Summary and Conclusion

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Biodiversity of natural resources has served not only for the primary human needs but also for healthcare, since time immemorial. The plant species mentioned in the ancient texts of Ayurveda and other Indian systems of medicines may be explored with the modern scientific approaches for better leads in healthcare.

The decrease in the use of whole animals in experimentation in the research field over the last two decades can be attributed to a number of factors. It is possible to work towards a gradual replacement of animal-based studies without compromising on scientific quality as demonstrated by the three R's approach (Reduction, Refinement and Replacement). Advances in *in vitro* technology have permitted a large variety of cells and tissues to be cultured.

Cancer is one of the major causes of death worldwide. Apoptosis is a programmed cell death, which is induced in the cancer cells to kill them. But the present therapies used for cancer have many side effects. For instance, they induce apoptosis in normal cells also. Prevention or minimization of side effects caused by the therapeutic drug is important at this moment.

Nature has diversifying plants and plant products. Among them, many are classified as under-exploited plants. The present study aimed at analyzing one such plant, *Rhinacanthus nasutus*. In spite of known uses in traditional medicine, not much documented evidence is available on the antioxidant and anti-apoptotic effects of the leaves of *Rhinacanthus nasutus*. The leaves of the plant were taken for all the analyses performed, which gains significance in that every time when the sample is collected for the analyses, uprooting the plant is not needed, thereby reducing the injury caused to the plants. This improves the biotechnological value of the study.

The importance of medicinal plants is now being widely recognized. Researchers believe natural herbs may help alleviate the harmful side effects of standard anticancer agents. Hence the development of anti-apoptotic drugs from plants or plant-derived products require special attention in this scenario. In these regards, the present study was formulated to analyze the influence of *Rhinacanthus nasutus* leaf extracts (aqueous, methanol and chloroform) on minimizing the oxidant (hydrogen peroxide in untransformed cells and etoposide in transformed cells) induced events in various *in vitro* models such as goat liver slices, cultured chick embryo fibroblasts cells, *Saccharomyces cerevisiae cells* and laryngeal carcinoma cells (Hep2).

The study was formulated in four phases. In the first phase, the leaves were analyzed for their antioxidant content. In phase II, the antioxidant efficacy 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. The effect of the leaf extract was analyzed on live cells, which included normal (untransformed) cells as well as cancer (transformed) cells, in the third phase of the study. In the final phase, phytochemical analysis of the leaves was done to identify the bioactive components present in the leaves of the candidate plant that evoke the responses against the oxidant induced damage to biomolecules.

It is evident from the results of phase I that *Rhinacanthus nasutus* leaves contained both enzymic (SOD, CAT, Px and GST) and non-enzymic antioxidants (vitamin C, E, A and reduced glutathione) and thus have a potential candidature as a source of antioxidants. Therefore, it can be used for further analysis in search of agents that can combat the various diseases associated with oxidative stress.

The efficiency of the leaf extracts in counteracting oxidative stress was analysed in phase II of the study. *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. The 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.

An array of radicals (DPPH, ABTS, and hydroxyl) has been tested against the *Rhinacanthus nasutus* leaf extracts. The analysis of the results obtained in the free radical scavenging assays showed that the leaves are very effective in counteracting pre-formed radicals and oxidants (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.

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. 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 lipids) 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. This observation substantiates the biomolecular protection of the leaves.

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 (SOD, CAT, Px and GST) and non-enzymic (ascorbate, tocopherol, vitamin A and GSH) antioxidants were assessed in the liver slices subjected to oxidative stress under the influence of plant extracts (water, methanol and chloroform).

The toxic effect of H_2O_2 in the goat liver slices was negated by the concordant treatment with the methanolic extract of the candidate plant. Thus, the study revealed that the methanolic extract of *Rhinacanthus nasutus* leaves could influence the antioxidant defense status of the oxidatively stressed (H_2O_2) goat liver slices to a considerable extent. Since, in the earlier phases of the study and in the liver slices, the methanolic extract of the leaves evoked the maximum response, only this extract was analysed in the subsequent systems.

Chick embryo fibroblasts constitute a primary cell culture system, which possesses a very high proliferative potential and a high rate of metabolism. These cells, unlike the liver slices, can be maintained alive in culture for a longer duration. Therefore, the combined and individual effects of oxidative stress and *Rhinacanthus nasutus* leaf extract was studied using these cells.

The antioxidant status assessed using enzymic (SOD, CAT, Px and GST) and non-enzymic (vitamin C, E, A and reduced glutathione) antioxidants were significantly reduced upon H_2O_2 assault in the chick embryo fibroblasts. Treatment with the methanolic extract of *Rhinacanthus nasutus* leaves increased the antioxidant potential in the primary cells. The leaf extract was found to be very effective in alleviating the toxicity of H_2O_2 in the oxidatively injured primary chick embryo fibroblasts.

In the same cells, the methanolic extract addition elicited a stronger DPPH scavenging activity over the controls, which is indicative of the protective effect of *Rhinacanthus nasutus* leaves against oxidant-induced damage. Treatment with the methanolic extract of *Rhinacanthus nasutus* in H_2O_2 treated primary cells depressed the LPO in these cells, suggesting the antioxidant protection of the leaf extracts.

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 in the present study. 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 of giemsa staining 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.

Giemsa stained cells (primary cells and yeast cells) treated with hydrogen peroxide showed well-defined apoptotic morphology, which was strongly hinderedwith by the treatment with the methanolic extract of *Rhinacanthus nasutus* leaves. When the cells were exposed to etoposide and the leaf extract, the extent of cell death was comparable to that induced by etoposide alone, suggesting that *Rhinacanthus nasutus* leaf extract does not influence the extent of apoptosis induced by etoposide.

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 treated with etoposide.

From the cell viability assays (MTT and SRB), 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, showing that *Rhinacanthus nasutus* leaves are capable of decreasing the toxicity of etoposide. Thus, our results reveal that *Rhinacanthus nasutus* leaves can render the cancer cells susceptible to the action of chemotherapeutic drugs, while protecting the normal cells from the toxic effects. This observation reveals that the leaves can be used as a supportive therapy to minimize the harmful side effects of cancer chemotherapeutic drugs.

The present study also 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 augment the anticancer action of chemotherapeutic agents.

In the fourth and final phase of the dissertation, an attempt was made to identify the active principles in the leaf extract. Preliminary qualitative analyses of the leaves revealed phenolics and alkaloids to be the major components in the leaves, which were confirmed by TLC. To confirm the nature of these components, spectral analysis (GC-MS and IR) were also conducted.

GC-MS spectrum of the sample revealed the presence of alkaloid and polyphenolic components as seen from the m/z of the fragment ions. Base peak at m/z 129 in the mass spectrum of the GC at retention time 4.14 min corresponds to the presence of alkaloid component. Base peak at m/z 141.5 in the mass spectrum

of the GC at retention time 7.8 min corresponds to the presence of polyphenolic compounds. IR spectral results reveal that the leaf extract must possess compounds that are having a hydroxyl and carbonyl group, and the presence of lactone rings indicates the presence of flavonoids or coumarins.

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 substituting the use of live animals in research, and in turn, avoiding ethical issues.

The outcome of the present study highlights the protective effects rendered by the leaf extract of *Rhinacanthus nasutus* against oxidative stress. These observations scientifically validate and strengthen the candidature of the leaves in the preparation of medicinal aids to combat the wide spectrum of myriad diseases caused by oxidative stress.

SUGGESTIONS FOR FUTURE RESEARCH

The outcome of the study has opened up several promising insights of possible research. Some of them that can be suggested for active research are given below.

- Novel drugs for alleviating the side effects of chemotherapy can be designed once the active components in the leaves are isolated.
- The active components identified can be tested for the anti-apoptotic effect.
- The anticancer potential can be further probed using various other cell lines such as KB oral carcinoma and HeLa.
- A comparative analysis of the antioxidant potential of other parts of the plant like flowers, stem and roots can also be taken up.