Discussion

5. DISCUSSION

Antioxidant properties and other bioactivities of secondary metabolites of medicinal plants are of great interest in many fields such as pharmacology and food industry. It is a growing tendency that natural antioxidant compounds are being used to replace synthetic antioxidants due to their higher efficacy and lesser side effects after long term usage (Gao *et al.*, 2007a).

Medicinal plants are considered to be an important source of antioxidant compounds and the therapeutic benefit of many medicinal plants is often attributed to their antioxidant properties (Hasan *et al.*, 2007). Natural antioxidants strengthen the endogenous antioxidant defenses from ROS ravage and restore the optimal balance by neutralizing reactive species. They are gaining immense importance by virtue of their critical role in disease prevention (Venukumar and Latha, 2002).

Antioxidants that are present in herbs and spices are responsible for inhibiting or preventing the deleterious consequences of oxidative stress (Khalaf *et al.*, 2007) and are known to play an important role in the chemoprevention of diseases and aging.

There is an increasing demand for natural antioxidants to replace synthetic additives in the food industry. Plants have been considered as valuable sources of medicinal agents for the treatment of many diseases. Several epidemiological studies have indicated that a high intake of plant products is associated with a reduced risk of a number of diseases and disorders (Gosslau and Chen, 2004).

Epidemiological studies have consistently shown that regular consumption of fruits and vegetables is strongly associated with reduced risk of developing chronic diseases such as cancer and CVD. It is now widely believed that the actions of the antioxidant nutrients alone do not explain the observed health benefits of diets rich in fruits and vegetables, because when taken alone, the

individual antioxidants studied in clinical trials do not appear to have consistent preventive effects. It has been shown that a combination of phytochemical and nutraceuticals may exhibit a considerably stronger antioxidant property and that a major part of total antioxidant activity is from the combination of phytochemicals (Ray *et al.*, 2006).

Oxidant damage has been shown to be the root causative factor in several diseases like diabetes, cancer, cardio vascular diseases, neurodegenerative disorders and scurvy (Ko *et al.*, 2005). Thus, it becomes evident that strong antioxidant activity associated with plants, their extracts and purified components can be extrapolated to their medicinal properties against the myriad diseases associated with oxidative damage, and such plants can be exploited for the preparation of pharmaceuticals to combat these diseases.

Many plants with such activity have indeed been identified. However, many other plants, especially widespread in the biodiversity–rich countries like India, remain under-exploited, predominantly due to the inadequacy in the scientific validation of such plants.

One such plant is *Rhinacanthus nasutus,* commonly called Nagamalli (Tamil). The roots of this plant are being used in some Ayurvedic medicinal preparations and there are some sparse reports about the possible antioxidant potential of the leaves.

Hence, in the present study, it was undertaken to conduct an elaborate and systematic study on the antioxidant and anticancer activity of the leaves of *Rhinacanthus nasutus,* which is an under-exploited plant present in some parts of South India and Thailand.

The study spanned four phases. In the first phase, the leaves were analyzed for their antioxidant content. In phase II, the antioxidant potential of the leaves

were probed into, using free radicals and known oxidants, as well as in cell-free systems and in isolated biomolecules subjected to oxidative stress. In the third phase, the effect of the leaf extract was analyzed on live cells, which included normal (untransformed) cells as well as cancer (transformed) cells. In the fourth and final phase, phytochemical analysis of the leaves was done to identify the additional phytochemical components present in them.

PHASE I

In this phase, the leaves were analyzed for the activities of enzymic antioxidants and the levels of non-enzymic antioxidants. The results showed that the leaves of *Rhinacanthus nasutus* were rich sources of the enzymic antioxidants, namely superoxide dismutase, catalase, peroxidase, glutathione S-transferase and polyphenol oxidases. Thery were also found to contain considerable levels of the non-enzymic antioxidants ascorbate, tocopherol, total carotenoids, lycopene, reduced glutathione, phenols and chlorophyll.

These results reveal that *Rhinacanthus nasutus* leaves have a potential candidature as a source of antioxidants. The literature is rich with reports that correlate the antioxidant content of plants with their medicinal properties.

It is evident from the results reported by Mohamed *et al.* (2007) that there is more than a 1000-fold difference among total antioxidants in various plant sources. These plant species could be used as a very good source of both water-soluble and lipid-soluble antioxidants. The antioxidant property of the pomegranate flowers (Kaur *et al.*, 2006) and *Salix caprea* (Alam *et al.*, 2006) have been suggested to be responsible for their hepatoprotective property. Alcoholic extracts of *Swertia diffusa*, *Berberis aristata*, *Withania somnifera* and *Pterocarpus santalinus* were reported to have appreciable amount of vitamin C and phenolics (Gacche and Dhole, 2006).

Plants having vitamins, flavonoids and polyphenols have been reported to possess remarkable antioxidant activity (Gupta and Sharma, 2006). Emami *et al.* (2007) have reported the strong antioxidant potency of *Cuprescus semiprevivenseven*, which was higher than that of tocopherol. Quantitative analysis of the total phenolic content of the seaweeds indicated that *Gelidella acerosa* and *Haligra* species have high phenolic contents, which correlated with their respective antioxidant and antimicrobial activity (Devi *et al.*, 2008).

Cervantes *et al.* (2007) have found that the aqueous extract of *Cissus quandrangularis* showed the highest levels of ascorbic acid. The high content of phenolic compounds, antioxidant activity, and antiproliferative activity of apple peels indicate that they may impart health benefits when consumed and should be regarded as a valuable source of antioxidants (Wolfe *et al.*, 2003).

Aegiceras corniculatum extracts were found to possess pronounced antioxidant effect that may be at least in part related to its anti-inflammatory and hepatoprotective activities (Roome *et al.*, 2008). It has been reported that a 50% aqueous methanol extract of *Rosa hemisphaerica J.herrm.*, *Prunus cerasus* L. and *Rosa canina* are potential sources of natural antioxidants. These antioxidant properties were also shown to depend on the concentration of the sample (Serteser *et al.*, 2008).

In the present study, *Rhinacanthus nasutus* leaves also proved to be very good sources of several well-known antioxidants, supporting the hypothesis that the leaves can be exploited for the preparation of medicinal aids to combat oxidative stress-induced diseases and disorders.

PHASE II

Having shown that the antioxidants were present in the leaves of *Rhinacanthus nasutus,* in this phase, the efficiency of the leaf extracts in

counteracting oxidative stress was analyzed. *In vitro* assays and cell free systems were used to evaluate the radical scavenging and antioxidant effects of different solvent extracts of *Rhinacanthus nasutus* leaves.

Solvent extraction is frequently used for the identification and isolation of the antioxidants. The extraction yield and antioxidant activity of the extracts are strongly dependent on the solvent, due to the differences in the antioxidant potential of compounds with different polarity. For these reasons, comparative studies for selecting the optimal solvent providing maximum antioxidant activity will be highly useful (Kang *et al.*, 2003).

In the present study, *Rhinacanthus nasutus* leaves were extracted into three different solvents with different polarity and were tested for their ability to scavenge free radicals in H_2O_2-induced oxidatively damaged systems. The solvents used were water, methanol and chloroform.

Hydrogen peroxide has two contradictory functions in cells, one as an oxidant responsible for lipid peroxidation, membrane injury, pigment bleaching, protein/enzyme inactivation, and the other as an inducer of antioxidant enzymes involved in the amelioration of oxidative stress. The increased production of toxic oxygen derivatives is considered to be a universal feature of stress conditions. Plants and other organisms have evolved wide range of mechanisms to contend with this problem, with a variety of antioxidant molecules and enzymes (Shirwaikar *et al.*, 2004).

The antioxidant efficacy of a compound or a plant extract cannot be accurately defined by studying the extent of scavenging using a single radical. Therefore, in the present study, an array / battery of radicals and oxidants (DPPH, H_2O_2, ABTS and hydroxyl) have been tested against the *Rhinacanthus nasutus* leaf extracts.

DPPH RADICAL SCAVENGING EFFECTS

DPPH is a relatively stable radical. The assay is based on the measurement of the scavenging ability of antioxidants towards the stable radical DPPH, which reacts readily with suitable reducing agent (Baskar *et al.*, 2007).

In the present study, the effects of *Rhinacanthus nasutus* leaves on DPPH were assessed as a measure of the radical scavenging ability of the leaf extracts. The aqueous, methanolic and chloroform extracts of the leaves were tested for their DPPH-scavenging potential, among which, the methanolic extract exhibited the highest activity.

DPPH scavenging has been used as a routine parameter in several studies establishing the antioxidant activity of natural products from plant and microbial sources (Shyur *et al.*, 2005).

The methanolic extracts of green tea and *Ficus bengalensis* exhibited antioxidant property as reflected by their higher hydrogen donating ability, which was implied to be the result of higher levels of total phenolic contents (Manian *et al.*, 2008). A crude methanolic extract of *Solanum pseudocapsicum* plant was found to possess very potent antioxidants, which was expressed by their ability to scavenge DPPH, ABTS radical cation and hydrogen peroxide (Badami *et al.*, 2005). Coban and Konuklugil, (2005) have reported that the methanolic extract of *Linum arboreum* had a strong scavenging effect on DPPH radical and this antioxidant activity was related to the presence of lignans in *Linum arboreum* (Hemmati *et al.*, 2004).

Methanolic extract of *Port Oxford cedar* (Gao *et al.*, 2007b) and a methanolic *Cherry blossom* extract (Lee *et al.*, 2007a) exhibited promising DPPH radical-scavenging activity. The ability of both crude and ethanolic extracts of palm fruit to act as hydrogen donors is indicated by their ability to scavenge DPPH

free radicals (Balasundram *et al.*, 2005). Another study postulated that *Annona* species reduces the radical to corresponding hydrazine when it reacts with hydrogen donors in antioxidant principle (Baskar *et al.*, 2007).

A crude methanolic extract of *Acerginnala max* (Han *et al.*, 2004), a methanolic extract of *Grateloupia filicina* (Athukorala *et al.*, 2003), *Curculigo orchiodes* (Bafna and Mishra, 2005) and the extracts of *Terminalia belleria*, *Terminalia chebula* and *Mangifera indica* (Aquil *et al.*, 2006) had good DPPH radical scavenging activity.

Cumin extract exhibited a dose-dependent scavenging of DPPH radicals, which was found to be 2.6 times more potent than the standard butylated hydroxy toluene (Ani *et al.*, 2006). The results reported by Patil *et al.* (2005) revealed that the extract of *Mellilotus officinalis*, which contained a high amount of flavonoid and phenolic compounds, exhibited potential antioxidant activity. The high scavenging property of *M.officinalis* was attributed to the hydroxyl groups existing in the phenolic compounds. The methanolic extract of *Ipomoea obscura* was found to possess significant free radical scavenging activity (Srinivasan *et al.*, 2007) and the chloroform extract of *Carissa spinarum L.* displayed strong DPPH activity (Rao *et al.*, 2005).

A concentration dependent scavenging effect of *Andrographis paniculata* was demonstrated against DPPH and ABTS showing its ability to convert unpaired electrons to paired ones (Tripathi and Kamat, 2007). A crude aqueous extract of *Chlorophytum borivilianum* (Kenjale *et al.*, 2007) has been shown to scavenge DPPH free radicals and TBARS revealing that it is a promising adaptogen or anti-stress agent as well as a potential antioxidant.

In the present study, the methanolic extract of *Rhinacanthus nasutus* leaves exhibited an effective scavenging ability when compared to the other two extracts. Differential responses between aqueous and methanolic extracts of *Citrus*

paradise seeds have been reported by Giamperi *et al.* (2004), which are in agreement with our findings.

In the backdrop of the above studies, the ability of *Rhinacanthus nasutus* leaf extracts in effectively scavenging DPPH radicals reveals the strong antioxidant and the radical scavenging potential of the leaves.

HYDROGEN PEROXIDE SCAVENGING EFFECTS

Hydrogen peroxide is a reactive oxygen species responsible for cytotoxic damage through the formation of hydroxyl radicals (Geracitano *et al.*, 2005). The antioxidant property, measured as hydrogen peroxide scavenging has been shown to correlate with the antimutagenicity of phytochemicals (Geetha *et al.*, 2005).

The ability of *Rhinacanthus nasutus* leaf extracts to scavenge hydrogen peroxide in an *in vitro* system was also followed in the present study. The results revealed that the methanolic extract contained a strong scavenging effect against hydrogen peroxide.

Methanolic and hotwater extracts of *Phellinus baumii* exhibited good inhibition rates, of about 80-90%, by hydroxyl radical scavenging activity and hydrogen peroxide scavenging activity (Shon *et al.*, 2006). The methanolic extract of *Mucuna pruriens* seeds showed strong antioxidant activity by inhibiting DPPH, hydroxyl radical and hydrogen peroxide (Rajeswar *et al.*, 2005). Methanolic and ethyl acetate extracts of the medicinally valuable *Chionanthus virginicus* scavenged H_2O_2 to a significant extent (Gulcin *et al.*, 2007a).

Gulcin *et al.* (2007b) have reported that the water and ethanolic extracts of *Ocimum basilicum* had strong antioxidant activity as reflected by their effectiveness in scavenging H_2O_2. An aqueous extract of *Piperitae folium* exhibited hydrogen peroxide scavenging activity (Sroka *et al.*, 2005). H_2O_2 was

also scavenged in a dose-dependent manner by the aqueous extract of Taiwan folk medicine (Wang *et al.*, 2004).

A crude aqueous and ethanolic extracts of *Piper nigrum* seeds (Gulcin, 2005) and garlic (Sato *et al.*, 2006) exhibited strong H_2O_2 scavenging effects. Several phytochemicals are also effective in scavenging H_2O_2. Mono dermosides from *Leontice amirnowii* tuber (Gulcin *et al.*, 2006) and a polysaccharide from *Ocimum sanctum* (Subramanian *et al.*, 2005) were shown to possess strong H_2O_2 scavenging activity.

Glei *et al.* (2006) have shown that the arabinoxylans from wheat bran and the fermentation products mediated by the gut flora protect the human colon cells from the genotoxic damage caused by H_2O_2. H_2O_2 scavenging activity was also associated with cinnamic acid, benzoic acid (Mansouri *et al.*, 2005) and several non-steroidal anti-inflammatory drugs (Costa *et al.*, 2005). Glutathione found in most tissue cell and subcellular compartments scavenged H_2O_2 reaction non-enzymatically with singlet oxygen (Simroff *et al.*, 2004). The ascorbate present in plant leaves directly scavenged hydroxyl radical and reduced H_2O_2 to water via ascorbate peroxidase (Smirnoff, 2005). Agents that induce catalase activity in the cells can also effectively scavenge H_2O_2 (Yashioka *et al.*, 2006).

In the present study, the observation that *Rhinacanthus nasutus* leaf extracts can very effectively scavenge H_2O_2 shows the strong antioxidant activity of the leaves.

ABTS RADICAL SCAVENGING EFFECTS

The leaf extracts of *Rhinacanthus nasutus* were then analyzed by ABTS assay, another *in vitro* antioxidant screening method, applicable for both lipophilic and hydrophilic antioxidants. The assay is based on the inhibition of the absorbance of radical cation, $ABTS^+$ that has a characteristic long wavelength

absorption spectrum (Malpure *et al.*, 2006). Also, the products of ABTS scavenging reaction may have a higher antioxidant capacity and can further react with the remaining ABTS radicals (Arts *et al.*, 2004). Thus, ABTS radical is approximately twice as sensitive as the DPPH radical (Summa *et al.*, 2006).

The methanolic extract of *Rhinacanthus nasutus* leaves showed the maximum extent of scavenging of ABTS radical cation. While DPPH is a pre-formed radical, $ABTS^{+}$ is generated during the assay. Thus, this test reveals the ability of the extract in preventing the formation of free radicals.

Several studies have reported the ABTS inhibiting action of plant extracts. A methanolic extract of *Terminalia arjuna* was highly significant in inhibiting the formation of ABTS radicals (Tilak *et al.*, 2003). *Annona squamosa* extract (Shirwaikar *et al.*, 2004) and most of the Thai culinary plants such as chillies and holy basil (Wangcharoen and Morasuk, 2007) were found to be very effective in quenching ABTS radical cation.

Hutadilok-Towatano *et al.* (2006) have demonstrated that a methanolic extract prepared from *Nelumbo nucifera* leaves and *Teucrium sauvegei* (Salah *et al.*, 2006) exhibited a pronounced activity in inhibiting ABTS radicals. An extract prepared from *Calendula officinalis* exhibited strong antioxidant potential *in vitro* against the free radical ABTS cation (Preethi *et al.*, 2006).

Kim *et al.* (2002) demonstrated that plant phenolics have strong antioxidant capacity as reflected by their ability to inhibit ABTS radicals. Other studies have used this inhibitory assay to prove the antioxidant activity of flavonoids (Lee *et al.*, 2002) and ascorbic acid and its derivatives (Takebayashi *et al.*, 2006). ABTS assay has also been valuable in reporting the free radical scavenging activities of poly herbal formulations, as shown for triphala (Naik *et al.*, 2006).

In the present study also, *Rhinacanthus nasutus* leaf extracts possessed strong ABTS inhibitory activity, which substantiates their antioxidant action.

HYDROXYL RADICAL SCAVENGING EFFECTS

Hydroxyl radicals are extremely reactive species that readily oxidize all cellular macromolecules including proteins, sugars, lipids and DNA (Bhupinder *et al.*, 2004). DNA is susceptible to oxidative damage and hydroxyl radicals oxidize guanosine or thymine to 8-hydroxyl-2-deoxy guanosine and thymine glycol, which change DNA and lead to mutagenesis and carcinogenesis. Hydroxyl radicals generated by Fenton reaction can induce DNA strand breaks in calf thymus DNA and uncoiling of super coiled DNA (Sheeja *et al.*, 2006).

In the present study, the effect of *Rhinacanthus nasutus* leaf extracts on hydroxyl radical-induced damage to deoxy ribose was analysed as a measure of the hydroxyl radical scavenging activity. It was found that all the three extracts of *Rhinacanthus nasutus* leaves (aqueous, methanolic and chloroform) exhibited strong protection to deoxy ribose against hydroxyl radical-induced damage, with the methanolic extract performing better than the other two.

There is a lot of reported support for this observation in the literature. Hydroxyl radical scavenging activity in the deoxy ribose decomposition method suggested that the methanolic extracts of *Rhus coriaria* had considerable antioxidant activity against hydroxyl radicals (Candan and Sokeman, 2004). A crude methanolic extract of celery (*Apium graveolens*) leaves and roots were found to be good scavengers of hydroxyl radicals (Popovic *et al.*, 2006). A methanolic extract of *Geranium niveum* roots showed powerful hydroxyl radical scavenging effect as reported by Maldonado *et al.* (2005).

The antioxidant effect of the methanolic extract of *Mucuna pruviens* seeds (Rajeswar *et al.*, 2005) and the chloroform extract of *Solanum trilobatum* (Sini and Devi, 2004) have been reported to have hydroxyl radical scavenging abilities.

The polyphenols present in bitter cumin extract might react with hydroxyl radicals by donating hydrogen atoms and convert the radicals to more stable non-radical products and thus the hydroxyl radical quenching ability of polyphenolic compounds of *Bitter cumin* has been suggested to be responsible for the protection rendered against oxidative damage caused to DNA (Ani *et al.*, 2006). DNA damage by hydroxyl radicals could also be effectively prevented in the presence of plant extracts such as *Ludwigia octovalvis*, *Vitis thunbergii*, *Rubus parvifoliu*, *Lindernia galli* and *Zanthoxylum nitidum* (Shyur *et al.*, 2005).

Triphala, an ayurvedic formulation, exhibited potent inhibition of hydroxyl radicals. The antioxidant activity of *Triphala* provide a mechanistic basis for relieving stress by way of combating oxidative damage (Shivaprasad *et al.*, 2008).

Our results showed a trend comparable to that reported by Maldonado *et al.* (2005) that a methanolic extract of *Rhinacanthus nasutus* leaves possessed strong hydroxyl radical scavenging activity when compared to the aqueous and chloroform extract, which indicates that *Rhinacanthus nasutus* exhibited better protective effects towards deoxyribose, an important constituent of the monomer of DNA.

Thus, the results obtained revealed that H_2O_2-induced production of hydroxyl radicals can be effectively inhibited by the leaf homogenate of *Rhinacanthus nasutus*.

In similar lines, the aqueous and methanolic extracts of *Cassia fistula* Linn bark (Ilavarasan *et al.*, 2005) and acetone and ethyl acetate extracts of *Kappahyeas alavezzi* (Fayaz *et al.*, 2005) effectively scavenged the hydroxyl radicals.

Teucrium species possessed free radical and hydroxyl radical scavenging activity as well as antioxidant activity *in vitro* (Panovska *et al.*, 2005).

The aqueous extracts of pepper (Oboh *et al.*, 2006), *Teucrium polium* (Ljubuncic *et al.* 2006) and *Mangifera indica* (Pardo-andreu *et al.*, 2006) were shown to be potent inhibitors of hydroxyl radicals. Park *et al.* (2006) have reported that *Acanthopanax senticosus* may work by providing hydrogen atoms from their phenolic hydroxyl groups to scavenge hydroxyl radicals generated from hydrogen peroxide.

In the present study, the analysis of the results obtained in the free radicals scavenging assays shows that the leaves are very effective in counteracting pre-formed radicals (DPPH and H_2O_2), inhibiting the formation of new radicals, ($ABTS^+$) and preventing the damage to biomolecules by the radicals (hydroxyl). Thus, the leaves possess a broad spectrum of antioxidant action.

EFFECT OF *Rhinacanthus nasutus* LEAF EXTRACTS ON DNA DAMAGE

Oxidative assault to biological system manifests at the cellular level on important biomolecules. Of these, the lipid molecules form the primary target and the DNA molecules, the ultimate target. Therefore, as a next step, after ascertaining the free radical scavenging properties of the *Rhinacanthus nasutus* leaf extracts (water, methanol and chloroform), their effects on DNA and lipids were followed *in vitro*.

The protective effects of *Rhinacanthus nasutus* leaf extracts on oxidative damage inflicted upon herring sperm DNA were analyzed. H_2O_2 exposure (oxidative stress) caused severe damage to herring sperm DNA, which was effectively counteracted by the presence of *Rhinacanthus nasutus* leaf extracts. The extracts, by themselves, did not cause any DNA damage.

A combined antioxidant supplementation has been shown to be very effective, even at moderate doses, in preventing oxidative DNA damage (Volkovova *et al.*, 2006). Supplementation with β-carotene, α- carotene and ascorbate have been shown to decrease oxidative DNA damage (Kim *et al.*, 2007a). In our study, the protection rendered to DNA by *Rhinacanthus nasutus* leaf extract is probably mediated by such antioxidants, which are present in leaves as shown by our results reported in phase I.

Methanolic extracts of *Nelumbo nucifera* inhibited H_2O_2-induced damage to pUC 18 DNA (Wang *et al.*, 2003). Methanolic extracts of *Celastrus paniculatus*, *Picorhiza kurao* and *Withania somnifera* protected the DNA cleavage induced by H_2O_2-photolysis in non-immortalized human fibroblasts (Russo *et al.*, 2001).

The consumption of plant based foods has been reported by several workers to protect against oxidative DNA damage. A methanolic extract of rice hulls showed a protective effect against oxidative DNA damage induced by H_2O_2 (Jeon *et al.*, 2006). Green tea catechins partially protected DNA from hydroxyl radical induced strand breaks and base damage (Anderson *et al.*, 2001).

Sugar molasses exerted protection to DNA against damage induced *in vitro* (Guimaraes *et al.*, 2007). Gill *et al.* (2007) have shown that the supplementation of water cress in the diet reduced lymphocyte DNA damage in healthy adults.

Acanthopanax senticorus, referred to as Siberian ginseng, effectively protected DNA against oxidative damage induced by H_2O_2 (Park *et al.*, 2006). Heo *et al.* (2005) showed that the antioxidant activity of the enzymic extracts of the brown seaweed *Ecloniacava* was associated with a reduction in oxidative DNA damage, which was dose-dependent.

Similar DNA protective effects have been reported for *Polygonum aviculare* extract (Hsu, 2006), *Satureja hortensis* extract (Mosaffa *et al.*, 2006) and *Mesona procumbens* extract (Yang, 2006). Plant antioxidant components like grape seed polyphenols (Fen and Lou, 2004) and anthocyanins (Weisel *et al.*, 2006) have been reported to protect DNA against oxidant-induced damage.

The plant phenolic compounds such as genistein and resveratrol showed their ability by inhibiting the damage induced by ROS in phi x-174 plasmid DNA (Win *et al.*, 2002). In the same plasmid, UV-induced strand cleavage was completely inhibited by the phenolic components of *Calocedrus formosana* (Wang *et al.*, 2004). Hydroxylated-4-thiaflavans, a group of antioxidants, showed protection against oxidative DNA damage in herring sperm DNA induced by cumene hydroperoxide (Lodovici *et al.*, 2006).

Our results reveal that *Rhinacanthus nasutus* leaves render strong protection to DNA against oxidant-induced damage *in vitro.* This observation substantiates the biomolecular-protection of the leaves.

EFFECT OF *Rhinacanthus nasutus* LEAF EXTRACTS ON LPO

Lipid peroxidation is a free-radical chain reaction and the reactive oxygen species can accelerate lipid oxidation. LPO is the most common index of oxidative stress and Thio Barbituric Acid Reactive Substances (TBARS), the end product of LPO, is routinely used as a marker of this process (Ozgen *et al.*, 2006).

Mondal *et al.* (2006) showed that the methanolic extract of *Diospyros malabarica* showed a dose-dependent prevention of the generation of lipid peroxides. Dzingirai *et al.* (2007) have reported that the plant extracts gave a dose dependent protection against lipid peroxidation in rat brain homogenate with the highest protection detected from *Elionurus muticus* whole plant and the least from *Hypoxis hemerocallidea.* In another study (Bafna and Mishra, 2005), a novel

pentacyclic triterpenoid present in *Curculigo orchioides* was suggested to be predominantly responsible for the LPO inhibitory action of the methanolic extract of the plant.

The aqueous and methanolic extracts of *Phyllanthus niruri* were potent inhibitors of LPO induced by Fe^{2+} and ascorbate *in vitro* (Harish and Shivanandappa, 2006). Aqueous and methanolic extracts of *Achyrocline satureioides* possess significant free radical scavenging and antioxidant activity *in vitro*, reflected by their capacity to reduce lipid peroxidation (Desmarchelier *et al.*, 1998). Crude, boiled and ethanolic extracts of *Auricularia auricula* (Acharya *et al.*, 2004) and *Cissus quadrangularis* extract (Jainu and Devi, 2005) showed significant inhibition of LPO.

An ethanolic extract of *Zizipus mauritiana* leaves significantly lowered the LPO induced by CCl_4 (Dahiru *et al.*, 2005), which property was attributed to the probable presence of flavonoids, phenolics, tannins and saponins. A water extract of *Selaginella involvens* showed inhibition of lipid peroxidation in rat liver homogenate (Gayathri *et al.*, 2005).

The hydroalcoholic extract of the dried flowers of *Hibiscus sabdariffa* decreased the sodium arsenite induced LPO significantly in the liver (Usoh *et al.*, 2005). In a similar report (Pandey *et al.*, 2005), the aqueous ethanolic extract of *Saussarea costus* drastically reduced the extent of LPO, which was attributed to its chlorogenic acid content. An aqueous extract of *Scoparia dulcis* showed a marked antioxidant activity *in vitro*, which was assayed by measuring the TBARS in fowl egg yolk (Ratnasooriya *et al.*, 2005).

Stachys beckeana and *Stachys dinarica* were efficient in suppressing LPO (Kukic *et al.*, 2006). The antioxidant activity possessed by six plants used in Turkish traditional medicine was reflected by their LPO inhibitory action (Oboh and Gbolade, 2003). An aqueous extract of *Teucrium polium* (Ljubunic *et al.*,

2006) and a polyherbal formulation, Hingwashtak churna (Shirwaikar *et al.*, 2006b) exhibited strong inhibitory action on LPO.

Our results demonstrated that the *Rhinacanthus nasutus* leaf extracts exhibit powerful radical quenching ability against a team of radicals, albeit to different extents. They are also very effective in protecting the target biomolecules (DNA and lipid) against oxidant-induced damage, which process is the key event in the initiation of many diseased conditions. The maximum effect was mediated by the methanolic extract of *Rhinacanthus nasutus* leaves.

PHASE III

The use of alternative systems for the analyses of biomolecules, with the view of minimizing and/or eliminating tests on live animals, has been the thrust of bioscientists for quite a few years now. Also there is a growing awareness regarding the ethical concerns involved in the use of live animals in biological research (Goldberg and Hartung, 2006).

Precision-cut tissue slices allow the investigation of the effects of various substances on biological tissues under *in vivo*–like conditions over a limited time span (Kern *et al.*, 2006). Precision-cut liver slices have proved useful for several pharmacological and toxicological investigations, since the model permits the maintenance of normal lobular architecture and cell-cell interactions within their original matrix. Precision-cut liver slices have been increasingly used in both descriptive and mechanistic studies for xenobiotic and liver specific toxicity (Onderwater *et al.*, 2004).

In tune with this background, in phase III of the study, precision-cut slices from goat liver were employed to evaluate the protective effects rendered by *Rhinacanthus nasutus* leaves *in vitro* against hydrogen peroxide induced toxicity. Enzymic and non-enzymic antioxidants were assessed in the liver slices subjected

to oxidative stress under the influence of the leaf extracts (water, methanol and chloroform).

ENZYMIC ANTIOXIDANTS

SOD, CAT, POD and GST were the enzymes analyzed in goat liver slices. One of the adaptations of the stress tolerant species / cultivars is the dominance of a defense system of antioxidant systems. These enzymes include SOD, which scavenges superoxide radicals and converts this to oxygen and H_2O_2, which is then detoxified by catalase. In addition, non-specific peroxidases play an important role in antioxidative protection.

SOD

Among the multileveled interdependent antioxidant systems, which aerobic cells have evolved to protect against oxidant injury, is the family of SODs, which catalyses the conversion of the superoxide ion into hydrogen peroxide (Milhavet *et al.*, 2000).

In the present study, the activity of SOD decreased significantly when challenged with the oxidant (H_2O_2). Treatment with *Rhinacanthus nasutus* leaf extracts brought higher SOD activities in the slices, with the methanolic extract showing the maximum effect. Our results show that *Rhinacanthus nasutus* extracts improved the activities of SOD in the oxidant stressed group, indicative of their antioxidant potential.

Eventhough the literature is rich with the reports of antioxidant activities of several compounds and extracts, not many reports are available that use liver slices (or any other organ slice) for the assessment of this activity. Many studies are, however, available in intact, live experimental animals like rats.

An investigation by Shin *et al.* (2001) reported that both water and methanolic extracts of *Paecilomyces japonica* were found to cause significant increase in rat liver cytosolic SOD, catalase and glutathione peroxidase activities and also a significant decrease in malondialdehyde (MDA) production in TBA reactant assay in rats.

Ugochukwu and Babadu (2002) demonstrated that the aqueous and ethanolic extracts of *Gangonema latifolium* leaves increased the activities of SOD and glutathione peroxidase in rats. The activity of SOD increased significantly in liver slice culture in *Gmelina arborea* bark and counteracted the H_2O_2 and paraquat induced oxidative stress (Sinha *et al.*, 2006).

Rats treated with the aqueous extracts of *Ocimum sanctum* showed significant increase in the activity of SOD, catalase and GSH with a decrease in MDA level. These enzymes are known to quench the superoxide radical and thus prevent the damage of the cells caused by free radicals (Shetty *et al.*, 2007).

Our results showed that *Rhinacanthus nasutus* leaf extracts improved the activities of SOD in the oxidant stressed group, indicative of their antioxidant potential.

CATALASE

By catalyzing the conversion of H_2O_2 to oxygen and water, catalase has been shown to shift the redox balance towards antioxidant and leading to increased antioxidant capacity, which may alleviate the detrimental effect of H_2O_2 (Ren *et al.*, 2004).

In the present study, during H_2O_2 intoxication, the catalase activities were found to be significantly reduced, which may be due to their utilization in the removal of H_2O_2 generated by the action of SOD. Treatment with the leaf extracts,

however, improved the status and the effect was more pronounced in the groups treated with the methanolic extract of *Rhinacanthus nasutus* leaves.

Pandit *et al.* (2004) have reported that an aqueous extract of *Adhatoda vasica* leaves enhanced CAT activities in the liver homogenate of rats. Meera and Rana (2006) reported that the pretreatment with hydroalcoholic extracts of *Taraxacum officinale* roots improved the levels of catalase and peroxidases in rats intoxicated with CCl_4.

Co-administration of grape seed oil improved the activity of catalase in CCl_4 treated rats (Maheswari and Rao, 2005). Dibenzylbutyrolactone lignans of *Torreya nucifera* significantly preserved the activities of SOD, CAT, Px and GR in the CCl_4 injured rat hepatocytes (Kim *et al.*, 2003). The catalase activity in the rat liver decreased with CCl_4 treatment and upon treatment with *Strychnos potatorum* extracts, the CAT activity improved significantly (Sanmugapriya and Venkataraman, 2006).

Our results clearly demonstrate that the extracts of *Rhinacanthus nasutus* increased the activity of CAT, which would have brought about effective and complete scavenging of the superoxide-peroxide oxidants with the combined action of SOD. The effect elicited by the methanolic extract was very effective.

PEROXIDASE

The standard oxidant (H_2O_2) caused a decrease in the activity of peroxidase in the goat liver slices. The adverse effects of H_2O_2 were reverted by *Rhinacanthus nasutus* leaf extracts.

Oral administration of methanolic extracts of *Phyllanthus* significantly elevated the activity of hepatic glutathione peroxidase during CCl_4 stress in rats (Lee *et al.*, 2006a, b). Frei and Higdon (2003) have reported that rats supplemented with green tea extract in drinking water attenuated the ethanol-

associated decrease in liver GPx activities. The rhizomes of *Curculigo orchioides* improved the GPx activity in CCl_4 treated liver (Venukumar and Latha, 2002).

The activity of SOD, though very vital in detoxifying superoxide, releases hydrogen peroxide, which is also a strong oxidative species. Thus, improvement in the activity of SOD, unaccompanied by an increase in hydrogen peroxide detoxifying enzymes like CAT and GPx will be meaningless (Ramasarma, 1990). Thus, the observation that *Rhinacanthus nasutus* leaf extracts are effective in increasing the CAT and GPx activities gains significance, implying their ability to scavenge both superoxide radicals and hydrogen peroxide.

Lead induced decrease in glutathione peroxidase activity has been reported by Jeyaprakash and Chinnaswamy (2007). This could be probably due to either increased utilization of GSH by the cells to act as scavengers of free radicals caused by toxic chemical agents or enhanced utilization of GSH by GPx.

All these reports validate our results, wherein the treatment with the extracts has significantly improved the activities of SOD, CAT and Px which form the first level of defense against ROS.

GST

Glutathione S-transferase consists of a large family of GSH utilizing enzymes that play an important role in the detoxification of xenobiotics in mammalian system (Sohini and Rana, 2007). GSTs are involved in the nucleophilic attack of electrophilic substances, thereby decreasing their reactivity with cellular macromolecules (Singh *et al.*, 2005).

The results showed that the activities of GST in the goat liver slices were significantly reduced in the presence of H_2O_2. GST is an enzyme that is regarded as a marker of liver cell damage because its activity changes rapidly in response to cellular damage (Tredger and Sherman, 1997). In the present study also, drastic

reduction in the GST activity evidences the liver damage caused by the oxidant (H_2O_2) treatment. The activites were restored to normal levels by the administration of *Rhinacanthus nasutus* leaf extracts, which prove that the leaf extracts readily protected the cells from liver damage.

In the physiological system, glutathione and related enzymes play a key role in the defense against oxidative stress (Ray *et al.*, 2007). *Piper betle* leaf extract attenuated the total glutathione S-transferase activity and GST alpha isoform activity and protected the liver from the damage induced by CCl_4 in rat (Young *et al.*, 2006). An alcoholic extract of *Aloe vera* leaves resulted in a significant increase in GST activity in the tissues of diabetic rats (Rajasekaran *et al.*, 2006). The effect of an ethanolic extract of *Hibiscus sabdariffa* in improving the GST activity in sodium arsenate induced oxidative stress in rats has been reported by Usoh *et al.* (2005).

Higher levels of antioxidant enzymes must be necessary to detoxify increased concentrations of lipid peroxidation products that are generated from oxidative stress. The aqueous extracts of the roots of *Declepis hamiltonii* inhibited the ethanol-induced oxidative stress in the liver of rats by increasing the activities of SOD, CAT, GPx, GR and GST in a dose-dependent manner (Srivastava and Shivanandappa, 2006). The activities of glutathione disulfide reductase and GST, which normally decreased in CCl_4 injured rat hepatocytes were significantly preserved by the treatment with the phenylpropanoids isolated from the roots of *Scrophularia buergeriana* (Lee *et al.*, 2002).

Our results are corroborative with these reports, emphasizing the antioxidant response evoked by all the leaf extracts of *Rhinacanthus nasutus*.

NON-ENZYMIC ANTIOXIDANTS

The body has an effective mechanism to prevent and neutralize the free radical induced damage. This is accomplished by a set of endogenous antioxidants such as vitamins C, E, A and reduced glutathione.

ASCORBIC ACID

Ascorbic acid is a key antioxidant, partially protecting lipids from peroxidative damage and it has many biological activities in the human body (Linster and van Schaftingen, 2007). Vitamin C, a major extracellular non-enzymic antioxidant plays a crucial role in scavenging several reactive oxygen species (Pavana *et al.*, 2007). A medline research indicates that vitamin C is the most investigated antioxidant responsible for the elimination of free radicals. It is the most important antioxidant as it is water soluble and thus capable of penetrating into each cell of the human body to scavenge free radicals and prevent their potential damage (Tariq, 2007). Vitamin C depletion leads to the formation of hydroperoxides even when other antioxidants are still present (Narendhirakannan *et al.*, 2005).

H_2O_2 exposure decreased the levels of vitamin C in the goat liver slices. This reduction was effectively reversed by the leaf extracts of *Rhinacanthus nasutus*. Among the three extracts assessed, aqueous extract of *Rhinacanthus nasutus* caused the maximum increase in the levels of ascorbic acid.

Vitamin C can not only eliminate hydroxides and peroxides, but also restore the activity of glutathione peroxidase, and thus counteract free radical action (Zhou *et al.*, 2005). Ascorbic acid influences many enzyme activities and minimizes the damage caused by oxidative process through synergic function with other antioxidants. Vitamin C regenerates vitamin E and they both act synergistically in scavenging a wide variety of ROS (Pourcel *et al.*, 2007).

Sivalokanathan *et al.*, (2006) have reported a significant increase in the levels of vitamin C on treating nitrosodiethylamine treated livers with ethanolic extract of *Terminalia arjuna*. *Withania somnifera* extracts effectively, counteracted the damage produced by H_2O_2 and replenished vitamin C levels in hydrogen peroxide induced goat liver slices (Sumathi and Padma, 2008).

The extracts of *Nigella sativa* elevated the levels of vitamin C in CCl_4 treated rats (Kanter *et al.*, 2005). CCl_4 treatment to rats has been reported to significantly deplete plasma and tissue antioxidants, including vitamin C. This effect was counteracted by the oral administration of curcumin (Kamalakkannan *et al.*, 2005).

Thus, in the present study, the increase brought about in the depleted levels of vitamin C upon H_2O_2 treatment, by *Rhinacanthus nasutus* leaf extracts validates the use of these leaves in medicinal preparations for disorders and diseases caused by oxidative stress.

TOCOPHEROL

Tocopherol is used in combating free radicals and the most active form of vitamin E is present in cellular membrane and acts as a protective lipid soluble agent, generating the poorly reactive tocopherol radicals (Llances *et al.*, 2005).

Exposure of goat liver slices to H_2O_2 caused a significant decrease in the levels of tocopherol. These levels were improved on treating the H_2O_2 exposed liver slices with the leaf extracts. Methanolic extract offered the maximum protection. α-tocopherol scavenges and quenches various ROS and lipid oxidation products, stabilizes membranes and modulates signal transduction (Shao and Chu, 2005; Noctor, 2006).

Tocopherol has to be maintained in its native state, to be functionally active, for which ascorbate contributes. Ascorbate converts tocopheroxyl radical to its native state. Therefore, loss of tocopherol interferes in the tocopherol regeneration and leads to impaired membrane function. A decrease in the antioxidant vitamins C and E in *Plasmodium vivax* and *Plasmodium falciparum* infected patients might be due to the counteraction of the increased oxidative stress during the exposure to oxidants and by inhibiting membrane lipid peroxidation or due to their increased utilization as plasma antioxidants (Prasannachandra *et al.*, 2006).

Piperine, the major alkaloid present in *Piper nigrum* and *Piper longum* significantly elevated the vitamin E level in lung and liver of cancer bearing mice (Selvendiran *et al.*, 2003). An extract of *Hippophea rhamnoides* improved the levels of vitamin E to overcome the oxidative stress induced by nicotine in rats (Gumustekin *et al.*, 2003).

Co-administration of vitamin E with the extracts of *Withania somnifera*, *Ocimum sanctum* and *Zingiber officinalis* enhanced the protective effects against the oxidative stress imposed in rats (Misra *et al.*, 2005). Vitamin E treatment decreased the elevated TBARS levels in plasma and RBCs, and increased the reduced vitamin E levels, thus preventing RBC membrane destruction and hemolysis in acetone induced oxidative stress in rats (Armutcu *et al.*, 2005).

Thus, it is perceivable that the increase in vitamin E levels by the leaf extracts of *Rhinacanthus nasutus* proves its antioxidant potential.

CAROTENOIDS

Carotenoids have the capacity of quenching singlet oxygen and acting as free radical scavengers and antioxidants *in vitro* (Pavia and Corcepcion, 2006). Carotenoids and other antioxidant pigments are involved in several physiological

processes and signaling in animals, which cannot synthesize them and therefore, must acquire them from food (Biard *et al.*, 2005).

The ability of carotenes as antioxidants is attributed to the stabilization of organic peroxide free radicals within its conjugated alkyl structure (Murray, 1998). Carotenoids can protect lipids from peroxidative damage (Valko *et al.*, 2004).

Vitamin A levels in the liver slices decreased significantly when assaulted with H_2O_2. *Rhinacanthus nasutus* leaves were very effective in maintaining the level of carotenoids. The methanolic extract was more effective than the aqueous extract in this regard.

The results of Gill *et al.* (2007) support the theory that consumption of water cress (cruciferous vegetables) can decrease the damage to DNA and cause a possible modulation of antioxidant status by increasing carotenoid concentration. This study is in agreement with our observations.

Administration of β-carotene during CCl_4 treatment reduced several signs of fibrosis (Knook *et al.*, 1995). β-carotene attenuated liver cirrhosis induced by thioacetamide, which was suggested to be due to the scavenging of free radicals by β-carotene (Wardi *et al.*, 2001). Seifen *et al.* (1995) reported that β-carotene administration could prevent the long term loss of retinoids from the CCl_4 injured liver in rats.

Murthy *et al.* (2005) reported that the carotenoids obtained from *Spirulina platensis* exerted higher antihepatotoxic effect. Vitamin A administration has been reported to prevent hepatic injury caused by CCl_4 treatment (Noyan *et al.*, 2006).

It is evident from these studies that an increase in carotenoid concentration can be effective in the defense against oxidative stress. In the present dissertation, the administration of *Rhinacanthus nasutus* leaf extracts increased the levels of

carotenoids in the oxidant-assaulted liver slices, proving the strong protective action of the leaves.

REDUCED GLUTATHIONE

Glutathione plays a major role in the cellular protection against oxidative damage. Depletion of GSH renders the cell more susceptible to oxidative stress (Das and Vasudevan, 2005). Hepatic GSH level was sufficiently maintained in epaltes treated mice to counteract the increased formation of free radicals (Hewawasam *et al.*, 2004).

In the present study, when goat liver slices were exposed to the oxidant (H_2O_2) alone, the levels of GSH were depleted. The co-exposure to *Rhinacanthus nasutus* leaf extracts reverted this trend and increased the GSH levels to a statistically significant extent.

The administration of *Indigofera oblongifolia* prevented the decrease in SOD, CAT, GPx and GSH caused by CCl_4 and restored the levels towards normalcy in the liver of rats (Shahjahan *et al.*, 2005). An ethanolic extract of *Thymbra spicata* increased the levels of GSH in hypocholesterolemic mice (Avci *et al.*, 2006). Yeon *et al.* (2005) have reported that the methanolic extracts of *Saururus chinensis* enhanced the GSH levels to counteract the oxidative stress buildup in murine macrophages.

Long term administration of *Salvia miltiorrhiza* increased the level of hepatic GSH in CCl_4 induced liver injury. Sanchinone, a lignan from *Saururus chinensis*, attenuated the CCl_4 induced toxicity by increasing GSH, SOD and GPx in rat hepatocytes (Sung *et al.*, 2000).

In our study, an increase in GSH by the leaf extracts of *Rhinacanthus nasutus* presents very significant implications in the manifestation of the antioxidant defense by the leaves.

LPO

Lipid peroxidation is a free radical chain reaction and the reactive oxygen species can accelerate lipid oxidation (Ani *et al.*, 2006). Exposure to H_2O_2 significantly elevated the extent of LPO in the goat liver slices which implies the severity of oxidative stress, with the methanolic extract offering maximum protection.

Tilak *et al.* (2003) have reported that the methanolic extract of *Terminalia arjuna* rendered maximum protection against lipid peroxidation by inhibiting TBARS formation by 100% and LOOH formation by 90%. A methanolic extract of *Stachys* species was found to be more effective in an enzyme-independent lipid peroxidation system (Haznagy-Radnai *et al.*, 2006). *Annona squamosa* extracts showed better dose-dependent prevention towards the generation of lipid peroxides (Baskar *et al.*, 2007). The chloroform extracts of *Grateloupia filicina* reduced the levels of lipid peroxidation (Athukorala *et al.*, 2003).

Treatment with the root extract of *Withania somnifera* dose dependently and significantly reduced the lipid peroxidation induced by reserpine in rat brain homogenate (Naidu *et al.*, 2006). An extract of *Ganoderma lucidum* decreased the level of hepatic MDA level in CCl_4 intoxicated rats (Lin and Lin, 2006). Mandal and Das (2005) reported that the two fold increase in conjugated diene by the induction of CCl_4 was decreased to normal level by galactosylated liposomal quercetin pre-treatment.

In tune with these reports, the present study revealed that the methanolic extract of the leaves of the candidate plant could influence the antioxidant defense status of the oxidatively stressed (H_2O_2) goat liver slices to a considerable extent.

Therefore, for the subsequent analyses of the study, only the methanolic extract of *Rhinacanthus nasutus* leaves was employed.

ANTIOXIDANT STATUS ASSESSED IN CHICK EMBRYO FIBROBLASTS

The next *in vitro* model used in the present study is the fibroblast cells isolated from the chick embryo. Primary cells harvested directly from an organism and can be grown for several weeks *in vitro* in specialized cell culture medium before the cells undergo senescence. Primary cells are especially sensitive to chemicals, toxins and viruses and are often used for various research and industrial applications (http://www.oie.inteleng/ norms/ manual /A_00081.htm)

Apoptosis represents the physiological way to eliminate excessive cells during embryogenesis and tissue remodelling. Under these conditions, apoptosis occurs in a controlled environment where dying cells are promptly removed by phagocytosis and replaced by new cells generated by mitosis (Guicciardi and Gores, 2007).

Therefore, in the present study, the methanolic extract of *Rhinacanthus nasutus* leaves was analysed for its antioxidant potential as well as antiapoptotic effects in oxidatively stressed (H_2O_2) chick embryo fibroblasts.

The enzymic (SOD, CAT, Px and GST) and non-enzymic (vitamins C, E, A and reduced glutathione) were assessed in the primary cultured cells after exposure to H_2O_2, in the presence and the absence of *Rhinacanthus nasutus* leaf extract.

The results showed that both enzymic and non-enzymic antioxidants significantly decreased upon H_2O_2 stress. This stress induced effect was effectively nullified by the leaf extract of *Rhinacanthus nasutus*. The analysis of LPO in the cells also reiterated these findings.

The toxicity caused by H_2O_2 is normally accompanied with the increase of lipid peroxides. Pretreatment of the intestinal epithelial cells with *Rheum*

tanguticum polysaccharides were reported to significantly elevate cell survival, SOD activity and decreased the level of MDA, LDH activity and cell apoptosis (Liu *et al.*, 2005b).

Ramprasath *et al.* (2006) have reported that in carcinoma-bearing animals, the activities enzymic and non-enzymic antioxidants were increased. Carotenoid ROS quenching capacities control, both *in vitro* and *in vivo*, the polymorphonuclear neutrophils ROS generation and probably protect these cells against DNA, membrane lipid and protein damages during oxidative burst (Walrand *et al.*, 2005). Exposure of human bladder cancer cells to a *Ginkgo* extract produced an adaptive transcriptional response, augmenting its antioxidant status and inhibiting DNA damage (Ertekin, 2004).

Glutathione levels increased significantly when compared with the control group in mouse liver when the animals were treated with an ethanolic extract of *Nigella sativa* seeds. These results suggested that the increase in glutathione levels in plant treated group was due to lowering of the toxicity of free radicals in animal tissues (Musa *et al.*, 2004).

Individual antioxidants such as vitamins C, E and carotenoids can decrease the oxidative damage to DNA in *in vitro* model systems, in cultured cells and in human supplements (Collins and Harrington, 2003). *Salvia officinalis* extracts modulated the antioxidant status of the cells by increasing glutathione content against H_2O_2 induced DNA damage in CaCo-2 cells (Aherne *et al.*, 2006). Kumar and Gupta (2003) have demonstrated that a significant decrease in MDA and an increase in glutathione and catalase levels were observed in oxidatively stressed rats treated with the aqueous extract of *Centella asiatica*.

The observations of Geetha *et al.* (2002) suggest that the alcoholic extracts of leaves and fruits of *Seabuckthorn* have marked cytoprotective properties, which could be attributed to the antioxidant activity. Cell-based examinations (YPEN-1

cells, a rat prostate endothelial cell line, and HEK 293T cells, human kidney epithelial cells) of EtOAc fraction of *Sophora flavescens* was carried out via the inhibition of *t*-BHP (*tert*-butylhydroperoxide)-induced intracellular ROS generation. The study revealed that the increased antioxidant effects of the EtOAc fraction of *Sophora flavescens* were primarily implemented by the prenylated flavonol (Jung *et al.*, 2008).

The findings of Jaksa *et al.* (2005) suggested that the supplementation of *Strobilanthes crispus* extract on diethylnitrosamine and 2-acetyl-aminofluorene induced rats reduced the severity of hepatocarcinogenesis as shown by the increase in the tumor marker enzyme GST.

The antioxidative activity of geranium (*Pelargonium inquinans* Ait) was investigated *in vitro* by Piao *et al.* (2008) in oxidatively stressed renal epithelial LLC-PK(1) cells by the radical generator, 2,2'-azobis(2-amidinopropane) dihydrochloride, which suggested that geranium had an excellent antioxidative potential.

Crude methanol extracts of leaves and twigs from *Ledum groenlandicum* showed a strong antioxidant activity using a cell based-assay (DLD-1 colon carcinoma and A-549 lung carcinoma cells) (Dufour *et al.*, 2006). Oral administration of ethanol extract of *Bauhinia variegata* effectively suppressed liver tumor in rats induced by N-nitrosodiethylamine as revealed by a decrease in lipid peroxidation, glutathione peroxidase and glutathione S-transferase. The extract produced an increase in enzymatic antioxidant (superoxide dismutase and catalase) levels and total proteins (Rajkapoor *et al.*, 2005).

Mera *et al.* (2001) showed that the treatment with *Nigella sativa* decreased the elevated MDA concentrations, increased the lowered GSH concentrations, and prevented lipid peroxidation-induced liver damage in diabetic rabbits. Intratracheal administration of bleomycin to rats reduced the body weight,

enzymic antioxidants (superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase) and non-enzymic antioxidants (reduced glutathione, vitamin C, vitamin E and vitamin A). Intraperitoneal administration of epigallocatechin-3-gallate significantly improved the body weight, enzymic and non-enzymic antioxidants and considerably decreased the lipid peroxidation marker levels (Sriram *et al.*, 2008). These results correlate with our own, wherein oxidative stress decreased the antioxidant levels, which were significantly improved by the plant extract treatment.

EFFECT OF *Rhinacanthus nasutus* LEAF EXTACTS ON OXIDATIVE STRESS-INDUCED APOPTOTIC DEATH IN VARIOUS TYPES OF CELLS

Prolonged oxidative stress in the cellular environment that cannot be counteracted by the system's endogenous antioxidant defense, results in the cell committing itself to the programmed death process namely apoptosis. This process can be followed experimentally by various tell-tale cellular and nuclear events. Several agents, especially plant extracts or their components, have been known to influence the process of apoptosis.

In the present study, the effect of *Rhinacanthus nasutus* leaf extract on oxidative stress-induced apoptosis was analysed in untransformed (chick embryo fibroblasts and *Saccharomyces cerevisiae*) and transformed (Hep2 laryngeal carcinoma) cells. Oxidative stress was induced in the normal cells using H_2O_2, while the same was done in the cancer cells using etoposide, a standard chemotherapeutic drug known to act via oxidative stress-induced apoptosis. Apoptotic markers like morphological changes (by giemsa staining), nuclear changes (by EtBr, PI and DAPI staining), the extent of cell death (by SRB and MTT) and DNA fragmentation were followed. The results obtained are discussed below.

MORPHOLOGICAL CHANGES ASSOCIATED WITH APOPTOSIS

Morphological characteristics of apoptotic cells can be identified with membrane blebbing, clusters of membrane-bound apoptotic bodies and chromatin associated in compacted masses (Liu et al., 2005a). The most observable morphological changes that characterize apoptosis are cell shrinkage and membrane blebbing (Bortner and Cidlowski, 2002). Oxidative stress caused by H_2O_2 administration has been reported to induce cell damage and eventual cell death (Yashioka *et al.*, 2006).

In the present study, oxidative stress caused a steep increase in the number of cells exhibiting morphological changes associated with apoptosis. The leaf extract of *Rhinacanthus nasutus*, when administered alone, evoked a differential response depending on the cell type. In the normal cells (chick embryo fibroblasts and yeast cells), the extract did not induce apoptosis. However, there was a marked increase in the number of apoptosing cells in the cancer (Hep2) cells exposed to *Rhinacanthus nasutus* leaf extract. These results suggest that *Rhinacanthus nasutus* leaves possess anticancer activity, and this action is mediated by selective cytotoxicity towards cancer cells. Additionally, oxidative stress-induced cellular death was effectively counteracted by the presence of *Rhinacanthus nasutus* leaf extracts in the normal cells. But in the cancer cells, the leaf extract caused no change in the extent of cytotoxicity of etoposide.

These observations are highly significant as it can be inferred that *Rhinacanthus nasutus* exerts anticancer effects; it protects normal cells against stress-induced death, while exerting no such protection to cancer cells.

Several studies have been reported in the literature, wherein apoptosis has been followed using the typical morphological features. The plant extract of *Selaginella bryopteris* showed protective action against stress-induced (H_2O_2) cell death in a number of experimental systems including mammalian cells (Sah *et al.*,

2005). *Strontium ranelate* was shown to inhibit H_2O_2 induced apoptosis in cultured osteoblasts (Senkoylu *et al.*, 2008).

Cheng *et al.* (2007) quantified apoptosis in HL-60 cells treated with cat's claw. In the same cells myricetin caused apoptotic body formation, which is preceded by membrane blebbing (Ko *et al.*, 2005). Morphological changes were observed in human neuroblastoma cell line treated with thimerosal (Humphrey *et al.*, 2005). Celecoxib treatment induced apoptosis associated morphological changes in K562 cells (Subhashini *et al.*, 2005).

Occurrence of true apoptosis in yeast was demonstrated in oxygen stress-induced cell death in *Saccharomyces cerevisiae* (Madeo *et al.*, 2002). There are several reports indicating that low doses of H_2O_2 trigger apoptosis in yeast with similar morphological changes as mammalian apoptosis (Ahn *et al.*, 2005).

Curcuma aromatica extract induced apoptosis and inhibited angiogenesis in Ehrlich Ascites Tumour cells *in vivo* as shown in the typical apoptotic morphology including condensed kidney-shaped nucleus, membrane blebbing and formation of apoptotic bodies in the extract treated cells (Thippeswamy and Salimath, 2006).

BC-4, an isomeric compound isolated from the plant *Boswellia carteri Birdu* could induce apoptosis of human fibrosarcoma HT-1080 cells as proved by Wright–Giemsa staining (Zhao *et al.*, 2003). When T47D cells were treated with trans-resveratrol, apoptosis was established and the apoptotic morphology was viewed under microscope after giemsa staining (Alkhalaf, 2007).

Zhang *et al.* (2008) have demonstrated that wogonin, a mono flavonoid extracted from the root of *Scutellaria baicalensis* could effectively inhibit the proliferation of several cancer cell lines as determined by giemsa staining. Julibroside J8 isolated from the *Albizia julibrissin* induced apoptosis-associated morphological changes in HeLa cells (Zheng *et al.*, 2006). Liu *et al.* (2004)

suggest that diosgenyl saponins have the properties to induce mitotic arrest and apoptosis.

Hu *et al.* (2006) investigated the effect of adriamycin in Tca 8113 cells, which caused apoptotic body formation, which is preceded by membrane blebbing. The effect of lidamycin in human hepatoma BEL- 7402 cells, showed cell multinucleation observed by giemsa staining (Gao *et al.*, 2007a).

Icariin, a flavonoid isolated from *Epimedium* counteracts the apoptotic effect caused by hydrogen peroxide in human umbilical vein endothelial cell line ECV-304 in a concentration-dependent manner (Wang and Huang, 2005). Costa *et al.* (2008) have reported that pristimerin, a compound isolated from *Maytenis flicitolia*, induced necrosis in a concentration-dependent manner in human tumour cell line (HL-60). Compounds isolated from garlic were shown to induce apoptosis in SH-SY5Y cells, as reflected by the morphology visualized by Wright-Giemsa staining (Karmakar *et al.*, 2007).

The characteristic apoptotic morphology like membrane blebbing and cell volume shrinkage were studied in various cancer cells (Xu *et al.*, 2006). Similar effects were seen in human colon tumor HCT-15 cells (Park *et al.* 2002) as well as in retinal ganglion cancer N_{18} cells (Lin *et al.*, 2006). In another study (Hilemen *et al.*, 2004), 2-methoxy estradiol was shown to preferentially kill human leukemia cells (HL-60). Similarly, 6-formylpterin was shown to bring about heat induced apoptosis in U937 cells, as shown by giemsa staining (Wada *et al.*, 2005).

Ajith *et al.* (2006) confirmed that lovastatin induced apoptosis in ascites tumour cell lines from mice in a dose-dependent manner. *In vitro* studies of parthenolide was shown to inhibit the proliferation of human hepatocellular carcinoma cell line BEL-7402 as assessed by giemsa staining (Song and Zhang, 2006).

Ceramide derivatives A_2, B_3 and H_9 enhanced the 1,25-dihydroxy vitamin D3-induced differentiation in human HL-60 leukemia cells (Kim *et al.*, 2007b). Similar effects were brought about by gemicitabine in nine osteosarcoma cell lines (Ando *et al.*, 2005). Baatout *et al.* (2005) examined epigallocatechin-gallate (a tea component), resveratrol (a wine component) and curcuma on cell proliferation and radiation-induced apoptosis using giemsa staining in human leukemic cell line EOL-1. Etoposide induces apoptosis in U-937 GTB cells (Martinsson, 2001).

The results of the present study revealed that the methanolic extract of *Rhinacanthus nasutus* leaves can exert a differential response against the oxidative stress-induced apoptosis in different types of the cells.

NUCLEAR CHANGES ASSOCIATED WITH APOPTOSIS

The nuclear changes associated with apoptosis were quantified by EtBr staining, propidium iodide staining and DAPI staining. The results revealed that, in all the cell types studied, oxidative stress (imposed by H_2O_2 or etoposide) caused a steep increase in the number of cells that commit to apoptosis. *Rhinacanthus nasutus* leaf extract administration showed no cytotoxicity in the normal cells (chick embryo cells and yeast cells), but significant cytotoxicity towards Hep2 cells. These results show that the anticancer potential of the leaf extract is selectively exerted against the cancer cells, while sparing the normal cells. Additionally, it was also noticeable that *Rhinacanthus nasutus* leaf extract protected normal cells from the death induced by H_2O_2, while no such response was exerted against the cancer cells.

ETHIDIUM BROMIDE STAINING

A dual staining with acridine orange (AO)/ EtBr has been used by several researchers to study the apoptotic cells (Kalvelyte *et al.*, 2003). Studies have shown that pomegranate extracts and genistein had significant growth inhibition

by inducing apoptosis in human breast cancer cells MCF-7, which was assessed by AO / EtBr staining (Jeune *et al.*, 2005).

Several macrocyclic diterpenes isolated from the methanol extracts of *Hungarian euphorbia* were able to induce moderate apoptosis in human colon cancer cell line, which was evaluated using AO / EtBr (Engi *et al.*, 2007). Two phytochemicals, resveratrol and citroflavon-3-ol, and four plant extracts (grape seed polyphenols, olive oil extract, bearberry and *Echinacea*) examined for their effect on the viability by the EtBr assay provided evidence for strong protection against oxidative stress in H_2O_2 stress-induced U937 cells (O'Brien *et al.*, 2006).

A series of furanoacridones isolated from *Ruta graveolens* and two acridone alkaloids (arbosinine and evoxanthine) were investigated using the human cell lines HeLa, MCF-7 and A431, demonstrated by AO / EtBr. Among these, arbosinine proved best in inhibiting the proliferation of all three cell lines and the furanoacridones showed excellent potential as a new anticancer drug (Réthy *et al.*, 2007).

Ye *et al.* (2005) have reported that grifolin, an active substance isolated from *Albatrellus confluens* possessed apoptosis inducing activity in CNE1, HeLa, MCF-7 and SW480 cells. Curcuminoids isolated from *Zingiber cassumuna* were found to be effective in protecting the oxidatively stressed rat thymocytes (H_2O_2) as investigated using a flow cytometer and ethidium bromide (Nagano *et al.*, 1997).

In arsenic trioxide treated H9C2 cardiomyocytes, resveratrol significantly increased cardiomyocyte viability and attenuated cell apoptosis as measured by acridine orange / ethidium bromide staining (Zhao *et al.*, 2008). Moosavi *et al.* (2005) have documented the chromatin condensation in jurkat cells treated with 3-hydrogen wadaphnin isolated from *Dendrostellera lessertii* using acridine orange / ethidium bromide staining.

Results reported by Masuda *et al.* (2002) indicated that deoxy podophyllotoxin contributes to the cytotoxic action of the methanolic extract from the leaves of *Hernandia nymphacaefolia* on K562 cells using a flow cytometer with fluorescent probes, ethidium bromide and annexin V-FITC.

Ethidium bromide staining of Chinese Hamster Ovarian cells (CHO-K) showed that the extract of *Cochlospermum reguim* significantly reduced the cell proliferation and induced apoptosis (Ceschini and Campos, 2006). Fluorescent staining of cultured peripheral blood mononuclear cells with ethidium bromide showed the apoptotic morphology induced by cycloheximide (Baskic *et al.*, 2006).

The ethanol-soluble extract of *Letinula edodes* significantly decreased cell proliferation of murine skin carcinoma cells as revealed by ethidium bromide and acridine orange staining (Gu *et al.*, 2005; Gu and Belury, 2005). Hydrogen peroxide was shown to induce apoptosis or necrosis by ATP-dependent apoptosome formation in T-lymphoma jurkat cells (Saito *et al.*, 2006).

Jakopec *et al.* (2006) have reported that staining with AO / EtBr showed that HeLa cells commited apoptosis when treated with diazene N-phenyl-2(2-pyridinyl) diazene carboxamide.

PROPIDIUM IODIDE STAINING

Propidium iodide staining is another routine parameter used in most studies centering on apoptosis. Propidium iodide staining is based on the principle that apoptotic cells, among other typical features are characterized by DNA fragmentation and consequently loss of nuclear DNA content (Riccardi and Nicoletti, 2006). Addition of propidium iodide also helps to distinguish the early apoptotic cells from late apoptotic or necrotic cells since propidium iodide cannot enter the cells in the early stages of apoptosis when the membrane integrity is intact (Homburg *et al.*, 1995).

The nuclear morphological analysis revealed that with a physalin isolated from *Acnistus arborescens* induced apoptosis in K562 cells (Rocha *et al.*, 2006). Pretreatment of human leukemic cells with the phenol, rutin, rendered them more susceptible to apoptosis induced by Natural Killer cells (Dedoussis *et al.*, 2005).

Propidium iodide staining has been used as a measure to characterize the conditions inducing programmed cell death in yeast (Knorre *et al.*, 2005). A carbazole alkaloid caused an increase in propidium iodide staining implicating death by apoptosis in human leukemic K562 cells (Cai *et al.*, 2005). Treatment with a phenol rich fraction showed increased cell viability and exerted an apparent suppressive effect on H_2O_2 induced cultured SH-SY5Y cells as assessed by propidium iodide staining (Jung *et al.*, 2008).

The findings of Kaviarasan and Anuradha (2007) demonstrated that fenugreek seed polyphenolic extract acts as a protective agent against ethanol-induced increased hepatocyte apoptosis as investigated by propidium iodide staining. Kitts *et al.* (2007) examined the cytotoxic activity of *Panax quinquefolius* leaf extract on THP-1 leukemia cells. Propidium iodide staining showed a significant increase in apoptosis.

Some Chinese herbs screened for antiproliferative properties in human HaCaT keratinocyte model induced cell arrest and death as shown by propidium iodide staining (Tse *et al.*, 2007). The isolated component, sitibinin, from the crude seed extract of silymarin increased the apoptotic rate in Geo and HCT 116 cell lines (Hoghan *et al.*, 2007). An ether extract of *Cremanthodium humile* brought about the same effect in HeLa, A549, Hep G2 and SW 40 cell lines (Li *et al.*, 2007a). Isoflavones, including genistein, from the flower of Lupine (*Lupinus leteus*) at high concentration, induce apoptosis in Chinese hamster ovary cells as assessed by PI staining of cells (Rucinska *et al.*, 2007).

The extent of propidium iodide staining (along with other techniques) has been used to study the antiproliferative, antioxidative and apoptosis-inducing effects in cultured cells (Moongkarudi *et al.*, 2004). England *et al.* (2004) used the same technique to ascertain that N-acetyl-L-cysteine inhibited apoptosis in HL-60 tumor cells by the chemotherapeutic agent etoposide.

Increased apoptosis was seen in HepG2 cells treated with genistein (Chodon *et al.*, 2007). A similar effect was seen in human umbilical vein endothelial (HUVE-12) cells treated with genistein derivative, cytosine arabinoside Ara C, as determined by PI staining (Fu *et al.*, 2008).

PI staining of human promyelocytic leukemia cells (HL-60) showed increased apoptosis brought about by chelerythrine and its derivative dihydrochelrythrine in a dose-dependent manner (Vrba *et al.*, 2008). Ascorbic acid significantly inhibited adult retinal pigment epithelial cell proliferation by inducing apoptosis in a dose-dependent manner (Heckelen *et al.*, 2004). Aspirin also brought about the same effect in HeLa cells (Kutuk and Basaga, 2003).

Vijaya *et al.* (2007) examined the cytotoxic activity of saline extract prepared from ginger on Hep2 cell line. PI staining showed that the extract exerted a dose-dependent suppression of cell proliferation by chromosome condensation.

Propidium iodide stained cells in the present study showed that the methanolic extract of *Rhinacanthus nasutus* leaves reduced apoptosis in primary chick embryo fibroblasts and *Saccharomyces cerevisiae*, while not influencing Hep2 cells, which reiterates its anti-apoptotic property against normal cells and its anticancer property.

DAPI STAINING

DAPI is a fluorescent stain that is used to highlight the nuclear changes during apoptosis. Cell number and density analyses can also be carried out using DAPI staining (Konduri *et al.*, 2007).

DAPI staining was performed only in etoposide induced Hep2 cells. Upon staining with DAPI, the apoptotic nucleus exhibits much stronger fluorescence than the nucleus of normal (control) cells. Intense staining of apoptotic nucleus may result from the increased permeability of the dye and higher binding to DNA owing to altered chromatin configuration (Cohen *et al.*, 2003).

DAPI staining was used to assess curcumin induced nuclear condensation, a typical feature of apoptosis, in two human colon cancer cell lines SW480 and SW620. Curcumin was found to induce apoptosis in SW620 cells more efficiently than SW480 cells (Rashmi *et al.*, 2003).

The results of DAPI staining in our studies indicate that etoposide exposure caused a very significant number of Hep2 cells to commit to apoptosis. The presence of the methanolic extract of *Rhinacanthus nasutus* leaves brought the proportion of apoptosing cells down, showing its protective effect against the toxicity of etoposide. However, the extract by itself, increased the apoptotic death in the cells.

Parekkadan *et al.* (2007) and Zhang *et al.* (2007) observed a marginal increase in internucleosome degradation in untreated hepatocytes as measured by DAPI. Li *et al.* (2007b) performed DAPI staining and found zoledronic acid treatment caused line-1 cells to commit to apoptosis. Ho *et al.* (2007) also used DAPI to detect apoptosis in human THP-1 monocytic leukemia cell line treated with pigment epithelial–derived factor.

CELL VIABILITY ASSAY

MTT

Several studies showed that MTT assay has been used to assay the viability of cells subjected to oxidative stress and for establishing the protection rendered by medicinal plants. Many researchers have used MTT as a tool to measure the cell viability and proliferation rate (Somasundar *et al.*, 2005; Vona-Davis *et al.*, 2005) and also for analyzing a dose dependent inhibition of cell viability by various agents (Sreekanth *et al.*, 2007).

Our results clearly indicate a marked decrease in the cultured primary fibroblast cell survival rate upon exposure to the oxidant. However, methanolic extract of *Rhinacanthus nasutus* leaves increased the cell viability. H_2O_2 exposure also brought down the viability of *Saccharomyces cerevisiae* cells drastically. *Rhinacanthus nasutus* leaf extract increased the viability of the yeast cells subjected to oxidative stress.

Etoposide as well as *Rhinacanthus nasutus* leaf extract independently caused an increase in the extent of cell death in the cancer cells. However, the extent of survival increased slightly when etoposide and the extract were co-administered, showing that *Rhinacanthus nasutus* is capable of decreasing the toxicity of etoposide.

Zhen *et al.* (2006) analysed the cell viability in pC12 cells by MTT method, wherein the cell viability was reduced by H_2O_2 and *Cuscuta chinensis* increased the survival rate of pC12 cells and inhibited the apoptosis induced by H_2O_2. Strawberry phenolics were also reported to evoke the same results as determined by the MTT assay (Heo and Lee, 2005).

Cell proliferation estimated by the MTT assay showed that the dihydro cucurbitacin B inhibited cellular proliferation in a dose- and time-dependent

manner in human breast cancer cells (Yang *et al.*, 2007). Malikova *et al.* (2007) investigated the effects of several selected brassino steroids and related steroids on the viability of fibroblasts and human cancer cell lines. All of the tested sterols and related compounds had extremely weak or no detectable activity.

Human embryonic lung fibroblast cell line (MCC-5) and mouse macrophage cells were protected by a peptide from a protein of the oyster, *Crassostrea gigas*, as shown by the MTT assay (Qian *et al.*, 2008). Saponins from *Tribulus terrestris* induced apoptosis in hepatoma cells as shown by SRB and MTT assays (Sun *et al.*, 2004). Igenamine G, an alkaloid from *Pachychalina alcaloidifera* proved to be effective against human proliferating lymphocytes, as determined by the MTT assay (Cavalcanti *et al.*, 2008).

MTT assay also showed the antiproliferative effects of Panaxydol, from *Panax notogingseng* against rat malignant glioma cells in a dose-dependent manner (Hai *et al.*, 2007). Souli *et al.* (2008) established, using MTT, that indole-3-carbinol present in cruciferous vegetables inhibited the tumour growth in mouse PC cell line TRAMP-C2 and bovine capillary endothelial cells. Gambogic acid chemosensitizes BGC-823 / DOC gastric cancer cells to docetaxol, an anticancer drug, which was assessed by the MTT assay (Wang *et al.*, 2008a).

Human umbilical vein endothelial cells (HUVEL) subjected to oxidative stress were protected by the antagonist effect of telmisartan, an angiotensin II inhibitor as revealed by the MTT assay (Cianchetti *et al.*, 2008). K562 and Jurkat were the most sensitive cells for *Linum persicum* and in cultures of tumour cell lines treated with *Euphorbia cheiradevia*, the main inhibitory effect was for Jurkat cells after MTT assay (Amirgofran *et al.*, 2006).

The cell viability of an ethyl acetate extract of *Cudrania tricupsidata* stem bark treated HL-60 leukemic cells was determined by the MTT assay. The results

suggested that the cytotoxicity of the crude extract from the extract against HL-60 cells was due to apoptosis (Seo *et al.*, 2001).

HPS-1, an isolated polysaccharide component from the roots of *Hedyserum polybotrys* significantly inhibited the proliferation of human hepatocellular carcinoma Hep G2 cells and human gastric cancer MGC-803 cells, as evidenced by the MTT assay (Li *et al.*, 2007c). RGC-5 cells exposed to different durations and intensities of optical radiations were assayed for cell viability using MTT reduction assay. The study showed a decrease in the cell viability (Wood *et al.*, 2008).

Inhibition of Gli-1 mRNA in Huh7 cells through Gli-1 siRNA reduced the cell viability, where the viability was analyzed by MTT assay (Chen *et al.*, 2008). Cell viability, in the presence or absence of EGFR inhibition and cisplatin-induced apoptosis, were assessed by the MTT assay in SCCHN cells (Wang *et al.*, 2008b).

Thus, in the present study, quantification of the cell death using MTT assay showed the cytoprotective effects of *Rhinacanthus nasutus* leaves. This observation strengthens their antioxidant and antiapoptotic effects. MTT assay was effectively used to strengthen the data of apoptosis induced by H_2O_2 and its counteraction by *Rhinacanthus nasutus* leaf extract (methanol) in cultured primary fibroblast cells, *Saccharomyces cerevisiae* cells.

SRB

SRB assay is a colorimetric assay used to evaluate the cytotoxicity in cells subjected to oxidative stress and various chemical treatments. The assay for cytotoxocity in the present work was also done by the sulphorhodamine B assay, which estimates the amount of protein content, which is directly proportional to the cell viability.

The results obtained after the calculations for cytotoxicity as determined by the SRB assay showed that the viability reduced drastically in the H_2O_2 treated group of chick embryo fibroblasts, wherein the cell viability increased in the methanolic extract treated cells despite the apoptotic stress.

H_2O_2 exposure caused a significant decrease in the survival of *Saccharomyces cerevisiae* cells. The cytotoxicity of H_2O_2 was nullified by *Rhinacanthus nasutus* leaves in these cells.

The results of the SRB assay in Hep2 cells followed a similar trend to that of MTT assay. It can thus be deduced that the leaf extract can induce death in cancer cells.

Several studies have used the SRB assay to assess the extent of cytotoxicty of herbal extracts and products. The effect of liposomal incorporated sclareol and free sclareol in inducing apoptosis in the growth of human colon tumour (HCT 16) in SCID mice was confirmed with the SRB assay (Hatziantonion *et al.*, 2006). Wiyakrutta *et al.* (2004) found that 60 medicinal plants were effective against human oral epidermal carcinoma cells and breast cancer cells using SRB assay.

The cytotoxic effects of methanol extract of *Tulbaghia violacea* bulbs and leaves on cell growth and viability of cancer cell lines were established by SRB (Bungu *et al.*, 2006). Lopez-Lazaro *et al.* (2003) have reported that the methanolic extract of *Digitalis purpurea* screened for cytotoxic activity against three human cancer cells using SRB assay showed high cytotoxicity. Arecoline, the main areca alkaloid in the betel quid, is reported to have cytotoxic, genotoxic and mutagenic effects in various cells (Lee *et al.*, 2006a).

Konduri *et al.* (2007) measured the *in vitro* proliferation of human prostate carcinoma cells by SRB colorimetric analysis and observed a dose dependent growth stimulation when treated with testosterone. The effect of anthocyanidins

and anthocyanins against Dox-induced cytotoxicity was evaluated using SRB assay in H9C2 cells. The protective effect was higher in cyanidins and delphidin than other anthocyanidins (Choi *et al.*, 2007).

SRB assay also revealed that tamoxifen potentiates epirubin toxicity in sensitive and resistant breast cancer cell lines, MCF-7 and NCI-adr (Azab *et al.*, 2005). Dichloromethane and n-butanol extracts of *Hippeastrum vittatum* showed antiproliferative activity against five human cell lines (HT 29 colon adenocarcinoma, H460 non small cell lung carcinoma, RXF393 renal cell carcinoma, MCF-7 breast cancer and OVCAR-3 epithelial ovarian cancer cells) in *in vitro* studies (Silva *et al.*, 2008).

Harrington *et al.* (2000) analyzed the *in vivo* and *in vitro* activity of uncapsulated doxorubicin and cisplatin and their pegylated liposome encapsulated counterparts against human squamous cell cancer of head and neck using SRB assay. SRB assay has also shown the induction of apoptosis in HCT 16 colorectal cancer cells exposed to ruthenium II organometallic complex RM175 (Hayward *et al.*, 2005).

The combination of STI-571 with radiation or cisplatin had an additive antiproliferative effect in SKNMC cells. A similar effect was observed in human MCF-7 breast cancer cells, which was determined by sulphorhodamine B cytotoxicity assay (Yerushalmi *et al.*, 2007).

The cytotoxic effect of etoposide, vinblastine sulfate and decarbazine against b16-F10 and HMEC-1 cells was determined by SRB assay (Dandamudi and Campbell, 2007). The substituted pyridazino (4,5-b) phenzine-5,12-diones and tri / tetraazabenzo(a)fluorine-5,6-diones were evaluated for their cytotoxic activities by the SRB assay against several human cancer cell lines like A549 (lung), SK-OV-3 (ovarian), SK-MEL (melanoma) and HCT 15 (colon). Almost all

the synthesized compounds presented higher toxicity than that of doxorubicin against the cancer cell lines (Lee *et al.*, 2006c).

From the cell viability assays in the present study, it can be inferred that oxidative stress caused significant death in primary fibroblast cells, *Saccharomyces cerevisiae* cells and Hep2 cells. The leaves of *Rhinacanthus nasutus* offered significant protection against cell death in the oxidatively stressed normal cells, while not hindering with the cytotoxic effects of etoposide in cancer cells.

DNA FRAGMENTATION

Endonucleases such as caspase activated DNase C CAD and lysosomal DNase II has been found responsible for DNA fragmentation in apoptotic cells (Derakshan, 2007). The structural changes during apoptosis occur within the nucleus, where the chromatin condenses and aggregates and the DNA is degraded. DNA is cleaved in a systematic manner to provide nucleosomal fragments (Ribeiro *et al.*, 2006). The present study focused on following the apoptotic events induced by oxidative stress in various cell models. The DNA fragmentation data followed the same trend as the extent of apoptosis in the different treatment groups in all the cell types studied. These observations strengthen the conclusion that *Rhinacanthus nasutus* leaf extract can protect normal cells against oxidative stress, can exert anticancer action and render the cancer cells susceptible to the action of chemo-therapeutic agents.

Many studies have taken the extent of DNA fragmentation as a reliable index of establishing the protection rendered by medicinal plants and their components. DNA fragmentation was observed in Vero cells treated with T-2 toxin (Bouaziz *et al.*, 2006). An ethanolic extract of soybean induced DNA fragmentation in MCF-7 cells (Sebastian and Thampan, 2007).

Amirghofran *et al.* (2007) demonstrated the antitumour activity of an extract of *Dionysia terneana* against K562 leukemia cell line and Jurkat cells using DNA fragmentation as an index. In another study (Rho *et al.*, 2007), DNA laddering by fragmentation was shown to indicate the antitumor activity of the chloroform extract of the root bark of *Cudrania tricuspidata* against human cancer cells U937 (O'Callaghan *et al.*, 2001).

Inagaki *et al.* (2007) demonstrated that a compound purified from the ethyl acetate extract of black soybean vinegar induced the fragmentation of DNA and the development of apoptotic bodies in U937 cancer cells. *Duchesnea indica* phenolic fraction significantly inhibited SKOV-3 cell proliferation and markedly induced apoptosis by characteristic nuclear DNA fragmentation (Peng *et al.*, 2008).

Isolated, plant-derived components like 3-hydrogen wadaphnin diterpene ester isolated from *Dendrostellara lessertii* (Moosavi *et al.*, 2005), and oleanolic acid derived from *Fructus ligustri Lucidi* have been shown to influence DNA fragmentation in human cancer cells, reflecting their anticancer activity (Zhang *et al.*, 2007). The aqueous and alcoholic extracts of *Rhodiola* significantly inhibited both apoptosis and single stranded DNA breaks induced by tert-butyl hydro peroxide (Kanupriya *et al.*, 2005).

When OVCAR-3 cells were treated with 4-hydroxyphenyl retinamide, DNA fragmentation was found to occur in a dose dependent manner (Um *et al.*, 2001). Similarly, in HL-60 cells, fragmentation occurred when exposed to myricetin (Ko *et al.*, 2004).

Etoposide-induced oxidative stress was strongly reduced by olive leaf extract and bearberry (Carpenter *et al.*, 2006). Sermeus *et al.* (2008) have reported in their study that physiological hypoxia was shown to inhibit apoptosis induced in

HepG2 cells by etoposide. Indeed, hypoxia reduced DNA fragmentation, caspase activation and PARP cleavage.

These studies are in agreement with our results, wherein the pattern of DNA fragmentation matched those of the other apoptosis markers studied. In earlier studies conducted in our laboratory (Sreeja, 2006; Sathya, 2006), DNA damage was undetectable in yeast cells treated with H_2O_2. In both these studies, agarose gel electrophoresis was the method adopted for the analysis.

In the present study, however, observable DNA damage occurred in the yeast cells upon H_2O_2 exposure, as determined by the DPA method. There is evidence that yeast cells lack linker DNA (Lowary and Wildom, 1989), which is probably the reason that the DNA damage is not observable on the gel. Thus, it is possible that the damage observed in the DPA method involves other types of damage than inter nucleosomal cleavage (like single strand breaks, etc.). More studies are needed in this direction to confirm this suggestion.

PHASE IV

Phytochemicals and plant extracts, present in fruits, vegetables, plants, herbs, and beverages, have been shown to have antioxidant potential that may modulate the etiology of certain chronic diseases (Carpenter *et al.*, 2006). Thirupathi *et al.* (2008) have reported a wide range of compounds like pyrrolizidine alkaloids, cardoquinones, tannins, phenyl propanoid derivatives and triterpenes to be bioactive.

The preliminary phytochemical screening revealed the presence of alkaloids, phenols and flavonoids in the leaves of *Rhinacanthus nasutus*. To confirm the chemical nature of the active component present in *Rhinacanthus nasutus* leaves, spectral analysis (HPLC, GC-MS) were carried out, which

identified phenolics and alkaloids as the major components as evident by the presence of two major peaks observed in the GC-MS chromatogram.

Several studies have shown that the higher antioxidant activity associated with medicinal plants is attributed to the total phenolic compounds (Galati *et al.*, 2005; Popovic *et al.*, 2006; Manjunatha, 2006). The analysis of the phytochemicals in the methanolic extracts revealed phenolics and alkaloids to be the active components, which are probably the major players in the antioxidant responses evoked by the plant. Further studies are needed to be conducted to understand the structural features of the compounds predicted from the phytochemical analyses.

Flavonoids, anthraquinones, triterpenes, sterols and other compounds isolated from *Rhinacanthus nasutus* have been shown to have potent *in vitro* antifungal activity (Send *et al.*, 1996). Rhinacanthins-C, -N, and-Q, three main naphthoquinone esters, isolated from the roots of *Rhinacanthus nasutus* were found to induce apoptosis of human cervical carcinoma HeLa S3 cells (Siripong *et al.*, 2006). The ethanolic extracts of seven plant species found in Zimbabwe were found to possess phenolics as well as antioxidant activity (Muchuweti *et al.*, 2006).

In the present investigation, several antioxidants were identified in the first phase and two other components, which possessed alkaloidal and polyphenolic characteristics, were identified in the fourth phase. It is perceivable that the bioactivity, of antioxidant and anticancer nature, observed in the leaf extract is due to all these components. Thus the present study emphasizes the protective effects rendered by the methanolic extract of *Rhinacanthus nasutus* leaves, against oxidative damage induced in various *in vitro* models, which served the purpose of substitutes to the use of live animals in research, in turn, avoids ethical issues.

The findings of the present study, thus, validates and strengthens the leaves of *Rhinacanthus nasutus* as a potential candidate for the preparation of medicinal aids caused by the oxidants such as hydrogen peroxide and etoposide. They also highlight the anticancer activity of the leaf extract.

The results obtained in the present study are summarized and the conclusions that can be drawn from them are presented in the next chapter.