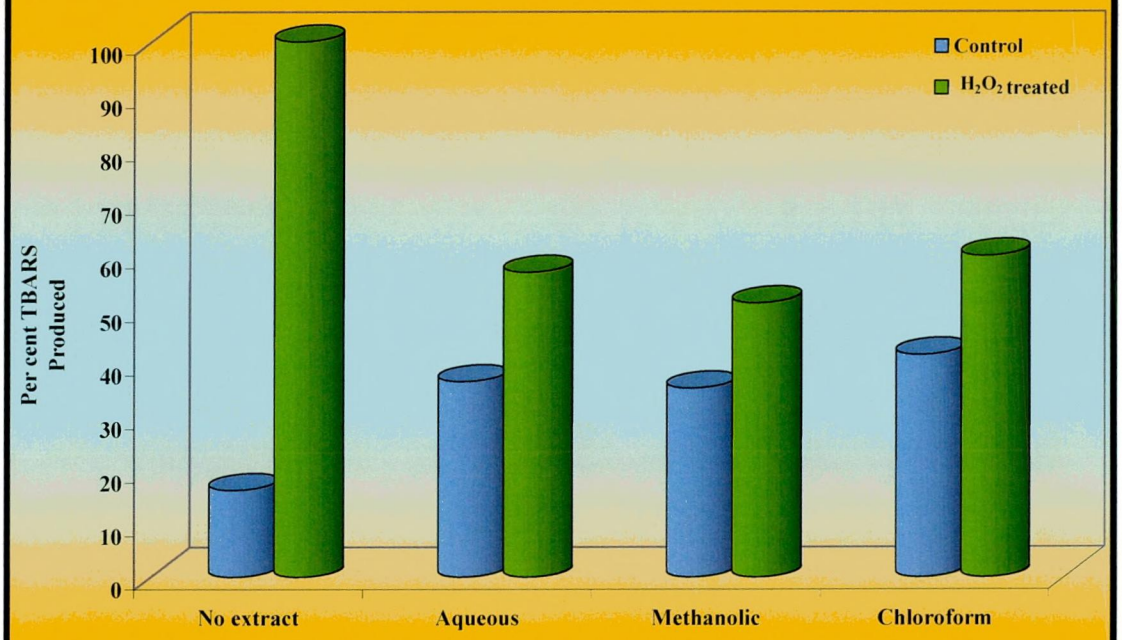
The ultimate target of persistent oxidant moieties are the DNA molecules, damage to which can lead to severe consequences in the cell (Ani *et al.*, 2006). Hence, in the present study, the effect of *in vitro* exposure of herring sperm DNA to *Rhinacanthus nasutus* leaf extracts was studied in the the presence and the absence of oxidative stress imposed by H_2O_2. The results obtained are schematically represented in Figure 3.

H_2O_2 caused an increased extent of damage to herring sperm DNA. The extent of damage decreased markedly in the presence of the leaf extracts of *Rhinacanthus nasutus*. The maximum protection was offered by the methanolic extract followed closely by the aqueous extract and then chloroform extract.

EFFECT OF *Rhinacanthus nasutus* LEAVES ON LPO

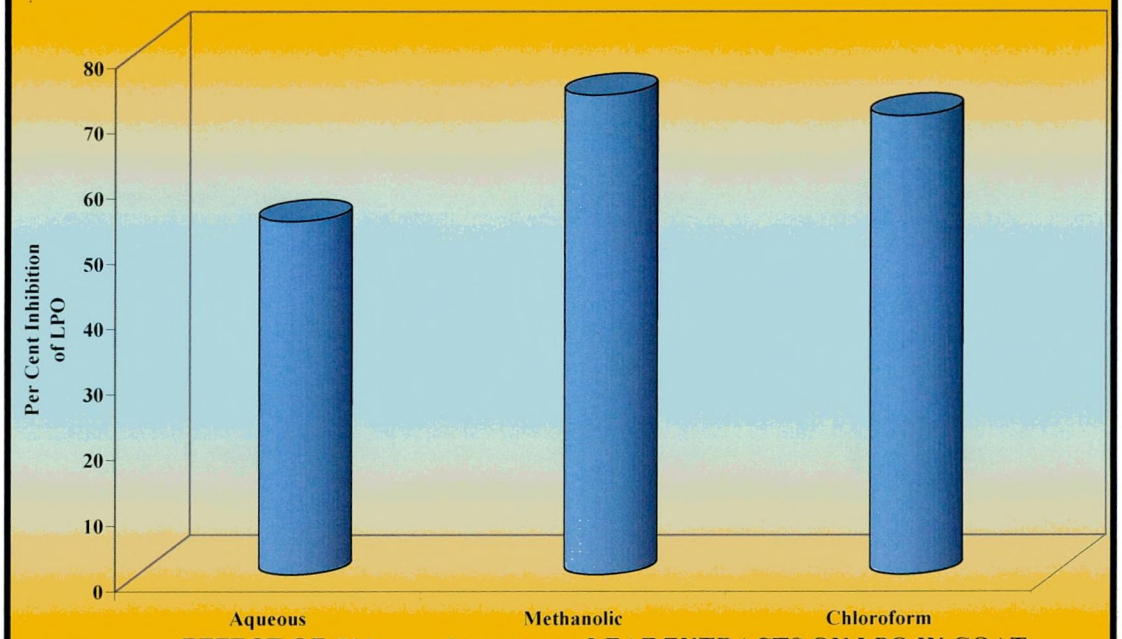
Excess generation of oxygen free radicals can cause oxidative damage to biomolecules resulting in lipid peroxidation (LPO), mutagenesis and carcinogenesis (Ozgen *et al.*, 2006).

FIGURE 3



EFFECTS OF *Rhinacanthus nasutus* LEAF EXTRACTS ON OXIDATIVE DAMAGE INDUCED IN HERRING SPERM DNA

FIGURE 4



EFFECT OF *Rhinacanthus nasutus* LEAF EXTRACTS ON LPO IN GOAT LIVER HOMOGENATE

The extent of lipid peroxidation was studied using goat liver homogenate, which constituted a combination of lipids from the plasma membrane and the intracellular membranes. The per cent inhibition of *in vitro* LPO by *Rhinacanthus nasutus* leaf extracts is depicted in Figure 4. All the three leaf extracts inhibited LPO to considerable extent. The extent of inhibition was greater in methanolic extract followed by the chloroform extract.

PHASE- III

The results thus far obtained, clearly indicated the strong free radical scavenging and biomolecule-protecting effects of *Rhinacanthus nasutus* leaves in various cell-free systems and tissue homogenate. Further to this, in the next phase, an elaborate study was formulated to analyze the effects of the leaf extract on live cells and tissues, in the presence and the absence of oxidative stress.

For this purpose, a wide spectrum of cellular and tissue systems were taken, all in tune with the global efforts of minimizing the use of live animals in biological research. The systems were chosen in such a way that they decreased / avoided the usage of animals, at the same time providing reliable and valuable scientific validation of the effects of the leaf extracts.

The *in vitro* models adapted were goat liver slices, primary cells of chick embryo fibroblasts, *Saccharomyces cerevisiae* cells and cell lines.

ANTIOXIDANT POTENTIAL OF *Rhinacanthus nasutus* LEAF EXTRACTS IN GOAT LIVER SLICES EXPOSED TO H_2O_2

Liver plays a major role in co-ordinating the internal environment of the body. Liver was the organ of choice in the present study, because it is the metabolic organ and is responsible for the metabolic clearance of many xenobiotics (James and Little, 2004).

Earlier studies done in our laboratory using goat liver slices as *in vitro* models, which simulate *in vivo* conditions, brought out successful results and the tissue slices provide a more representative model for the *in vivo* situation (Varier, 2002; Kiruthika, 2003; Saraswathi, 2006; Sumathi, 2007; Vidya, 2007). Precision-cut goat liver slices were subjected to oxidative stress in the presence and the absence of *Rhinacanthus nasutus* leaf extract, and the protective effect of the extract was analyzed by assessing the enzymic and non-enzymic antioxidants in the tissue slices.

ENZYMIC ANTIOXIDANTS

The enzymic antioxidants analysed in the slices were SOD, CAT, POD and GST.

SUPEROXIDE DISMUTASE

The activities of SOD in the slices exposed to H_2O_2 and/or *Rhinacanthus nasutus* leaf extract are represented in Table III.

TABLE III

EFFECT OF *Rhinacanthus nasutus* LEAF EXTRACTS ON SOD ACTIVITY IN GOAT LIVER SLICES EXPOSED TO H_2O_2 *in vitro*

SAMPLE	SOD ACTIVITY (Units /g tissue)	
	Without H_2O_2	With H_2O_2
No extract	25.49 ± 0.68	21.41 ± 0.27[a]
Aqueous extract	30.10 ± 0.04[a]	24.16 ± 0.23[abc]
Methanolic extract	28.13 ± 0.34[a]	38.66 ± 0.23[abc]
Chloroform extract	22.79 ± 0.26[a]	30.48 ± 0.56[abc]

The values are means ± S.D. of triplicates.

1 Unit = Amount of enzyme that causes 50% reduction in NBT oxidation.

a - Statistically significant ($P<0.05$) compared to untreated control

b - Statistically significant ($P<0.05$) compared to H_2O_2 alone treated group

c - Statistically significant ($P<0.05$) compared to the respective plant control

The activity of SOD decreased significantly ($P<0.05$) upon exposure of the liver slices to H_2O_2. Goat liver slices treated with the leaf extracts of *Rhinacanthus nasutus* showed an enhanced SOD activity compared to untreated controls. Co-treatment with the leaf extracts and H_2O_2 caused a significant elevation in the SOD activity. The methanolic extract elicited the maximum activity against H_2O_2 induced oxidative stress.

CATALASE

Catalase catalyses the decomposition of H_2O_2 to molecular oxygen and water, thereby protecting cells from the toxic effects of H_2O_2 (Hua *et al.*, 2007). The treatment of liver slices with H_2O_2 caused a significant ($P<0.05$) decline in the activity of catalase as shown in Table IV. This effect was very effectively counteracted by the administration of *Rhinacanthus nasutus* leaf extracts.

TABLE IV

EFFECT OF *Rhinacanthus nasutus* LEAF EXTRACTS ON CATALASE ACTIVITY IN GOAT LIVER SLICES EXPOSED TO H_2O_2 *in vitro*

SAMPLE	CAT ACTIVITY (Units /g tissue)	
	Without H_2O_2	With H_2O_2
No extract	169 ± 1.41	112 ± 1.70[a]
Aqueous extract	282 ± 1.70[a]	339 ± 2.12[abc]
Methanolic extract	225 ± 1.84[a]	424 ± 1.61[abc]
Chloroform extract	284 ± 1.98[a]	379 ± 1.72[abc]

The values are means ± S.D. of triplicates.

1 Unit = Amount of enzyme required to decrease the absorbance at 240nm by 0.05 units.

a - Statistically significant ($P<0.05$) compared to untreated control

b - Statistically significant ($P<0.05$) compared to H_2O_2 alone treated group

c - Statistically significant ($P<0.05$) compared to the respective plant control

The catalase activity was enhanced to a maximum extent in the presence of methanolic extract in the H_2O_2 treated group. Thus it was found to be effective in alleviating the toxicity of H_2O_2 in goat liver slices.

PEROXIDASE

SOD, CAT and GPx are important scavengers of superoxide ions and hydrogen peroxide. These enzymes prevent the generation of hydroxyl radicals and protect the cellular constituents from oxidant induced damage (Dash *et al.*, 2007). The effects of *Rhinacanthus nasutus* leaf extracts on the peroxidase activity in goat liver slices exposed to H_2O_2 *in vitro* are listed in Table V.

TABLE V

EFFECT OF *Rhinacanthus nasutus* LEAF EXTRACTS ON PEROXIDASE ACTIVITY IN GOAT LIVER SLICES EXPOSED TO H_2O_2 *in vitro*

SAMPLE	POD ACTIVITY (Units /g tissue)	
	Without H_2O_2	With H_2O_2
No extract	1.63 ± 0.02	1.23 ± 0.08[a]
Aqueous extract	2.02 ± 0.08[a]	1.30 ± 0.18[ac]
Methanolic extract	2.04 ± 0.06[a]	2.27 ± 0.04[abc]
Chloroform extract	1.90 ± 0.03[a]	1.92 ± 0.06[ab]

The values are means ± S.D. of triplicates.
1 Unit = change in absorbance at 430nm per minute
a - Statistically significant ($P<0.05$) compared to untreated control
b - Statistically significant ($P<0.05$) compared to H_2O_2 alone treated group
c - Statistically significant ($P<0.05$) compared to the respective plant control

A steep decrease in the activity of peroxidase was observed as a result of H_2O_2 induced oxidative damage. However, addition of the leaf extracts caused a significant ($P<0.05$) improvement in the peroxidase activity when compared to control. The methanolic extract was found to be more efficient in reverting back the decreased peroxidase activity due to hydrogen peroxide exposure.

GLUTATHIONE S-TRANSFERASE

Glutathione S-transferase and glutathione peroxidase are the most abundant detoxification enzyme families in the plant system. These enzymes play a pivotal role in inhibiting the cellular damage produced by a variety of stresses (Ali *et al.*,

2006). Table VI presents the activity of GST in the oxidant treated goat liver slices in the presence and absence of *Rhinacanthus nasutus* leaf extracts.

TABLE VI

EFFECT OF *Rhinacanthus nasutus* LEAF EXTRACTS ON GST ACTIVITY IN GOAT LIVER SLICES EXPOSED TO H_2O_2 *in vitro*

SAMPLE	GST ACTIVITY (Units /g tissue)	
	Without H_2O_2	With H_2O_2
No extract	0.16 ± 0.022	0.13 ± 0.005[a]
Aqueous extract	0.16 ± 0.005	0.18 ± 0.001[bc]
Methanolic extract	0.29 ± 0.012[a]	0.35 ± 0.006[abc]
Chloroform extract	0.12 ± 0.003[a]	0.17 ± 0.026[bc]

The values are means ± S.D. of triplicates.
1 Unit = nmoles of NADPH oxidized
a - Statistically significant ($P<0.05$) compared to untreated control
b - Statistically significant ($P<0.05$) compared to H_2O_2 alone treated group
c - Statistically significant ($P<0.05$) compared to the respective plant control

There was a significant decrease in the activity of GST upon H_2O_2 exposure, which were restored to normal in the groups treated with plant extracts. The methanolic extract of *Rhinacanthus nasutus* leaves augmented the GST activity in the oxidatively stressed groups over the untreated control values.

NON-ENZYMIC ANTIOXIDANTS

The levels of ROS are regulated by a variety of cellular defense mechanisms consisting of enzymic and non-enzymic antioxidant systems. Non-enzymic antioxidants (ascorbic acid, tocopherol, vitamin A and reduced glutathione) were estimated in oxidant challenged goat liver slices in the presence and the absence of the leaf extracts. The results obtained are given below.

ASCORBIC ACID

Ascorbic acid is the most investigated antioxidant responsible for the

elimination of free radicals (Tariq, 2007). The levels of ascorbic acid determined in the liver slices are presented in Table VII. Hydrogen peroxide caused a significant ($P<0.05$) reduction in the levels of ascorbic acid. When the tissue slices were exposed to both the oxidant and the *Rhinacanthus nasutus* leaf extracts, there was a significant ($P<0.05$) elevation in the levels of the vitamin. Among the three extracts, aqueous extract brought about the maximum increase. However, the levels of the vitamin remained lower than those in the untreated control group, even in the presence of the leaf extracts.

TABLE VII

EFFECT OF *Rhinacanthus nasutus* LEAF EXTRACTS ON THE LEVELS OF ASCORBIC ACID IN GOAT LIVER SLICES EXPOSED TO H_2O_2 *in vitro*

SAMPLE	ASCORBIC ACID (mg /g tissue)	
	Without H_2O_2	With H_2O_2
No extract	24.2 ± 0.070	10.7 ± 0.240^{a}
Aqueous extract	36.1 ± 0.318^{a}	18.7 ± 0.070^{abc}
Methanolic extract	27.0 ± 0.141^{a}	14.5 ± 0.014^{abc}
Chloroform extract	16.9 ± 0.001^{a}	15.1 ± 0.156^{abc}

The values are means ± S.D. of triplicates.

a - Statistically significant ($P<0.05$) compared to untreated control

b - Statistically significant ($P<0.05$) compared to H_2O_2 alone treated group

c - Statistically significant ($P<0.05$) compared to the respective plant control

TOCOPHEROL

Vitamin E has been regarded as the most potent, lipid soluble chain breaking antioxidant in nature and its use has been widely promoted to protect cell components from oxidative damage (Castellini *et al.*, 2007). As depicted in Table VIII, the tocopherol content depleted to a significant ($P<0.05$) extent upon exposure to hydrogen peroxide. However, treatment with *Rhinacanthus nasutus* leaf extracts caused a very steep increase, thereby compensating the vitamin

depletion due to the oxidant exposure. From the table values, it is evident that the methanolic extract evoked the maximum antioxidant response when compared to the other extracts.

TABLE VIII

EFFECT OF *Rhinacanthus nasutus* LEAF EXTRACTS ON THE LEVELS OF TOCOPHEROL IN GOAT LIVER SLICES EXPOSED TO H_2O_2 *in vitro*

SAMPLE	TOCOPHEROL (µg /g tissue)	
	Without H_2O_2	With H_2O_2
No extract	11.67 ± 0.231	14.95 ± 0.23[a]
Aqueous extract	12.65 ± 0.403[a]	16.80 ± 0.33[abc]
Methanolic extract	22.01 ± 0.283[a]	18.15 ± 0.24[abc]
Chloroform extract	18.30 ± 0.001[a]	18.10 ± 0.23[abc]

The values are means ± S.D. of triplicates.

a - Statistically significant ($P<0.05$) compared to untreated control

b - Statistically significant ($P<0.05$) compared to H_2O_2 alone treated group

c - Statistically significant ($P<0.05$) compared to the respective plant control

VITAMIN A

The levels of vitamin A observed in the different treatment groups are tabulated in Table IX.

TABLE IX

EFFECT OF *Rhinacanthus nasutus* LEAF EXTRACTS ON THE LEVELS OF VITAMIN A IN GOAT LIVER SLICES EXPOSED TO H_2O_2 *in vitro*

SAMPLE	VITAMIN A (mg /g tissue)	
	Without H_2O_2	With H_2O_2
No extract	161.23 ± 0.93	111.93 ± 0.16[a]
Aqueous extract	113.39 ± 1.12[a]	154.42 ± 0.25[abc]
Methanolic extract	235.43 ± 1.80[a]	211.64 ± 1.05[abc]
Chloroform extract	211.50 ± 1.30[a]	209.17 ± 0.28[abc]

The values are means ± S.D. of triplicates.

a - Statistically significant ($P<0.05$) compared to untreated control

b - Statistically significant ($P<0.05$) compared to H_2O_2 alone treated group

c - Statistically significant ($P<0.05$) compared to the respective plant control

The oxidant (H_2O_2) treatment caused a significant decrease in the levels of vitamin A when compared to untreated control. This declined level of vitamin A was very well counteracted by the administration of the leaf extracts.

REDUCED GLUTATHIONE

Reduced glutathione plays a crucial function in detoxification. Treatment of goat liver slices with hydrogen peroxide decreased the reduced glutathione content maximally (Table X), which was restored by the co-treatment with the leaf extracts of *Rhinacanthus nasutus*. The leaf extract caused a significant ($P<0.05$) increase in GSH levels, compared to untreated control, with the methanolic extract showing the maximum effect.

TABLE X
EFFECT OF *Rhinacanthus nasutus* LEAF EXTRACTS ON THE LEVELS OF REDUCED GLUTATHIONE IN GOAT LIVER SLICES EXPOSED TO H_2O_2 *in vitro*

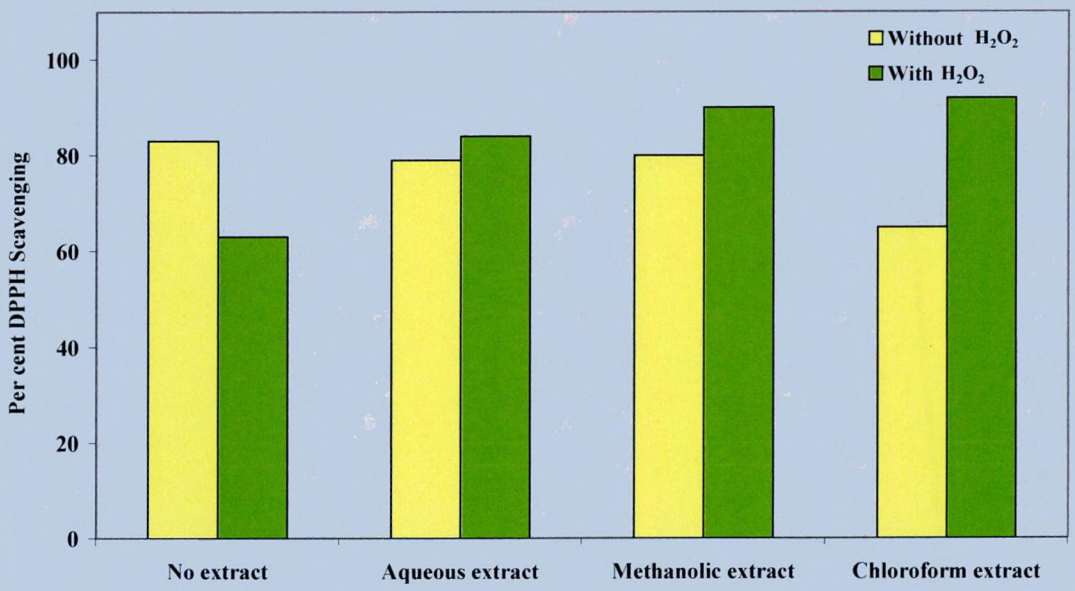
SAMPLE	GLUTATHIONE (nmoles /g tissue)	
	Without H_2O_2	With H_2O_2
No extract	182.26 ± 0.10	111.28 ± 0.24[a]
Aqueous extract	221.38 ± 0.70[a]	206.79 ± 0.11[abc]
Methanolic extract	236.97 ± 0.99[a]	226.84 ± 0.99[abc]
Chloroform extract	178.21 ± 0.11[a]	112.71 ± 0.16[abc]

The values are means ± S.D. of triplicates.
a - Statistically significant ($P<0.05$) compared to untreated control
b - Statistically significant ($P<0.05$) compared to H_2O_2 alone treated group
c - Statistically significant ($P<0.05$) compared to the respective plant control

EFFECT OF *Rhinacanthus nasutus* LEAF EXTRACTS ON THE DPPH SCAVENGING ABILITY IN GOAT LIVER SLICES EXPOSED TO H_2O_2 *in vitro*

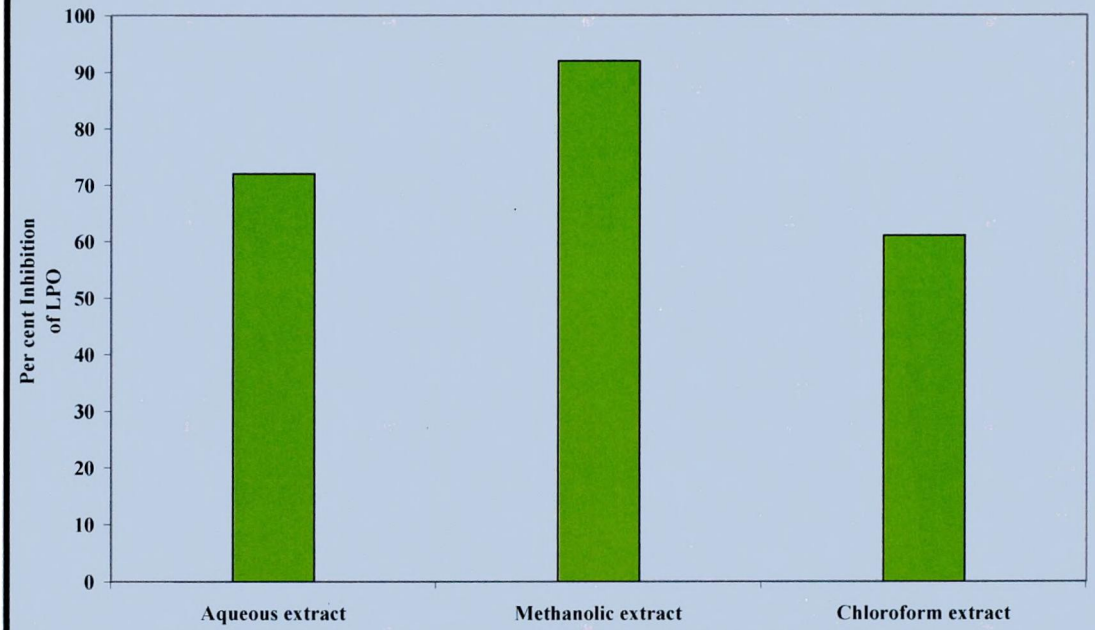
Figure 5 represents the DPPH scavenging activity of the homogenate prepared from the goat liver slices exposed to H_2O_2 and/or *Rhinacanthus nasutus* leaf extracts.

FIGURE 5



EFFECT OF *Rhinacanthus nasutus* LEAF EXTRACTS ON DPPH SCAVENGING ABILITY IN GOAT LIVER SLICES EXPOSED TO H_2O_2 *in vitro*

FIGURE 6



EFFECT OF *Rhinacanthus nasutus* LEAF EXTRACTS ON LPO IN GOAT LIVER SLICES EXPOSED TO H_2O_2 *in vitro*

Exposure to H_2O_2 caused a drastic reduction in the DPPH scavenging ability. The extent of scavenging effect was appreciably increased in goat liver slices treated with extracts after exposure to oxidant assault.

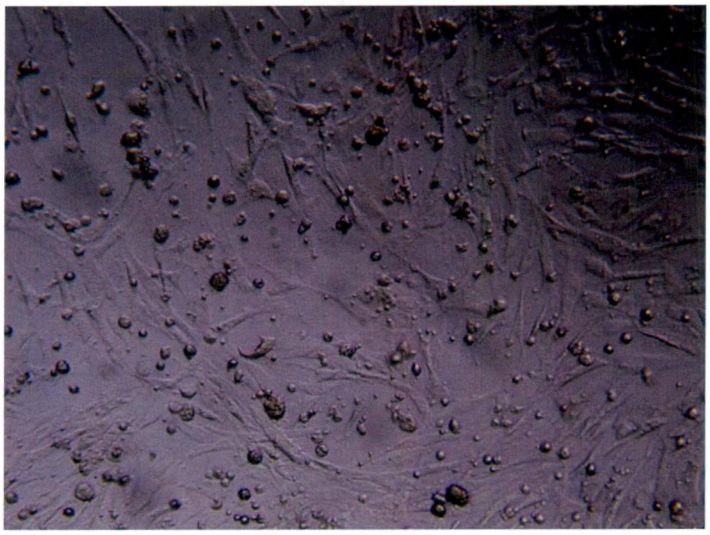
EFFECT OF *Rhinacanthus nasutus* LEAF EXTRACTS ON THE EXTENT OF INHIBITION OF LIPID PEROXIDATION IN GOAT LIVER SLICES EXPOSED TO H_2O_2 *in vitro*

The extent of LPO is considered as a direct measure of the extent of oxidant stress suffered by the system. Oxidative assault on lipids results in the formation of lipid peroxides, hydroperoxides and conjugated dienes. In tune with this, LPO was followed as the amount of TBARS formed in the oxidant treated goat liver slices and the results are given in Figure 6. The Figure illustrates the extent of inhibition of *in vitro* lipid peroxidation brought about by the leaf extracts of *Rhinacanthus nasutus*. The methanolic extract showed a greater inhibition of lipid peroxidation in the liver slices when compared to a moderate inhibition exhibited by the aqueous and chloroform extracts.

EFFECT OF *Rhinacanthus nasutus* LEAF EXTRACT ON THE ANTIOXIDANT STATUS OF CHICK EMBRYO FIBROBLASTS EXPOSED TO OXIDATIVE STRESS

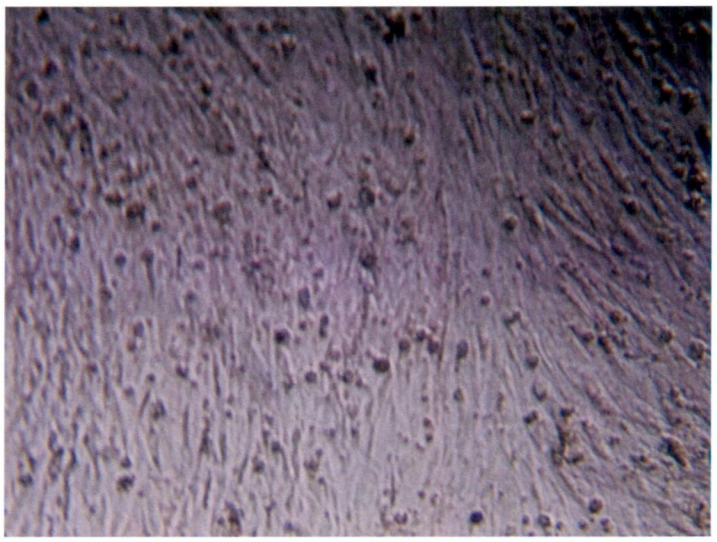
Chick embryo fibroblasts (Plate 2) constitute a primary cell culture system, which possesses a very high proliferative potential and a high rate of metabolism. These cells, unlike the liver slices, can be maintained alive in culture for a longer duration. Therefore, the combined and individual effects of oxidative stress and *Rhinacanthus nasutus* leaf extract were studied using these cells. Since, in the earlier phases of the study, and in the liver slices, the methanolic extract of the leaves evoked the maximum response, only this extract was analyzed in the subsequent systems.

PLATE 2a



CHICK EMBRYO FIBROBLASTS 24 HOURS AFTER SEEDING

PLATE 2b



CHICK EMBRYO FIBROBLASTS AT CONFLUENCE

ENZYMIC ANTIOXIDANTS

Rhinacanthus nasutus leaf extract was analysed for its antioxidant efficacy on oxidatively stressed chick embryo fibroblasts. The activities of the enzymic antioxidants were recorded in the primary chick embryo fibroblasts exposed to H_2O_2 in the presence and the absence of the methanolic extract of *Rhinacanthus nasutus* leaves and the results are presented in Table XI.

TABLE XI

EFFECT OF *Rhinacanthus nasutus* LEAF EXTRACT ON ENZYMIC ANTIOXIDANT ACTIVITIES IN PRIMARY CULTURED CHICK EMBRYO FIBROBLASTS EXPOSED TO H_2O_2 *in vitro*

ENZYME ACTIVITY	No extract		Methanolic extract	
	Without H_2O_2	With H_2O_2	Without H_2O_2	With H_2O_2
SOD ($^{\#}$Units /10^4 cells)	15.25 ± 0.25	14.0 ± 0.50	24.25 ± 0.25^{ab}	33.5 ± 0.50^{abc}
CAT (*Units /10^4 cells)	17.3 ± 0.05	8.6 ± 0.04^{a}	21.5 ± 0.03^{ab}	15.9 ± 0.04^{abc}
POD ($^{¢}$Units /10^6 cells)	16.0 ± 1.00	6.0 ± 1.00^{a}	28.0 ± 1.00^{ab}	39.0 ± 2.00^{abc}
GST ($^{\theta}$Units /10^6 cells)	7.5 ± 0.50	0.75 ± 0.30^{a}	8.0 ± 1.00^{b}	14.0 ± 1.00^{abc}

The values are means ± S.D. of triplicates.
#1 Unit = Amount of enzyme that causes 50% reduction in NBT oxidation.
*1 Unit = Amount of enzyme required to decrease the absorbance at 240nm by 0.05 units.
¢1 Unit = change in absorbance at 430nm per minute
θ1 Unit = nmoles of NADPH oxidized
a - Statistically significant ($P<0.05$) compared to untreated control
b - Statistically significant ($P<0.05$) compared to H_2O_2 alone treated group
c - Statistically significant ($P<0.05$) compared to the respective plant control

The activities of SOD, CAT, POD and GST were assessed and the results revealed that the oxidant (hydrogen peroxide) exposure caused a significant depletion in the enzyme activities in the chick embryo fibroblasts. *Rhinacanthus nasutus* leaves, by themselves, significantly ($P<0.05$) elevated all the enzyme

activities except GST, when compared to untreated control. The toxic effect of H_2O_2 was negated by the concordant treatment with the methanolic extract of the candidate plant.

NON-ENZYMIC ANTIOXIDANTS

Non-enzymic antioxidants are responsible for the protection of cells against oxidative stress and the parameters estimated included ascorbic acid, tocopherol, vitamin A and reduced glutathione. The results obtained are depicted in Table XII.

TABLE XII

EFFECT OF *Rhinacanthus nasutus* LEAF EXTRACT ON NON-ENZYMIC ANTIOXIDANT LEVELS IN PRIMARY CULTURED CHICK EMBRYO FIBROBLASTS EXPOSED TO H_2O_2 *in vitro*

LEVELS	No extract		Methanolic extract	
	Without H_2O_2	With H_2O_2	Without H_2O_2	With H_2O_2
Ascorbic acid (mg/10^4 cells)	16.0 ± 1.0	6.0 ± 1.00[a]	28.0 ± 1.00[ab]	39.0 ± 2.00[abc]
Tocopherol (µg/10^4 cells)	0.65 ± 0.05	0.32 ± 0.01[a]	1.14 ± 0.02[ab]	0.48 ± 0.02[abc]
Vitamin A (mg /10^6 cells)	1.57 ± 0.01	0.07 ± 0.03[a]	3.48 ± 0.02[ab]	1.51 ± 0.02[abc]
GSH (nmoles /10^6 cells)	330 ± 0.5	170 ± 0.6[a]	620 ± 0.3[ab]	310 ± 0.40[abc]

The values are means ± S.D. of triplicates.

a - Statistically significant ($P<0.05$) compared to untreated control

b - Statistically significant ($P<0.05$) compared to H_2O_2 alone treated group

c - Statistically significant ($P<0.05$) compared to the respective plant control

The non-enzymic antioxidants were significantly reduced upon H_2O_2 assault in the chick embryo fibroblast cells. Treatment with the methanolic extract of *Rhinacanthus nasutus* leaves increased the non-enzymic antioxidant content in the primary cells. The leaf extract was found to be very effective in alleviating the toxicity of H_2O_2 in the oxidatively injured primary chick embryo fibroblasts.

EFFECT OF METHANOLIC EXTRACT OF *Rhinacanthus nasutus* ON DPPH SCAVENGING ACTIVITY IN PRIMARY CHICK EMBRYO FIBROBLASTS EXPOSED TO H_2O_2 *in vitro*

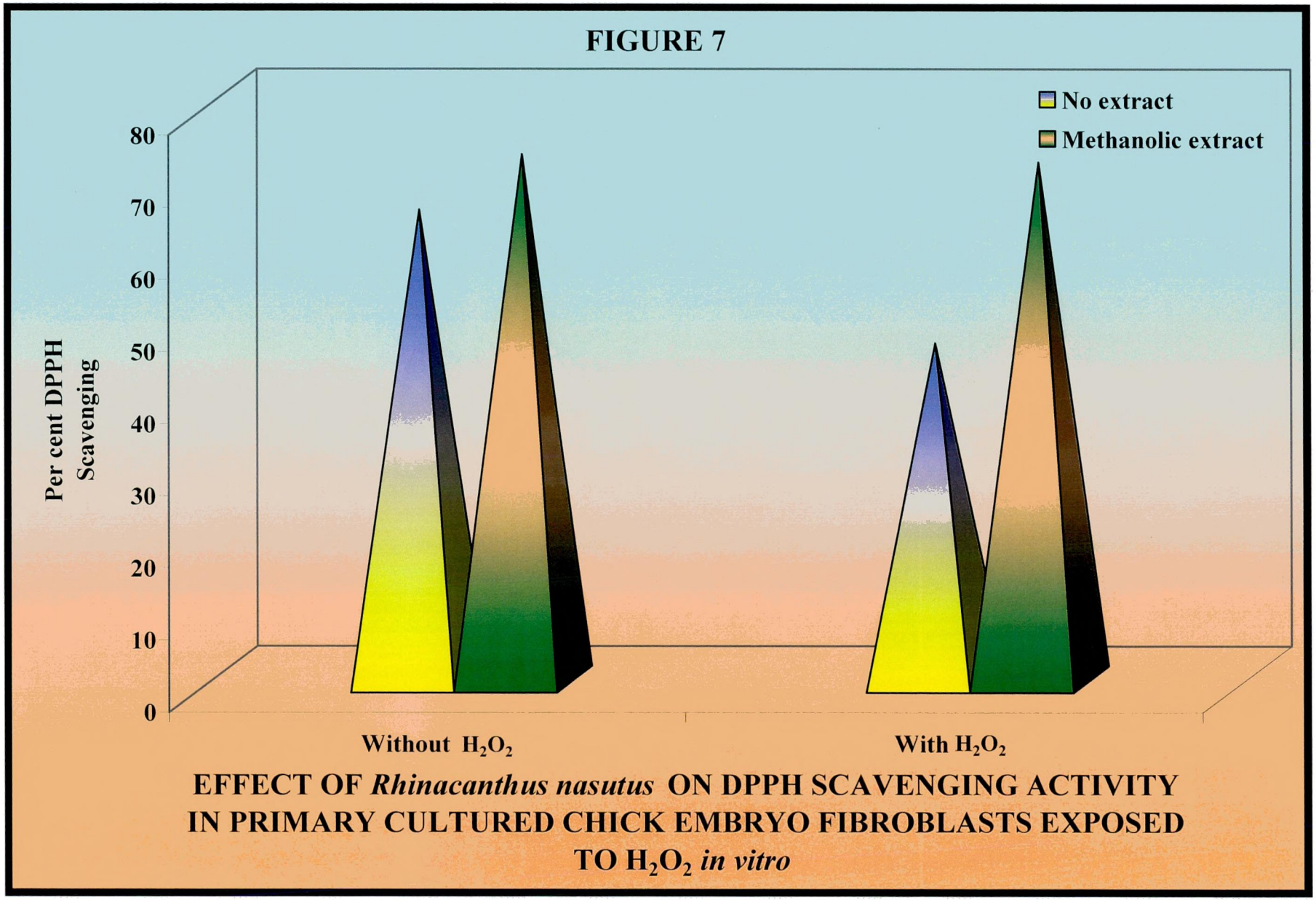
The total antioxidant potential of the cells treated with H_2O_2 in the presence and the absence of methanolic extract of *Rhinacanthus nasutus* leaves was assessed by the ability of the fibroblasts to scavenge the stable free radical DPPH and the per cent scavenging activity is presented in Figure 7. The DPPH scavenging ability decreased upon H_2O_2 insult. However, the methanolic extract addition elicited a better DPPH scavenging activity over the controls, which is indicative of the protective effect of *Rhinacanthus nasutus* leaves against oxidant induced damage.

LIPID PEROXIDATION

Lipid peroxidation is a free radical related process, which is potentially harmful because it is an uncontrolled, self-enhancing process, causing disruption of membranes, lipid and other cell components. Abnormally high levels of LPO can lead to the damage of cellular organelles and to oxidative stress (Mahboob *et al.*, 2005).

The oxidative stress imposed by H_2O_2 treatment was evident by the increased TBARS level of 82% in primary culture cells subjected to oxidant induced damage. Treatment with the methanolic extract of *Rhinacanthus nasutus* in H_2O_2 treated primary cells depressed the LPO in these cells suggesting the antioxidant protection of the leaf extracts.

FIGURE 7



EFFECT OF *Rhinacanthus nasutus* ON DPPH SCAVENGING ACTIVITY IN PRIMARY CULTURED CHICK EMBRYO FIBROBLASTS EXPOSED TO H_2O_2 *in vitro*

MORPHOLOGICAL CHANGES OBSERVED IN THE PRIMARY CELLS OF CHICK EMBRYO FIBROBLASTS

The morphological changes in chick embryo fibroblasts upon treatment with the standard oxidant hydrogen peroxide and the influence of methanolic extract of *Rhinacanthus nasutus* leaves on H_2O_2 induced oxidant insult was observed under phase contrast microscope.

In the present investigation, the morphological changes relating to apoptosis such as membrane blebbing, cell shrinkage accompanied by deformation and chromatin condensation were quantified per 100 cells in each of the treatment groups. The primary cells were stained with giemsa stain, the number of apoptotic cells to normal cells was counted and the values obtained are presented in Table XIII. The changes in the morphological features observed are shown in Plate 3.

TABLE XIII

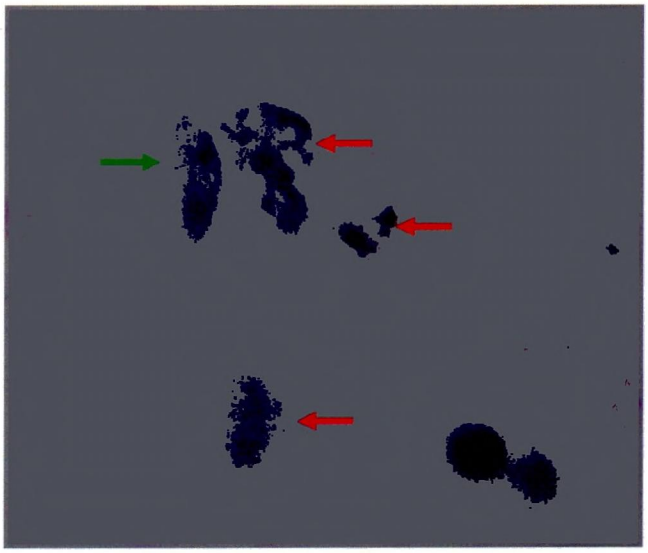
EFFECT OF *Rhinacanthus nasutus* LEAF EXTRACT ON THE MORPHOLOGICAL CHANGES IN CHICK EMBRYO FIBROBLASTS SUBJECTED TO OXIDATIVE STRESS AS DETERMINED BY GIEMSA STAINING

SAMPLE	No. of Normal cells / 100 cells		No. of Apoptotic cells / 100 cells	
	Control	H_2O_2 treated	Control	H_2O_2 treated
No extract	89	18	11	82
Methanolic extract	91	86	9	14

The values are means of triplicates

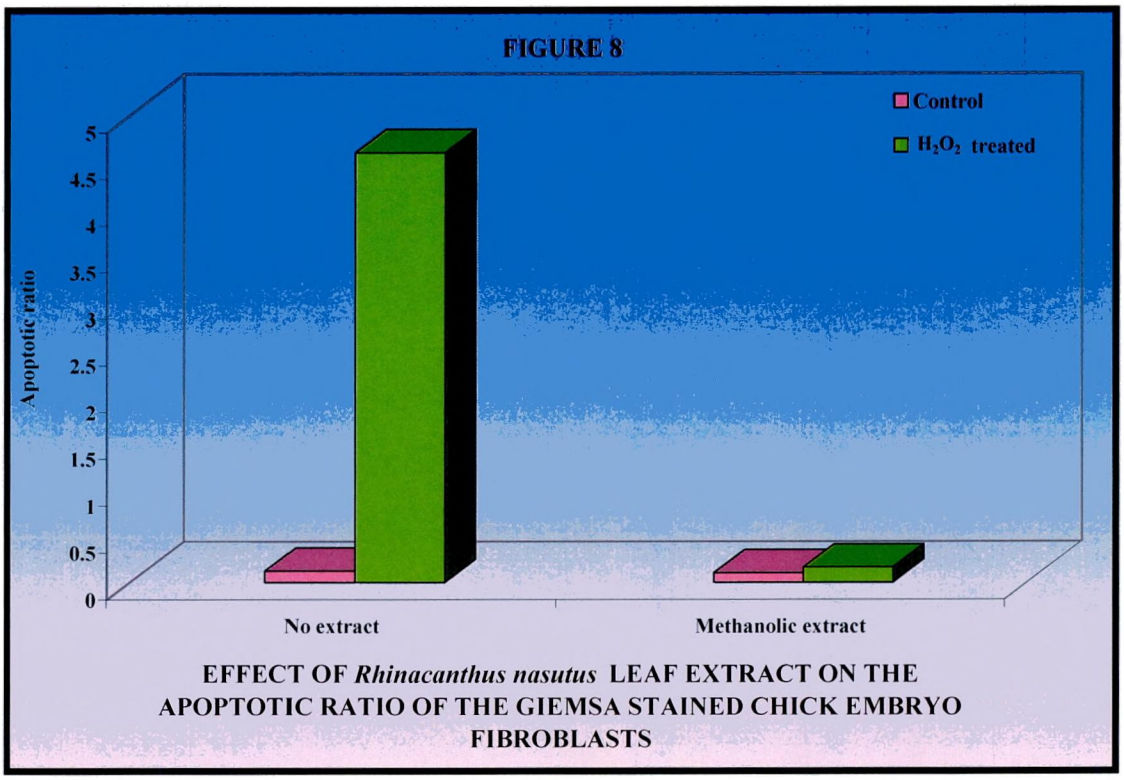
The number of apoptotic cells increased to higher levels in the oxidant treated groups. Administration of the methanolic extract of *Rhinacanthus nasutus* leaves drastically reduced the number of apoptosing cells.

PLATE 3



CHICK EMBRYO FIBROBLASTS STAINED WITH GIEMSA, SHOWING APOPTOTIC MORPHOLOGY OF MEMBRANE BLEBBING AND APOPTOTIC BODY ACCUMULATION

FIGURE 8



EFFECT OF *Rhinacanthus nasutus* LEAF EXTRACT ON THE APOPTOTIC RATIO OF THE GIEMSA STAINED CHICK EMBRYO FIBROBLASTS

The number of apoptosing cells to normal appearing cells was calculated for each group as proposed by Cantarella *et al.*, (2003). The ratios are expressed in Figure 8.

NUCLEAR CHANGES OBSERVED AFTER ETHIDIUM BROMIDE AND PROPIDIUM IODIDE STAINING

Nuclear changes, such as chromatin condensation and nuclear fragmentation are the confirmatory signs to any apoptotic studies. Ethidium bromide is a molecule that intercalates into nucleic acids and can be used to visualize the nuclear changes in apoptotic cells (Cury-Boaventura *et al.*, 2004). Ethidium bromide and propidium iodide staining methods were used to observe the nuclear and cellular changes in the cells undergoing apoptosis and the results obtained are depicted in Tables XIV and XV respectively.

TABLE XIV

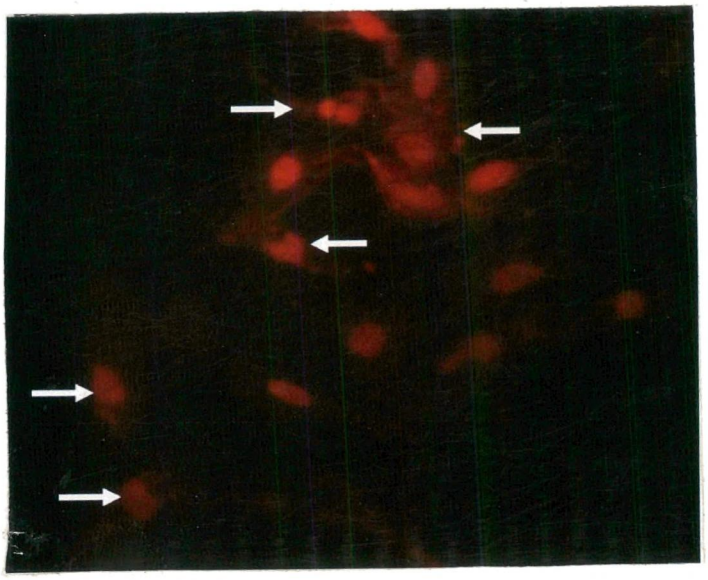
EFFECT OF *Rhinacanthus nasutus* LEAF EXTRACT ON THE NUCLEAR CHANGES IN CHICK EMBRYO FIBROBLASTS SUBJECTED TO OXIDATIVE STRESS AS DETERMINED BY EtBr STAINING

SAMPLE	No. of Normal cells / 100 cells		No. of Apoptotic cells / 100 cells	
	Control	H_2O_2 treated	Control	H_2O_2 treated
No extract	82	24	18	76
Methanolic extract	78	75	22	25

The values are means of triplicates.

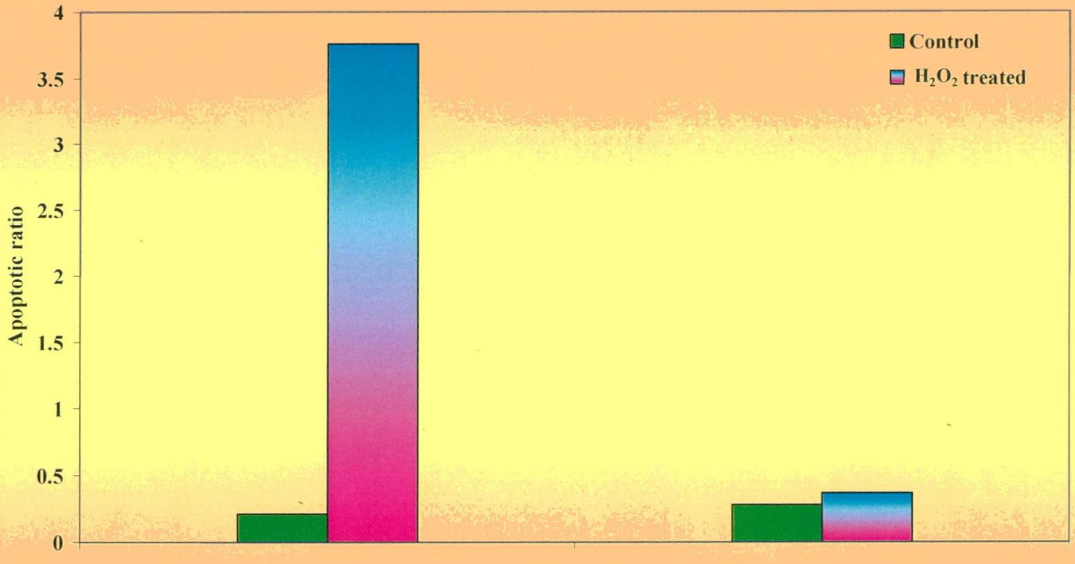
The apoptotic ratios of the EtBr and PI stained cells of chick embryo fibroblasts are presented in Figures 9 and 10 respectively.

PLATE 4



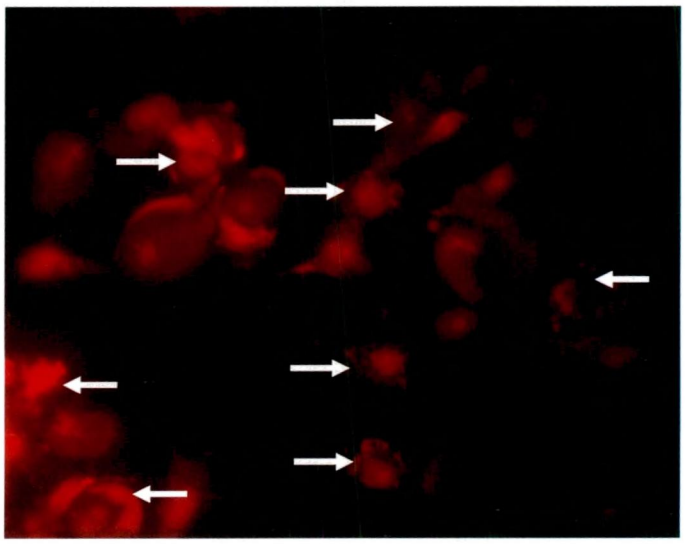
CHICK EMBRYO FIBROBLASTS STAINED WITH ETHIDIUM BROMIDE SHOWING NUCLEAR CHANGES OF APOPTOSIS

FIGURE 9



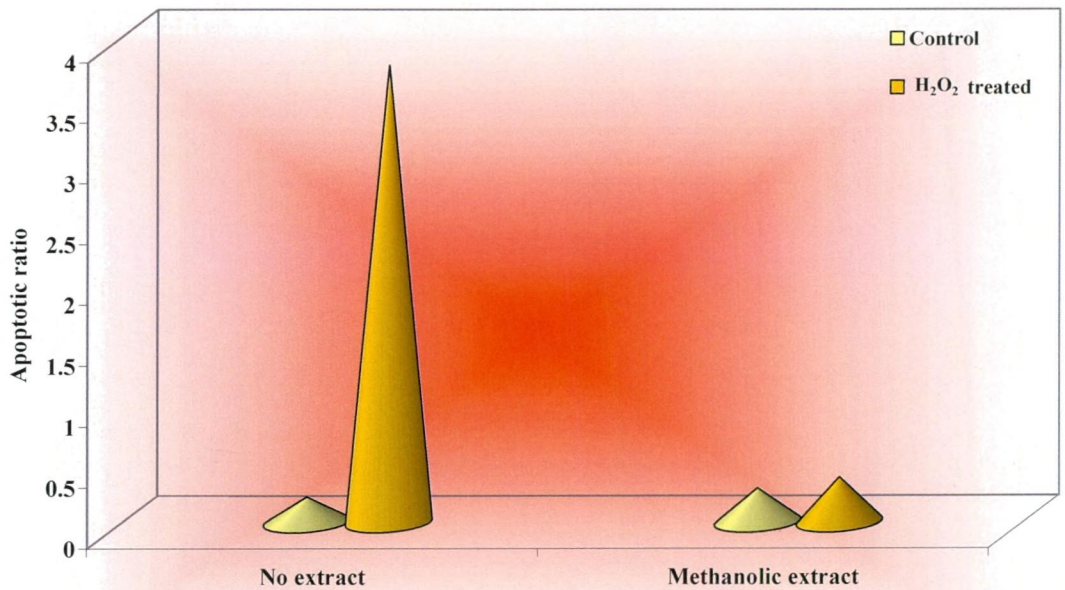
EFFECT OF *Rhinacanthus nasutus* LEAF EXTRACT ON THE APOPTOTIC RATIO OF THE EtBr STAINED CHICK EMBRYO FIBROBLASTS

PLATE 5



CHICK EMBRYO FIBROBLASTS STAINED WITH PROPIDIUM IODIDE SHOWING NUCLEAR AND CELLULAR CHANGES (APOPTOTIC BODIES) OF APOPTOSIS

FIGURE 10



EFFECT OF *Rhinacanthus nasutus* LEAF EXTRACT ON THE APOPTOTIC RATIO OF THE PI STAINED CHICK EMBRYO FIBROBLASTS

A steep increase in the number of cells undergoing apoptosis was observed in the ethidium bromide stained cells (Plate 4) when treated with H_2O_2, which was reverted by the presence of the leaf extracts, despite the oxidative injury imposed by H_2O_2. These results reveal that *Rhinacanthus nasutus* leaves can render protection against H_2O_2-induced cell death.

TABLE XV

EFFECT OF *Rhinacanthus nasutus* LEAF EXTRACT ON THE NUCLEAR CHANGES IN CHICK EMBRYO FIBROBLASTS SUBJECTED TO OXIDATIVE STRESS AS DETERMINED BY PROPIDIUM IODIDE STAINING

SAMPLE	No. of Normal cells / 100 cells		No. of Apoptotic cells / 100 cells	
	Control	H_2O_2 treated	Control	H_2O_2 treated
No extract	90	21	10	79
Methanolic extract	78	73	22	27

The values are means of triplicates

Propidium iodide staining is a routine parameter in most studies centering on apoptosis. The results of PI staining, as shown in Plate 5, indicated that upon H_2O_2 exposure, an increased number of apoptotic cells were observed. This number decreased sharply when exposed along with the methanolic extract of *Rhinacanthus nasutus* leaves.

CELL VIABILITY

MTT ASSAY

Cell viability assay gives a clear picture to the effect of the treatment with the leaf extract. The ability of the cells to convert MTT into formazan derivative was measured as an indicator of viability and the per cent viability was calculated. The values obtained are listed in Table XVI.

TABLE XVI

EFFECT OF *Rhinacanthus nasutus* LEAF EXTRACT ON CELL VIABILITY (MTT ASSAY) OF CHICK EMBRYO FIBROBLASTS SUBJECTED TO OXIDATIVE STRESS

SAMPLE	CELL SURVIVAL (%)	
	Control	H_2O_2 treated
No extract	100	68
Methanolic extract	145	129

The Values are means of triplicates.
The Values of the negative (untreated) control group were fixed as 100% viability and the per cent viabilities in the other groups were calculated relative to this

A marked decrease in the viability of oxidant stressed chick embryo fibroblasts was observed in the MTT assay. The methanolic extract of Rhinacanthus nasutus caused an increased survival of cells over the control group, indicating that the extract can counteract even the basal cell damage and cause maximum viability. An improvement in the extent of cell survival in the leaf extract treated group reiterates the protective influence of *Rhinacanthus nasutus* leaves on the apoptotic cell death induced by H_2O_2 in the primary cultured chick embryo fibroblasts.

SRB ASSAY

Cytotoxicity of the primary cultured cells as determined by the SRB assay (Table XVII) showed a drastic reduction in the cell viability after treatment with H_2O_2. The cell viability increased to a greater extent in the methanolic extract treated cells even in the presence of apoptosis- inducing stress. The per cent cell survival obtained for the assay revealed that the *Rhinacanthus nasutus* leaf extract exhibited good anti-apoptotic property and protected the primary cultured chick embryo fibroblasts against death caused by H_2O_2 induced oxidative stress.

TABLE XVII

EFFECT OF *Rhinacanthus nasutus* LEAF EXTRACT ON CELL VIABILITY (SRB ASSAY) OF CHICK EMBRYO FIBROBLASTS SUBJECTED TO OXIDATIVE STRESS

SAMPLE	CELL SURVIVAL (%)	
	Control	H_2O_2 treated
No extract	100	55
Methanolic extract	83	68

The values are means of triplicates.
The values of the negative (untreated) control group were fixed as 100% viability and the per cent viabilities in the other groups were calculated relative to this.

DNA FRAGMENTATION

Apoptosis is well characterized by DNA fragmentation. In the present study, the fibroblasts were treated with plant extract and / or H_2O_2 and the extent of DNA fragmentation was analysed by agarose gel electrophoresis. The migration pattern obtained is shown in Plate 6.

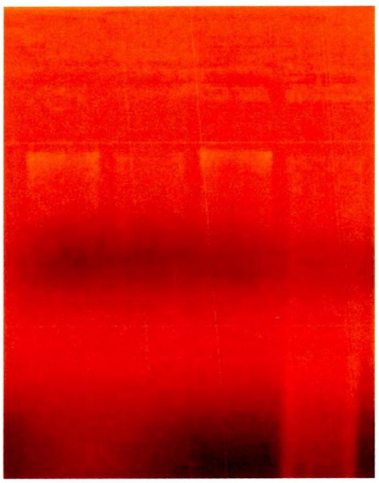
Exposure of the cells to H_2O_2 caused substantial DNA damage as evidenced by a lighter band (Lane 2). *Rhinacanthus nasutus* leaf extract by itself, did not cause significant damage (Lane 3). Additionally, the leaf extract decreased the extent of DNA damage caused by H_2O_2 (Lane 4). This was quantified using a digital gel documentation software (Alpha Ease FC of Alpha DigiDoc 1201) and the Integrated Density Values (IDV) obtained are presented in Table XVIII

TABLE XVIII

INTEGRATED DENSITY VALUES (IDV) OF THE BANDS IN THE AGAROSE GEL OF DNA FRAGMENTATION ASSAY

SAMPLE	IDV OF THE BANDS	
	Control	H_2O_2 treated
No extract	96250	69003
Methanolic extract	97911	96492

PLATE 6



DNA FRAGMENTATION PATTERN IN CHICK EMBRYO FIBROBLASTS

Lane 1 – Untreated group

Lane 2 – H_2O_2 treated group

Lane 3 – *Rhinacanthus nasutus* leaf extract treated group

Lane 4 – H_2O_2 + *Rhinacanthus nasutus* leaf extract treated group

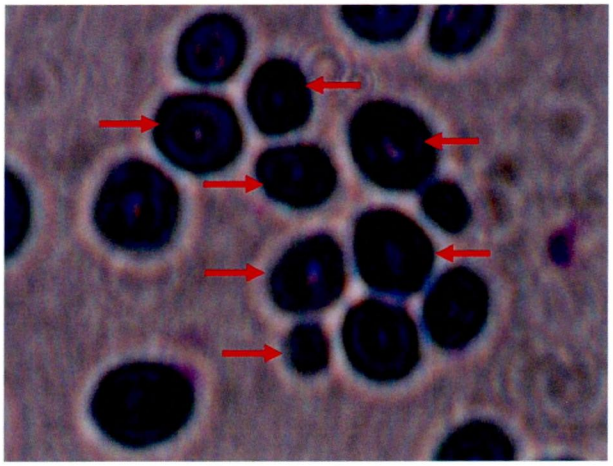
In the present study, besides enumerating the antioxidant properties of *Rhinacanthus nasutus* leaves, an effort was also made to validate alternative experimental systems that can replace/minimize the live animals used in biological research. In this pursuit, the goat liver slices and chick embryo fibroblasts were used. Even though the liver was obtained from the slaughterhouse and a whole set of analyses could be carried out in a small piece of liver, the source of the tissue involved the sacrifice of an animal. Also, in the case of the chick embryo fibroblasts, even though the cells could be multiplied in culture, the number of divisions are limited as the cells are untransformed (primary) cells. Thus, every time a new culture is started, an embryo is sacrificed.

In order to avoid this, and to have a reliable test system, our research group has been focusing on validating lower organisms as reliable test systems for analyzing the antioxidant activity of plant extracts. Previous studies in our laboratory (Sreeja, 2006; Sathya, 2006; Vijayachandran, 2007; Malathy, 2008) have shown and validated *Saccharomyces cerevisiae* cells as a very reliable model. In the present study, the effects of *Rhinacanthus nasutus* leaf extracts were studied on the apoptotic events induced in *Saccharomyces cerevisiae* cells by hydrogen peroxide. The observations made are presented below.

MORPHOLOGICAL CHANGES OBSERVED IN *Saccharomyces cerevisiae* CELLS

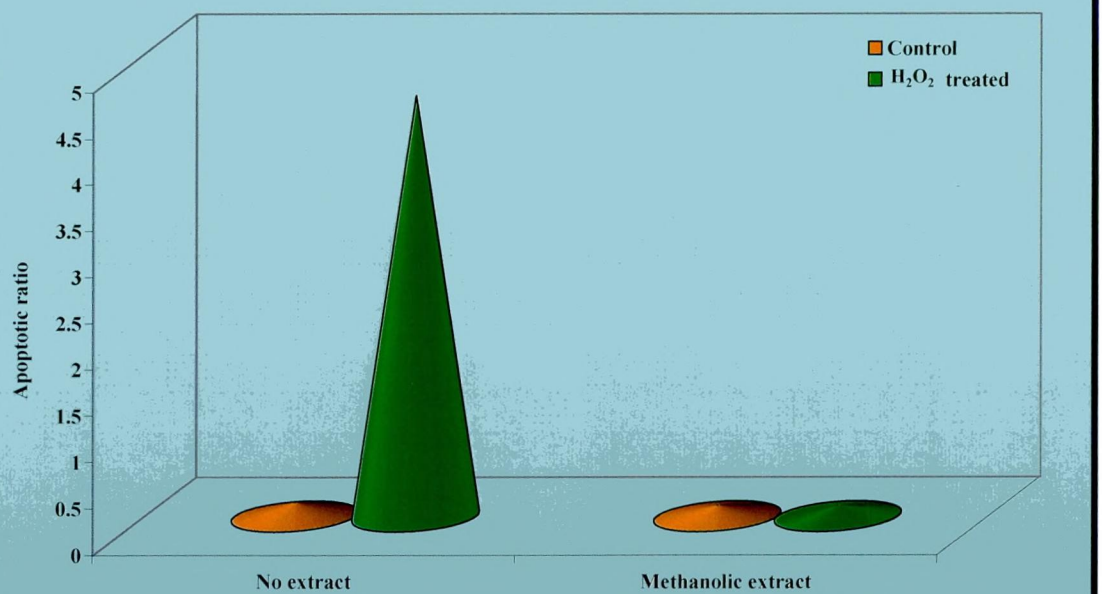
In order to understand the nature of the cellular death process and the molecular events involved in the process, studies were conducted on morphological and nuclear changes that occur during apoptotic death. The characteristic morphological changes in apoptotic cells were analysed by giemsa staining in the presence and the absence of H_2O_2 and plant extract. The numbers of apoptotic and non-apoptotic cells were counted under phase contrast microscope and the results are listed in Table XIX.

PLATE 7



Saccharomyces cerevisiae **CELLS STAINED WITH GIEMSA, SHOWING APOPTOTIC MORPHOLOGY OF MEMBRANE BLEBBING AND APOPTOTIC BODY ACCUMULATION**

FIGURE 11



EFFECT OF *Rhinacanthus nasutus* LEAF EXTRACT ON THE APOPTOTIC RATIO OF THE GIEMSA STAINED *Saccharomyces cerevisiae* CELLS

TABLE XIX

EFFECT OF *Rhinacanthus nasutus* LEAF EXTRACT ON THE MORPHOLOGICAL CHANGES IN *Saccharomyces cerevisiae* CELLS SUBJECTED TO OXIDATIVE STRESS AS DETERMINED BY GIEMSA STAINING

SAMPLE	No. of Normal cells / 100 cells		No. of Apoptotic cells / 100 cells	
	Control	H_2O_2 treated	Control	H_2O_2 treated
No extract	87	18	13	82
Methanolic extract	90	89	10	11

The values are means of triplicates

The ratios of apoptosing cells to normal cells were calculated in the giemsa stained treatment groups. The values are schematically represented in Figure 11.

Cells treated with hydrogen peroxide showed well-defined apoptotic morphology (Plate 7), which was strongly hindered by the treatment with the methanolic extract of *Rhinacanthus nasutus* leaves. Thus, it can be deduced the candidate plant possesses components that can counteract H_2O_2–induced death in yeast cells.

NUCLEAR CHANGES

The nuclear changes were visualized in the yeast cells subjected to oxidative stress in the presence and the absence of leaf extracts. The number of yeast cells showing nuclear apoptotic morphology was counted in ethidium bromide staining in each treatment group and the results are given in Table XX.

The ratios of apoptosing to normal cells were calculated and the values obtained are expressed in Figure 12. Hydrogen peroxide caused a significant proportion of the cells to commit to apoptosis. However, treatment with the

methanolic extract of *Rhinacanthus nasutus* leaves was highly efficient in combating this cell death due to oxidative damage by H_2O_2 exposure (Plate 8).

TABLE XX

EFFECT OF *Rhinacanthus nasutus* LEAF EXTRACT ON NUCLEAR MORPHOLOGY OF *Saccharomyces cerevisiae* CELLS SUBJECTED TO OXIDATIVE STRESS AS DETERMINED BY EtBr STAINING

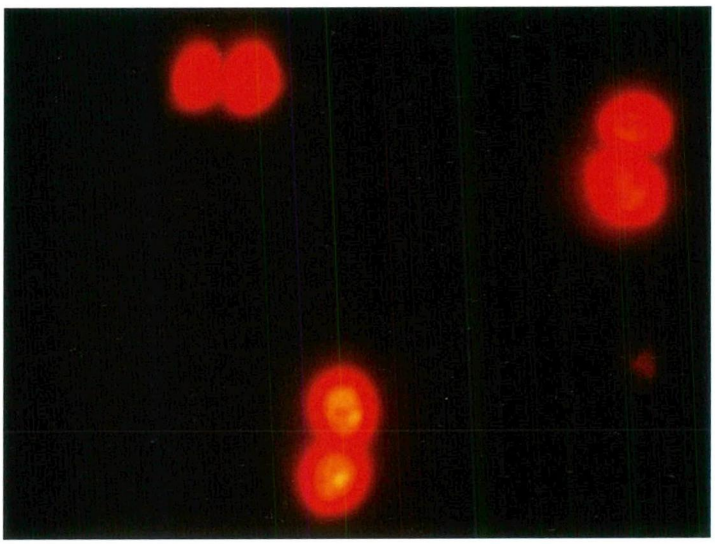
SAMPLE	No. of Normal cells / 100 cells		No. of Apoptotic cells / 100 cells	
	Control	H_2O_2 treated	Control	H_2O_2 treated
No extract	88	24	12	76
Methanolic extract	86	83	14	17

The values are means of triplicates

The apoptosis induction in *Saccharomyces cerevisiae* cells by H_2O_2 and its modulation in the presence of the methanolic extract of *Rhinacanthus nasutus* leaves was also quantified using propidium iodide staining method. The number of normal and dying cells in each treatment group was counted after PI staining and the values obtained are depicted in Table XXI. The ratios of apoptosing to normal cells were also calculated and represented in Figure 13.

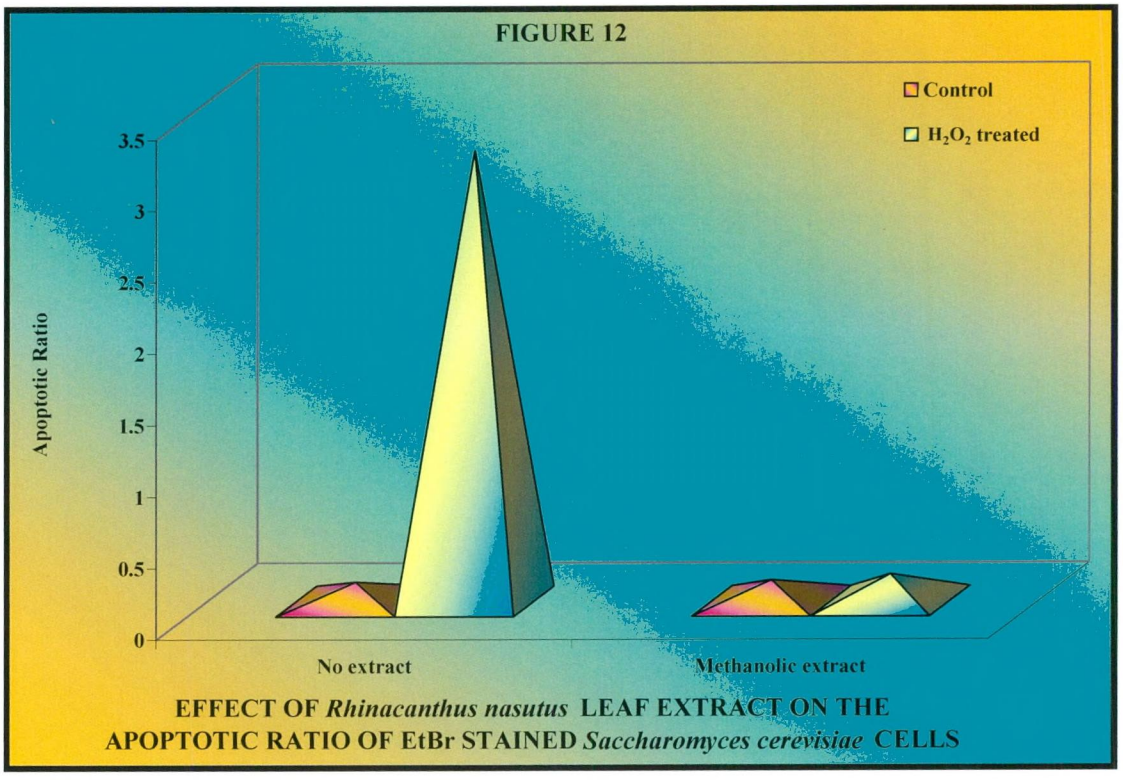
As deducible from the table values, the administration of hydrogen peroxide caused a very high number of yeast cells to become permeable to PI, indicating oxidation-induced apoptosis (Plate 9). The number of cells committed to apoptosis upon H_2O_2 exposure declined sharply in the presence of the methanolic extract of *Rhinacanthus nasutus* leaves.

PLATE 8



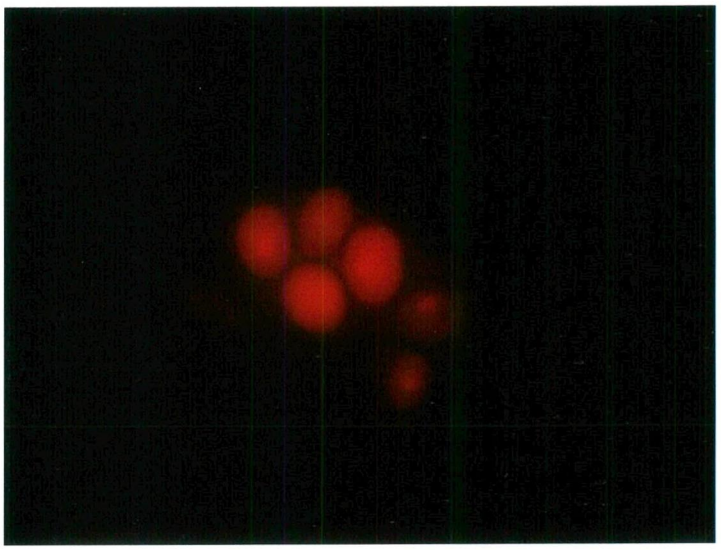
Saccharomyces cerevisiae CELLS STAINED WITH ETHIDIUM BROMIDE SHOWING NUCLEAR CHANGES OF APOPTOSIS

FIGURE 12



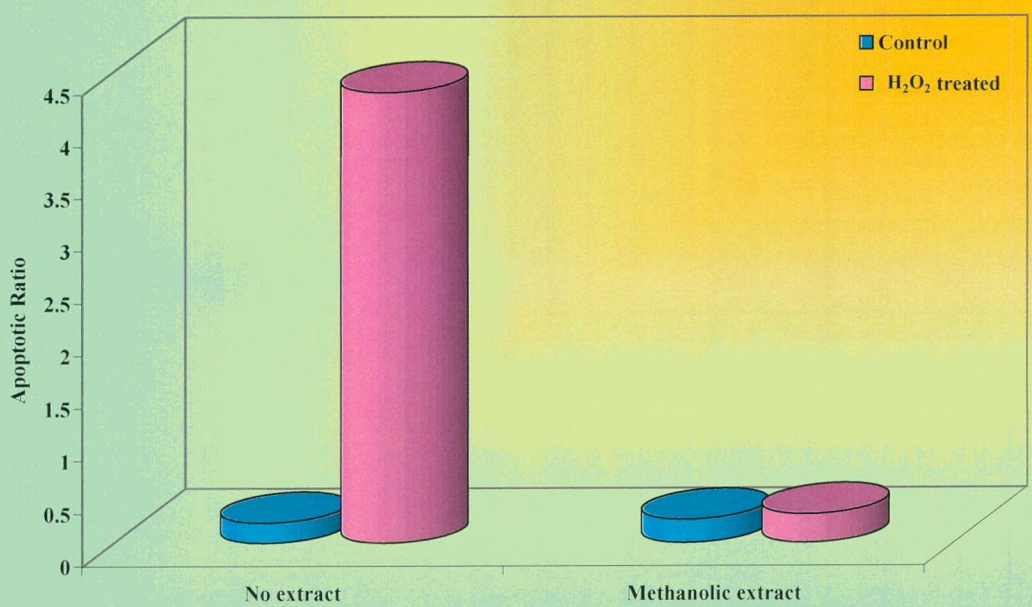
EFFECT OF *Rhinacanthus nasutus* LEAF EXTRACT ON THE APOPTOTIC RATIO OF EtBr STAINED *Saccharomyces cerevisiae* CELLS

PLATE 9



Saccharomyces cerevisiae CELLS STAINED WITH PROPIDIUM IODIDE SHOWING NUCLEAR CHANGES OF APOPTOSIS

FIGURE 13



EFFECT OF *Rhinacanthus nasutus* LEAF EXTRACT ON THE APOPTOTIC RATIO OF PI STAINED *Saccharomyces cerevisiae* CELLS

TABLE XXI

EFFECT OF *Rhinacanthus nasutus* LEAF EXTRACT ON NUCLEAR CHANGES IN *Saccharomyces cerevisiae* CELLS SUBJECTED TO OXIDATIVE STRESS AS DETERMINED BY PROPIDIUM IODIDE STAINING

SAMPLE	No. of Normal cells / 100 cells		No. of Apoptotic cells / 100 cells	
	Control	H_2O_2 treated	Control	H_2O_2 treated
No extract	84	19	16	81
Methanolic extract	82	79	18	21

The values are means of triplicates

These results suggest that the *Rhinacanthus nasutus* leaves can be used as a supplement to withstand the toxicity associated with cytotoxic agents.

CELL VIABILITY

MTT ASSAY

The cytotoxicity was assayed by MTT after treatment of *Saccharomyces cerevisiae* cells with leaf extract and/or H_2O_2. The results are tabulated below.

TABLE XXII

EFFECT OF *Rhinacanthus nasutus* LEAF EXTRACT ON CELL VIABILITY OF *Saccharomyces cerevisiae* CELLS SUBJECTED TO OXIDATIVE STRESS AS DETERMINED BY THE MTT ASSAY

SAMPLE	CELL SURVIVAL (%)	
	Control	H_2O_2 treated
No extract	100	54
Methanol extract	90	79

The values are means of triplicates.
The values of the negative (untreated) control group were fixed as 100% viability and the per cent viabilities in the other groups were calculated relative to this.

It is evident from the values presented in Table XXII that the administration of H_2O_2 drastically brought down the viability of *Saccharomyces cerevisiae* cells. The cytotoxicity of H_2O_2 was effectively counteracted by the methanolic extract of *Rhinacanthus nasutus* leaves.

SRB ASSAY

Sulphorhodamine B assay was also performed to assess the cell survival. The results of this assay (Table XXIII) showed a similar trend as that observed in the MTT assay.

TABLE XXIII

EFFECT OF *Rhinacanthus nasutus* LEAF EXTRACT ON CELL VIABILITY OF *Saccharomyces cerevisiae* CELLS SUBJECTED TO OXIDATIVE STRESS AS DETERMINED BY THE SRB ASSAY

SAMPLE	CELL SURVIVAL (%)	
	Control	H_2O_2 treated
No extract	100	54
Methanol extract	96	75

The values are means of triplicates.
The values of the negative (untreated) control group were fixed as 100% viability and the per cent viabilities in the other groups were calculated relative to this.

The results obtained with MTT and SRB assays, thus, confirmed the protective effects of *Rhinacanthus nasutus* leaves against oxidative stress- induced cell death in yeast cells.

DNA FRAGMENTATION

DNA fragmentation in apoptotic yeast cells were assayed using diphenylamine in a colorimetric assay and the per cent extent of fragmentation obtained are tabulated in Table XXIV.

TABLE XXIV

EFFECT OF *Rhinacanthus nasutus* LEAF EXTRACT ON DNA FRAGMENTATION IN *Saccharomyces cerevisiae* CELLS SUBJECTED TO OXIDATIVE STRESS

SAMPLE	EXTENT OF DNA DAMAGE (%)	
	Control	H_2O_2 treated
No extract	68	85
Methanolic extract	61	77

The values are means of triplicates

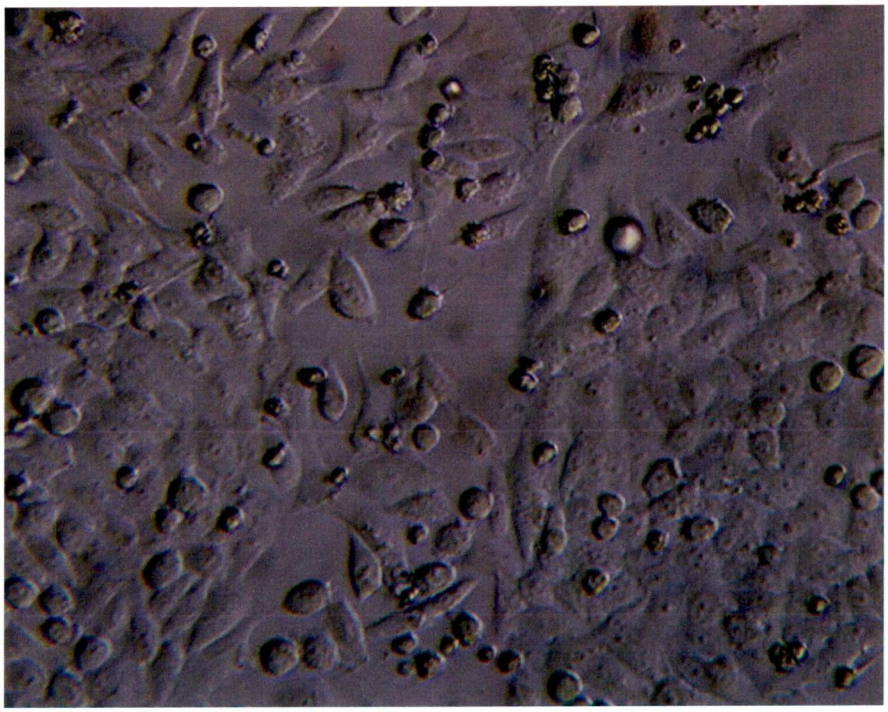
Earlier studies done in our laboratory (Sreeja, 2006; Sathya, 2006) implicate that H_2O_2 did not cause significant DNA fragmentation in *Saccharomyces cerevisiae* cells as followed by gel electrophoresis method.

Therefore, in the present study, DNA fragmentation was followed with the extent of colour formation with DPA. Exposure of H_2O_2 to the yeast cells caused significant DNA damage. The co-administration of the methanolic extract of *Rhinacanthus nasutus* leaves reduced the extent of DNA damage. However, the levels did not reach that observed in the control group.

ANTI-APOPTOTIC EFFECTS OF *Rhinacanthus nasutus* LEAF EXTRACT IN ETOPOSIDE INDUCED STRESS IN Hep2 CELLS

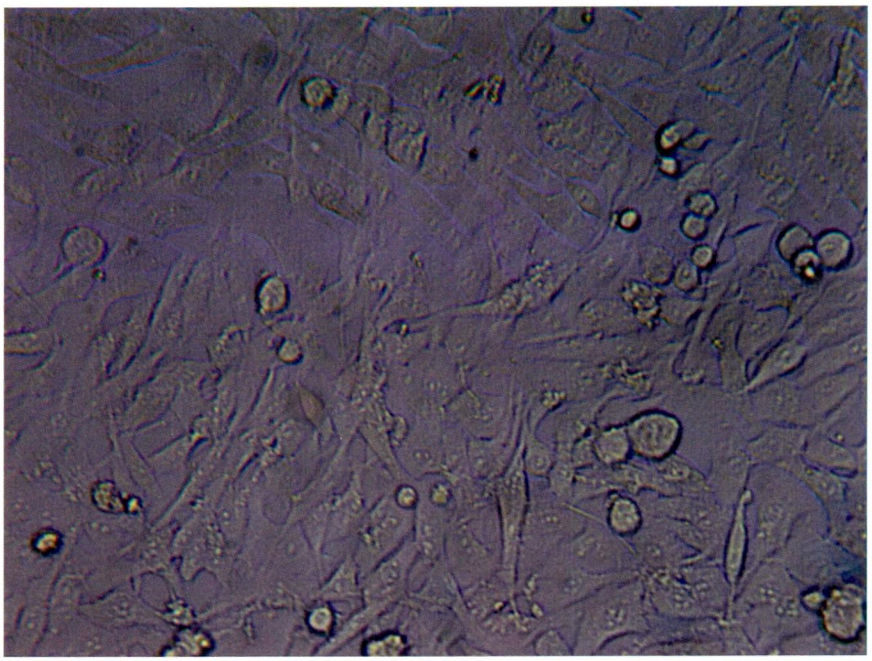
It is evident from the results obtained in the anti-apoptotic studies on chick embryo fibroblasts and *Saccharomyces cerevisiae* cells that *Rhinacanthus nasutus* can render protection to the untransformed (normal) cells against oxidative stress. As the next step of the study, it was felt imperative to study the effect of the leaf extract on transformed cells subjected to oxidative stress. For this purpose, Hep2 cell line (human laryngeal carcinoma) was employed (Plate 10).

PLATE 10a



Hep2 CELLS 24 HOURS AFTER SEEDING

PLATE 10b



Hep2 CELLS AT CONFLUENCE

Oxidative stress was imposed using a standard chemotherapeutic drug, etoposide, which is known to cause apoptotic death in cancer cells via an oxidative mechanism (Sermeus *et al.*, 2008). Apoptotic changes were observed as for the earlier test systems and the results are presented below.

MORPHOLOGICAL CHANGES

The morphological changes observed in giemsa stained Hep2 cells are depicted in Table XXV.

TABLE XXV

EFFECT OF *Rhinacanthus nasutus* LEAF EXTRACT ON THE MORPHOLOGICAL CHANGES IN Hep2 CELLS SUBJECTED TO OXIDATIVE STRESS AS DETERMINED BY GIEMSA STAINING

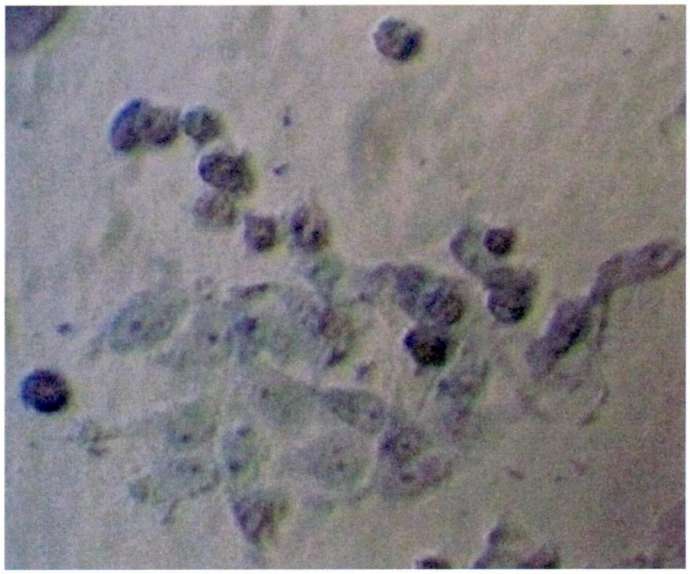
SAMPLE	No. of Normal cells / 100 cells		No. of Apoptotic cells / 100 cells	
	Control	Etoposide treated	Control	Etoposide treated
No extract	82	33	18	67
Methanolic extract	65	37	35	63

The values are means of triplicates

Addition of etoposide resulted in a marked increase in the proportion of apoptosing Hep2 cells. Surprisingly, the methanolic extract of *Rhinacanthus nasutus* leaf extract also caused an increase in the number of Hep2 cells committed to apoptosis (Plate 11). When the cells were exposed to etoposide and the leaf extract, the extent of cell death was comparable to that induced by etoposide alone, suggesting that *Rhinacanthus nasutus* leaf extract does not influence the extent of apoptosis induced by etoposide.

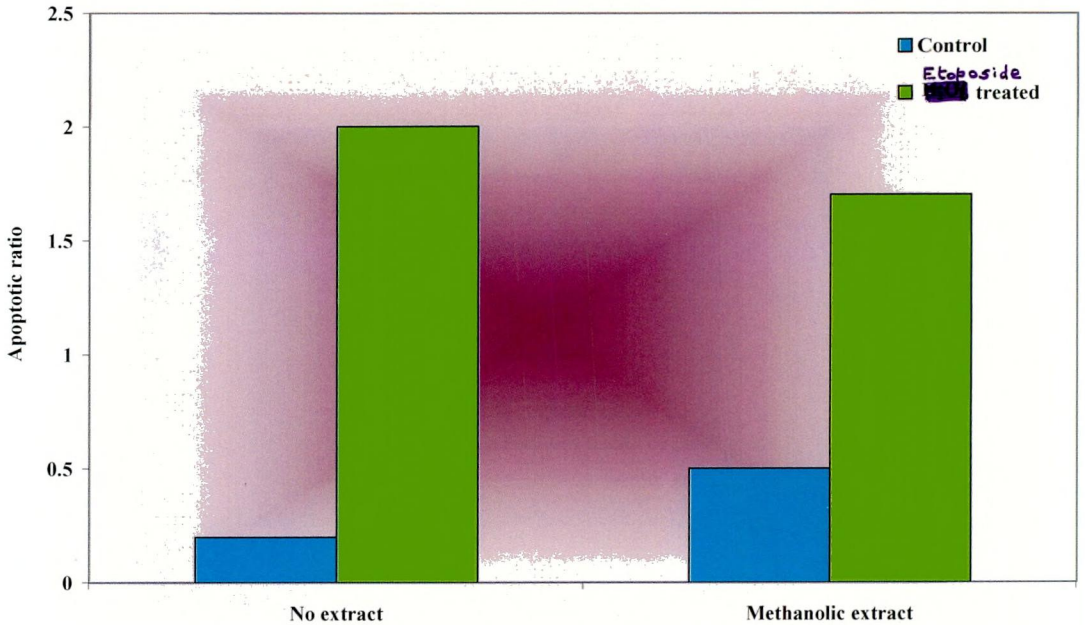
This was clearly evident when the ratio of apoptosing to normal cells was calculated in each treatment group, as depicted in Figure 14.

PLATE 11



Hep2 CELLS STAINED WITH GIEMSA, SHOWING APOPTOTIC MORPHOLOGY OF MEMBRANE BLEBBING AND APOPTOTIC BODY ACCUMULATION

FIGURE 14



EFFECT OF *Rhinacanthus nasutus* LEAF EXTRACT ON THE APOPTOTIC RATIO OF GIEMSA STAINED Hep2 CELLS

NUCLEAR CHANGES

The apoptosis induction in Hep2 cells by etoposide and its modulation in the presence of the methanolic extract of *Rhinacanthus nasutus* leaves was quantified using ethidium bromide and propidium iodide staining. The numbers of normal and dying cells in each treatment group after staining are tabulated in Tables XXVI and XXVII respectively. The results exhibited a trend similar to that obtained with giemsa staining.

TABLE XXVI

EFFECT OF *Rhinacanthus nasutus* LEAF EXTRACT ON NUCLEAR MORPHOLOGY OF Hep2 CELLS SUBJECTED TO OXIDATIVE STRESS AS DETERMINED BY EtBr STAINING

SAMPLE	No. of Normal cells / 100 cells		No. of Apoptotic cells / 100 cells	
	Control	Etoposide treated	Control	Etoposide treated
No extract	95	32	5	68
Methanolic extract	71	41	29	59

The values are means of triplicates.

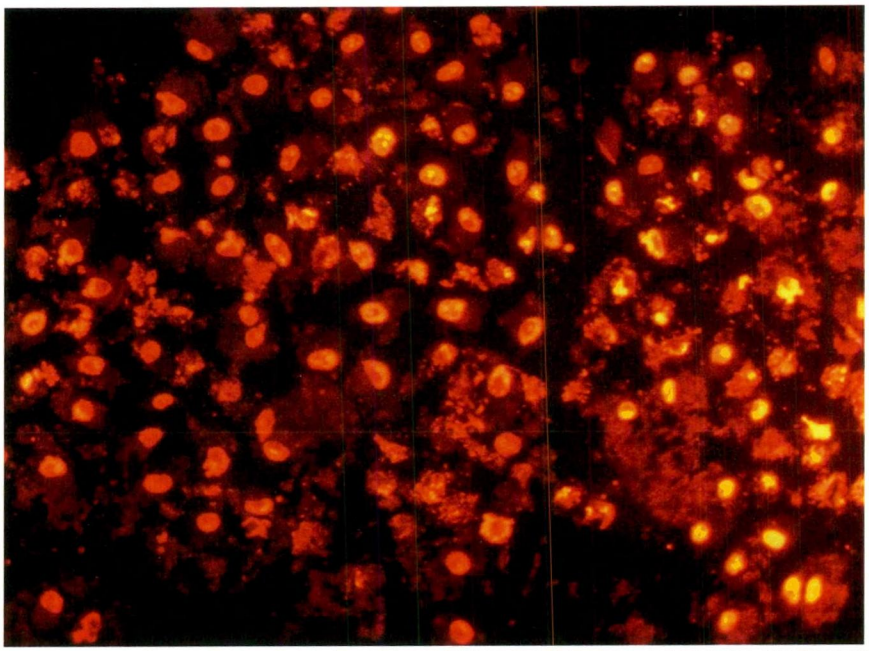
TABLE XXVII

EFFECT OF *Rhinacanthus nasutus* LEAF EXTRACT ON NUCLEAR CHANGES IN Hep2 CELLS SUBJECTED TO OXIDATIVE STRESS AS DETERMINED BY PROPIDIUM IODIDE STAINING

SAMPLE	No. of Normal cells / 100 cells		No. of Apoptotic cells / 100 cells	
	Control	Etoposide treated	Control	Etoposide treated
No extract	94	34	6	66
Methanolic extract	69	46	31	54

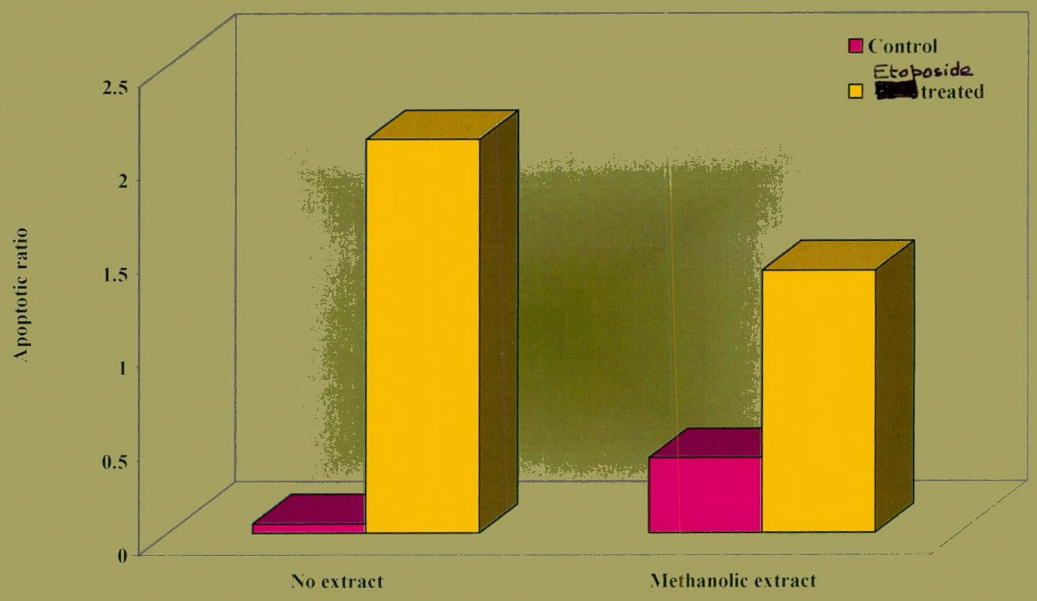
The values are means of triplicates

PLATE 12



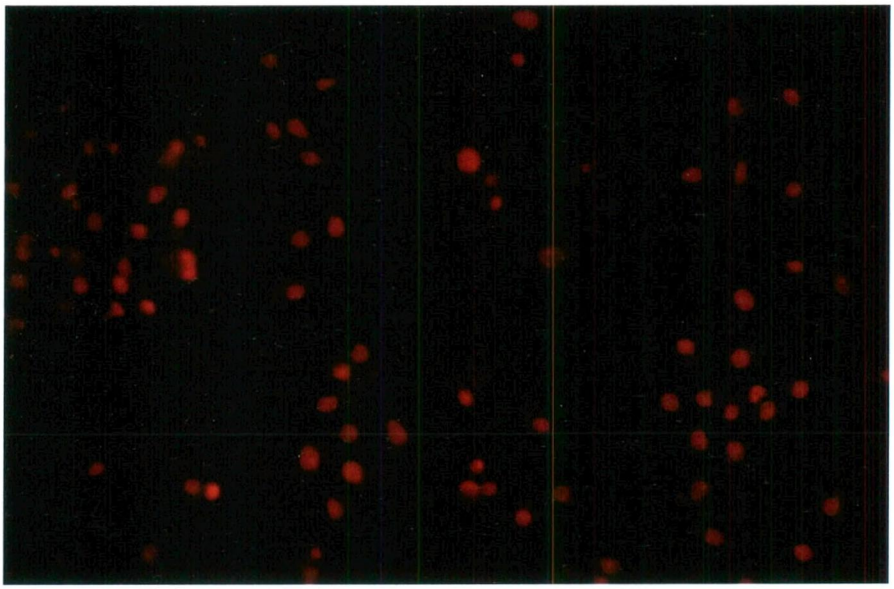
Hep2 CELLS STAINED WITH ETHIDIUM BROMIDE SHOWING NUCLEAR CHANGES OF APOPTOSIS

FIGURE 15



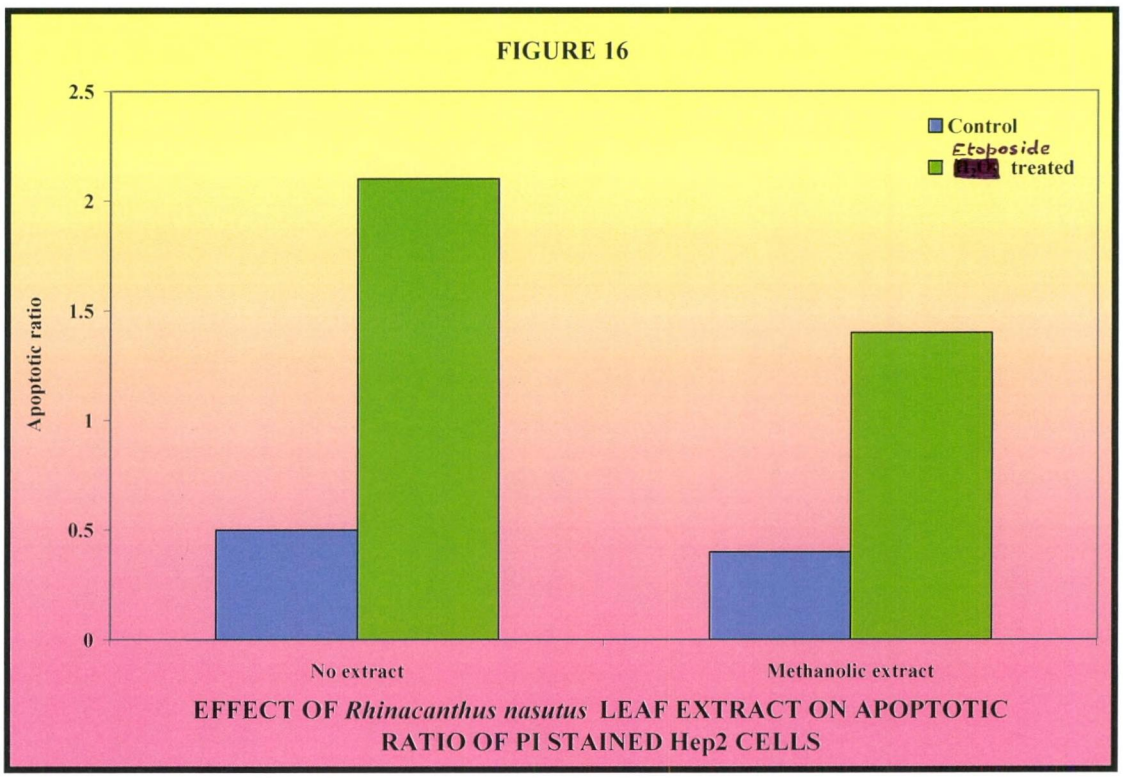
EFFECT OF *Rhinacanthus nasutus* LEAF EXTRACT ON APOPTOTIC RATIO OF EtBr STAINED Hep2 CELLS

PLATE 13



Hep2 CELLS STAINED WITH PROPIDIUM IODIDE SHOWING NUCLEAR AND CELLULAR CHANGES (APOPTOTIC BODIES) OF APOPTOSIS

FIGURE 16



EFFECT OF *Rhinacanthus nasutus* LEAF EXTRACT ON APOPTOTIC RATIO OF PI STAINED Hep2 CELLS

The ratios of apoptosing to normal cells were also calculated and the ratios are presented in Figures 15 and 16 respectively. Plates 12 and 13 are photographic records of the apoptosing cells in each of the staining methods.

DAPI specifically stains DNA and the extent of nuclear changes observed during etoposide induced apoptosis after staining in the different treatment groups of Hep2 cells are presented in Table XXVIII. The ratio of apoptosing to normal cells is illustrated in Figure 17.

TABLE XXVIII

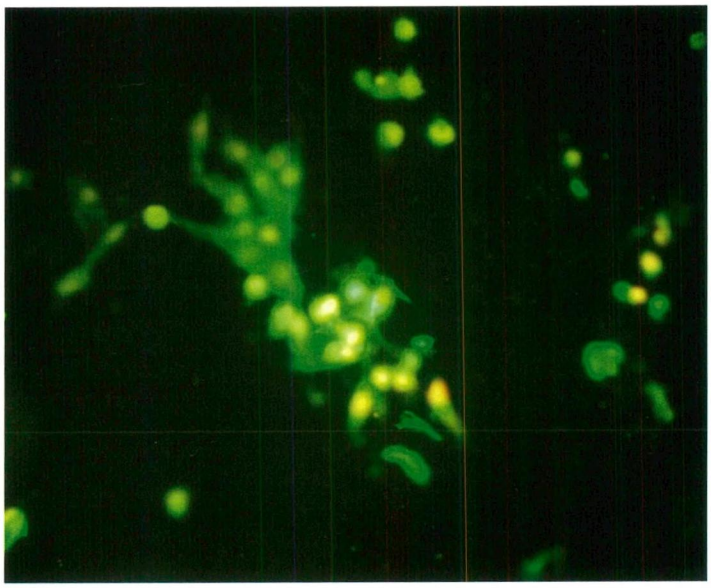
EFFECT OF *Rhinacanthus nasutus* LEAF EXTRACT ON NUCLEAR CHANGES IN Hep2 CELLS SUBJECTED TO OXIDATIVE STRESS AS DETERMINED BY DAPI STAINING

SAMPLE	No. of Normal cells / 100 cells		No. of Apoptotic cells / 100 cells	
	Control	Etoposide treated	Control	Etoposide treated
No extract	94	36	6	64
Methanolic extract	66	39	34	61

The values are means of triplicates.

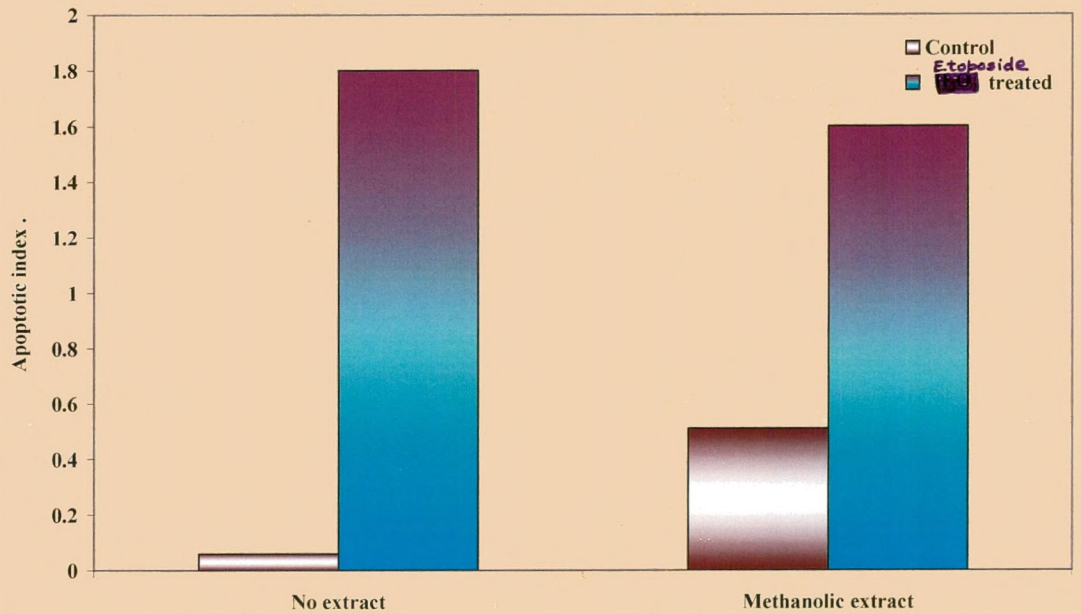
Etoposide treatment caused a significant proportion of the cells to show nuclear apoptotic morphology (Plate 13) as evident by their increased permeability to DAPI. The leaf extract (methanol) of *Rhinacanthus nasutus* also induced increased cell death, by itself, in Hep2 cells. However, the extract did not influence the extent of death induced by etoposide in Hep2 cells.

PLATE 14



Hep2 CELLS STAINED WITH DAPI SHOWING NUCLEAR CHANGES OF APOPTOSIS

FIGURE 17



EFFECT OF *Rhinacanthus nasutus* LEAF EXTRACT ON APOPTOTIC INDEX OF DAPI STAINED Hep2 CELLS

CELL SURVIVAL

MTT ASSAY

The cytotoxicity was assayed by MTT after treatment of Hep2 cells with leaf extract and/or etoposide. The results are tabulated below. The results presented in Table XXIX demonstrate the cytotoxic effect of etoposide administration to the Hep2 cells, which exerts a steep decline in the cell survival. The methanolic extract of *Rhinacanthus nasutus* also caused a marked decrease in the viability of Hep2 cells, which decreased further in the presence of etoposide.

TABLE XXIX

EFFECT OF *Rhinacanthus nasutus* LEAF EXTRACT ON CELL VIABILITY OF Hep2 CELLS SUBJECTED TO OXIDATIVE STRESS AS DETERMINED BY THE MTT ASSAY

SAMPLE	CELL SURVIVAL (%)	
	Control	Etoposide treated
No extract	100	33
Methanol extract	64	47

The values are means of triplicates.
The values of the negative (untreated) control group were fixed as 100% viability and the per cent viabilities in the other groups were calculated relative to this.

SRB ASSAY

The effects of the leaf extracts on the survival of Hep2 laryngeal carcinoma cells exposed to etoposide was also quantified using the SRB assay. The per cent cell viabilities in the various treatment groups are tabulated in Table XXX. The results revealed that the treatment with etoposide, both in the presence and the absence of *Rhinacanthus nasutus* leaf extract, caused a steep decline in the percent cell survival, which were on par with one another. It was also observed that the *Rhinacanthus nasutus* leaf extract, by itself (in the absence of etoposide) also caused a decrease in the Hep2 cell survival.

TABLE – XXX

EFFECT OF *Rhinacanthus nasutus* LEAF EXTRACT ON CELL VIABILITY OF Hep2 CELLS SUBJECTED TO OXIDATIVE STRESS AS DETERMINED BY THE SRB ASSAY

SAMPLE	CELL SURVIVAL (%)	
	Control	Etoposide treated
No extract	100	32
Methanol extract	53	37

The values are means of triplicates.
The values of the negative (untreated) control group were fixed as 100% viability and the per cent viabilities in the other groups were calculated relative to this.

DNA FRAGMENTATION

In the present study, the extent of DNA damage by fragmentation was assessed in the Hep2 cells exposed to etoposide in the presence and the absence of the methanolic extract of *Rhinacanthus nasutus* leaves. The extent of DNA damage was documented (Plate 14) and quantified using a digital gel documentation system and its software (AlphaEaseFC of Alpha Digidoc 1201).

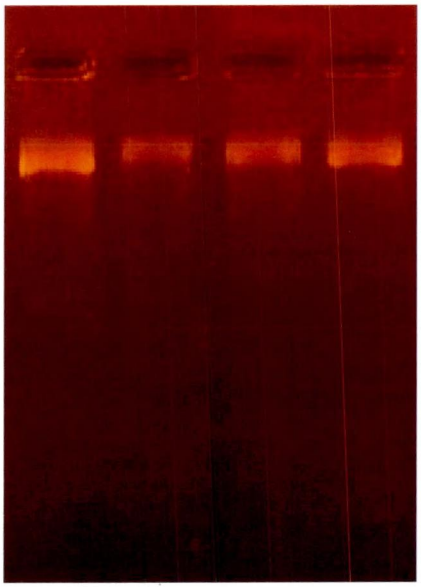
Hep2 cells treated with etoposide showed an increased DNA fragmentation as shown by the integrated density values (Table XXXI) and the administration of the plant extracts augmented the extent of DNA damage caused by etoposide as reflected by the decrease in the occurrence of DNA fragmentation.

TABLE XXXI

INTEGRATED DENSITY VALUES (IDV) OF THE BANDS IN THE AGAROSE GEL OF DNA FRAGMENTATION ASSAY

SAMPLE	IDV OF THE BANDS	
	Control	[redacted] Etoposide treated
No extract	69160	39320
Methanolic extract	62360	53295

PLATE 15



DNA FRAGMENTATION PATTERN IN Hep2 CELLS

Lane 1 – Untreated group

Lane 2 – Etoposide treated group

Lane 3 – *Rhinacanthus nasutus* leaf extract treated group

Lane 4 – Etoposide + *Rhinacanthus nasutus* leaf extract treated group

PHASE IV

The results of this study, as presented in the first three phases, clearly reiterated the protective effects of the methanolic extract of *Rhinacanthus nasutus* leaves against oxidative stress in a battery of experimental systems. It was then felt imperative to analyse the extract for its chemical composition, in order to identify additional components like secondary metabolite phytochemicals, other than the enzymic and non-enzymic antioxidants analysed in the first phase of the study.

For this, initially a preliminary phytochemical screening was conducted to identify the chemical nature of the principle component. Qualitative analysis of the extracts revealed the presence of alkaloids, phenolics and flavonoids in the leaf extracts of *Rhinacanthus nasutus.*

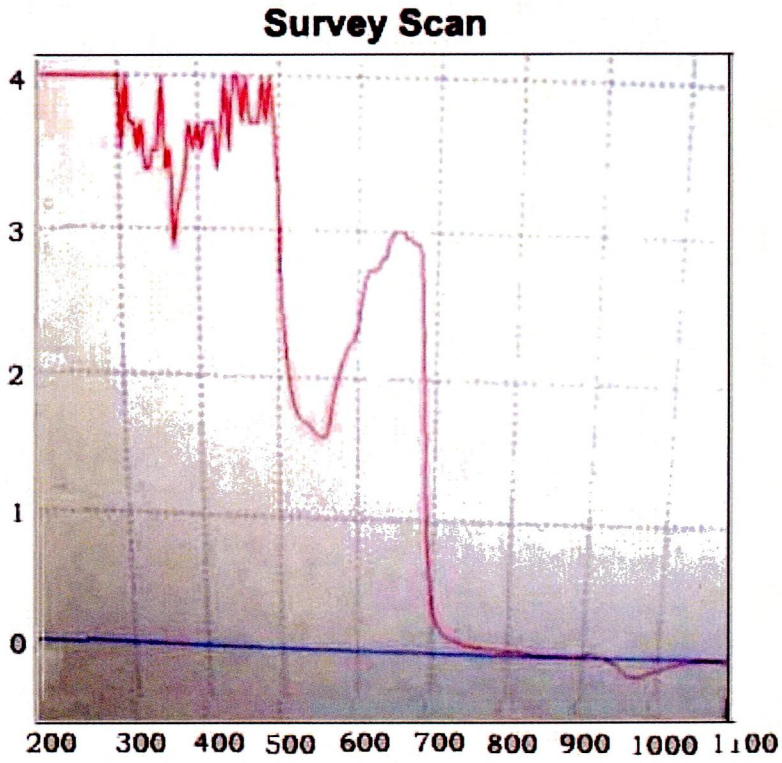
A preliminary absorbance survey scan of the methanolic extract of *Rhinacanthus nasutus* leaves in the wavelength ranging from 190nm-1100nm (Figure 18) revealed the presence of multiple components in the extract.

In order to confirm the results obtained after qualitative analysis, spectral analysis using HPLC, GC-MS and IR spectral analyses were carried out.

HPLC ANALYSIS

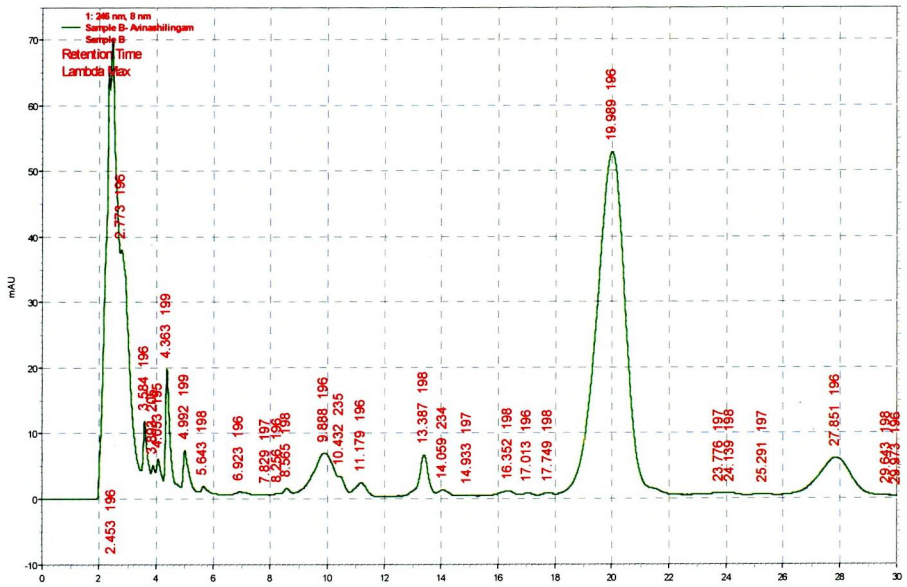
Two peaks (a major and a minor one) were observed in the spectrum (Figure 19) showing the presence of two principle components in the leaf extract of *Rhinacanthus nasutus* that might be responsible for its protective effect against oxidant induced damages in all the *in vitro* models studied.

FIGURE 18



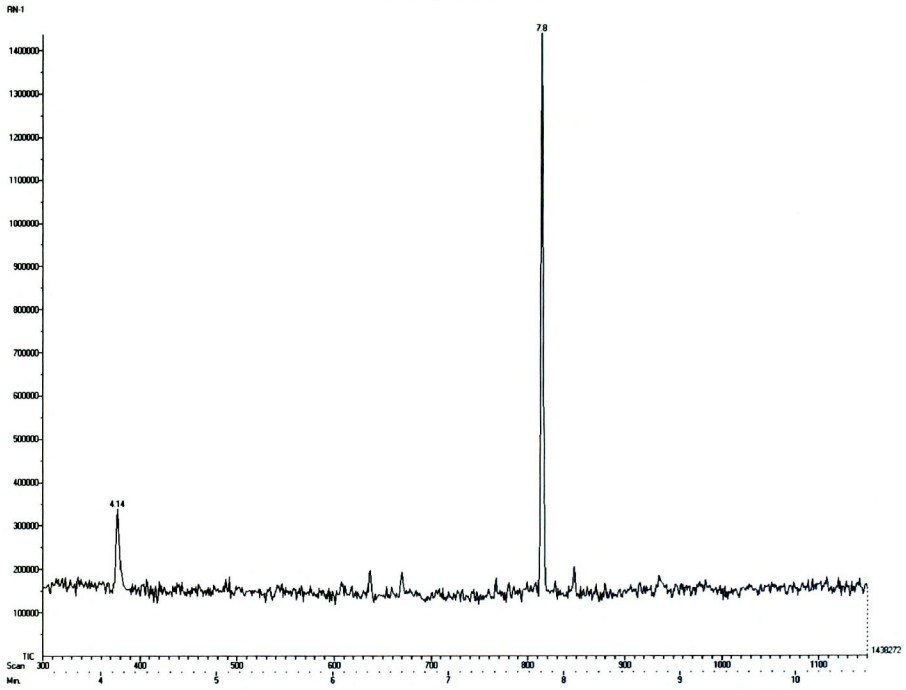
SURVEY SCAN OF THE METHANOLIC EXTRACT OF *Rhinacanthus nasutus* LEAVES

FIGURE 19



HPLC PROFILE OF *Rhinacanthus nasutus* LEAVES

FIGURE 20



GC MS PROFILE OF *Rhinacanthus nasutus* LEAVES

GC-MS ANALYSIS

The GC-MS analysis of the leaf sample of *Rhinacanthus nasutus* was carried out to identify the nature of the components present. The GC-MS output also showed the presence of two major components at retention times 4.14 and 7.8 (Figure 20). The respective fragmentation patterns of the components are shown in Figures 21a and 21b.

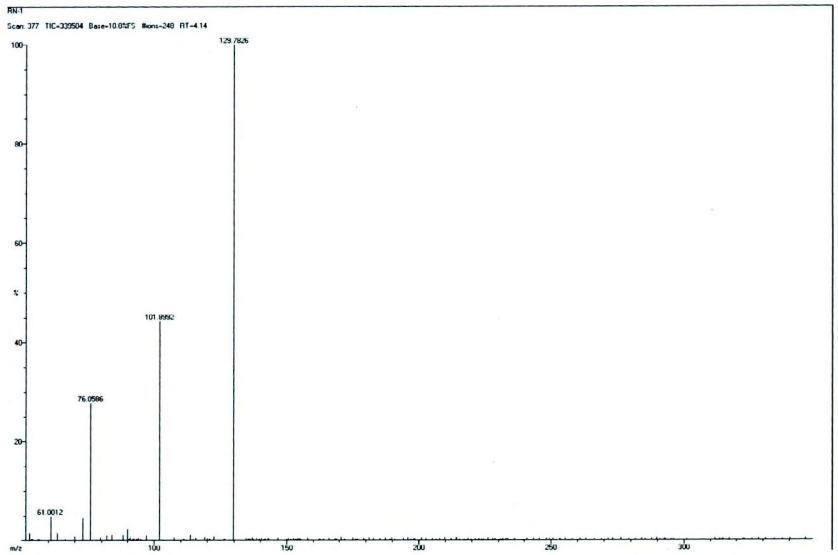
The mass spectrum of the compound with retention time 4.14 gave three major peaks (m/z) at 129.7826, 101.8992, and 76.0586. The element combination for the molecular ion peak at 129.7826 shows the probability for $C_8H_{19}N$, $C7H_5NO$, $C_6H_{11}NO_2$, C_9H_7N, $C_5H_7NO_3$. The above molecular formula indicates the possibility of an aromatic nitrogen containing compound that is an alkaloid. Also the fragmentation shows loss of CO, N_2, CH_2N or C_2H_4 (129-101-loss of 28) indicating the presence of nitrogen again. Therefore, it is perceivable that the compound at retention time 4.14 is possibly be an alkaloid.

The mass spectrum of the compound with retention time 7.8 gave eight major peaks (m/z) at 221, 203, 165, 147, 119, 102, 91 and 75. The fragmentation pattern of 119, 102, 91 and 75 is shown characteristically by aromatic alcohols or polyols. Also, the loss of m/z -18 (loss of H_2O) from molecular ion peak (221-203-18) is seen, which is again characteristic of alcohols or phenols. Therefore, it may be concluded that the compound with retention time 7.8 may be a polyphenolic compound.

IR ANALYSIS

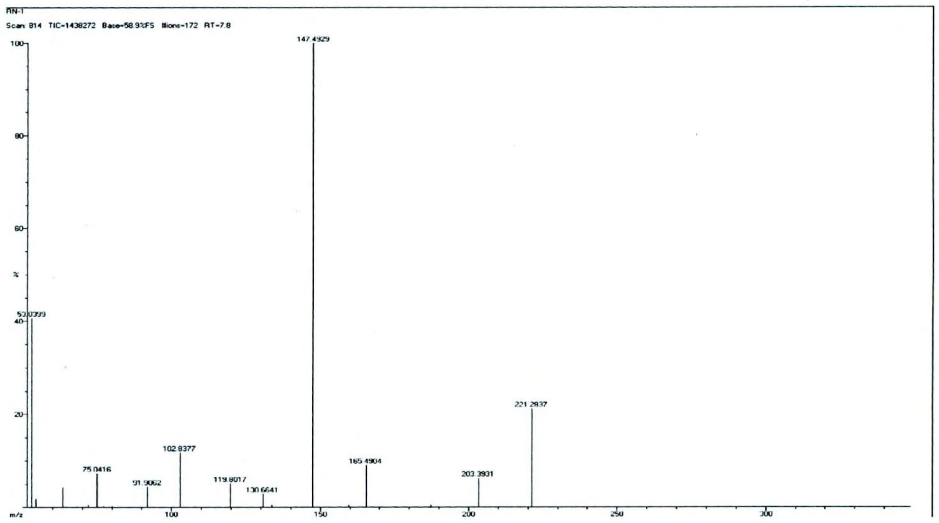
The results of IR spectrum of band 1 (Figure 22a) obtained from TLC showed major peaks at 3400cm^{-1} (broad and strong indication of $^{-}$OH); 2923cm^{-1}(indicative of –C-H stretching); 1741cm^{-1}(indicative of the presence of lactone ring); and, 1166 cm^{-1}(indicative of C-O stretching).

FIGURE 21a



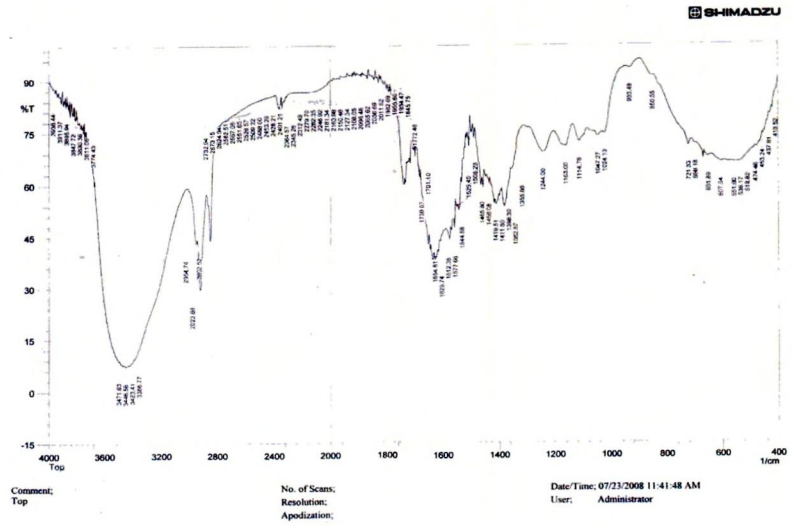
PEAK FRAGMENTATION OF GCMS SPECTRUM (4.14)

FIGURE 21b



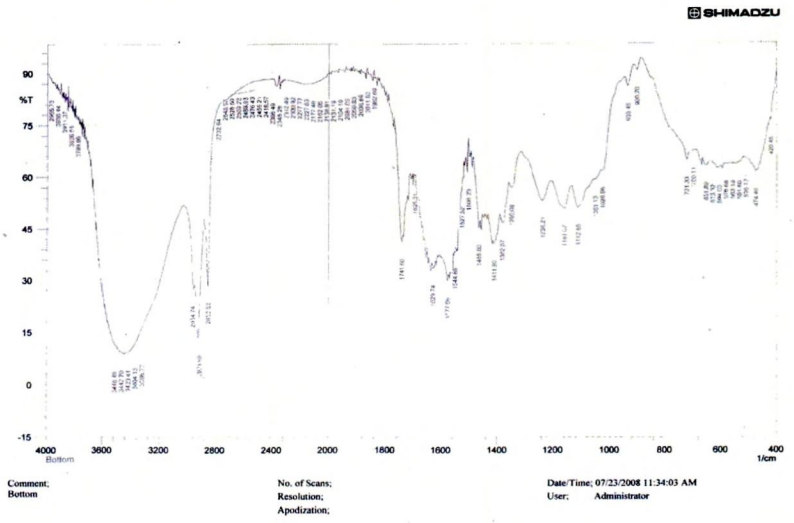
PEAK FRAGMENTATION OF GCMS SPECTRUM (7.8)

FIGURE 22a



IR SPECTRUM OF *Rhinacanthus nasutus* LEAVES (BAND 1 FROM TLC)

FIGURE 22b



IR SPECTRUM OF *Rhinacanthus nasutus* LEAVES (BAND 2 FROM TLC)

Hence, it may be concluded that band 1 contains a compound of polyphenolic nature. The presence of lactone rings indicates the presence of flavonoids or coumarins.

Band 2 was a single spot indicative of one compound. The IR spectrum of band 2 (Figure 22b) obtained from TLC showed a broad peak at 3440cm^{-1} indicative of hydroxyl group (-OH), a sharp peak at 1654cm^{-1} indicative of carbonyl group. Therefore, this band must possess compounds that are having a hydroxyl and carbonyl group.

Thus, the results and observations presented in this chapter clearly demonstrate the strong antioxidant and anticancer potential of *Rhinacanthus nasutus* leaves. These observations and results are discussed in the light of the available literature in the next chapter, drawing out the major conclusions that can be drawn from therein.