6. SUMMARY AND CONCLUSION

Free radicals are chemical species containing one or more unpaired electrons, like hydrogen atom, most transition metal ions, nitric oxide and oxygen, with two unpaired electrons. In living systems, free radicals are constantly generated and they can cause extensive damage to tissues and biomolecules, leading to various disease conditions, especially degenerative diseases, and extensive lysis.

The most effective way to eliminate free radicals which cause oxidative stress is with the help of antioxidants. Antioxidants prevent free radical-induced tissue damage by preventing the formation of radicals, scavenging them, or by promoting their decomposition.

Many synthetic drugs protect against oxidative damage, but they have adverse side effects. An alternative solution to the problem is to consume natural antioxidants from food supplements and traditional medicines. In almost all the traditional systems of medicine, medicinal plants play a major role and constitute their backbone. Medicinal plants are the most important source of life saving drugs for a majority of the world’s population.

One such medicinal plant is *Bacopa monnieri*, commonly known as “Brahmi”. It has been used for centuries in folklore and traditional systems of medicine as a memory enhancing, antiinflammatory, analgesic, antipyretic, sedative and anti-epileptic agent. Not many studies have been done on the antioxidant and apoptosis-modulating effects of *B. monnieri*. Hence, this study mainly focussed on assessing the antioxidant and apoptosis-modulating effects of *B. monnieri*.

The work was divided into four phases. In the first phase of the study, the different parts of the plant, namely leaves, stolon and roots, were assessed for their antioxidant status. The activities of superoxide dismutase, catalase, peroxidase, glutathione S-transferase and polyphenol oxidase, and the levels of vitamin C, vitamin E, total carotenoids, lycopene, glutathione, total phenols, flavonoids and chlorophyll were analysed.

The results obtained showed that all the three parts possessed considerable quantities of enzymic and non-enzymic antioxidants. Among the three parts tested, the
leaves possessed the maximum quantity of antioxidants analyzed, followed by the stolon and the roots. Therefore, in the further phases of the study, only the leaves of *B. monnieri* were used.

In order to identify the nature of the component rendering the maximum protection, three different extracts of the leaves were prepared in solvents of differing polarity namely, aqueous, methanol and chloroform. The extracts were tested for their radical quenching ability against a battery of radicals, namely DPPH, ABTS, H$_2$O$_2$, SO$^\bullet$, NO and OH$^\bullet$.

All the three extracts of the leaves effectively scavenged or inhibited all the radicals tested. Among the three extracts, the methanolic extract was the most effective radical scavenger, followed by the chloroform extract. The results obtained in the present study showed that *B. monnieri* leaves exhibited effective antioxidant and radical scavenging activity *in vitro*, implying their use in pharmacological and food industries due to its antioxidant properties.

In Phase II of the study, an effort was made to study the efficacy of the extracts in rendering protection to cellular biomolecules, namely lipids and DNA. For lipids, three different biological membranes of varying lipid composition were used. The membranes used were plasma membrane devoid of intracellular membranes (RBC ghosts), a mixture of plasma membrane and internal membranes (liver homogenate) and intact cells (liver slices). They were challenged with the oxidant H$_2$O$_2$ and the protection rendered by the extracts was ascertained.

The results revealed that the leaf extracts were very effective in inhibiting LPO in the different membrane systems. Since all the three systems comprise of plasma membrane lipids, the observation that a better protection occurs in homogenate than in plasma membrane and intact cell make it clear that some endogenous component in the cells may be involved. It implies that an endogenous factor in the cells, interacts synergistically with the leaf components to render better protection against lipid damage.

The DNA protective effects rendered by the extracts of *B. monnieri* leaves were studied using DNA from different sources and hierarchical levels. Both commercially available preparations and DNA from intact cells were used for the assay. They were $\lambda$ DNA (linear phage DNA), pUC18 DNA (circular plasmid DNA), herring sperm DNA
(genomic haploid DNA), calf thymus DNA (diploid eukaryotic DNA) and DNA from intact cells, namely human peripheral blood cells.

The results showed that all the three extracts were able to revert the damage caused by the oxidant, $H_2O_2$. The results obtained with the purified DNA preparations showed that the damage could not be completely reverted by the leaf extracts. The fact that the same dose of leaf extracts could completely revert the damage to basal levels in the intact cells suggests the possible involvement of some endogenous cellular component that works in conjunction with the leaf components to protect DNA.

Our results of the assays probing the extent of protection rendered by $B. monnieri$ leaf extracts to lipids and DNA strongly suggest the synergistic involvement of some, as yet unidentified, endogenous factor in the target cells, that acts in conjunction with the plant components to greatly reduce the extent of oxidative damage. Thus, our study shows that $B. monnieri$ leaves are an excellent source of naturally occurring antioxidant compounds with potent lipid and DNA damage inhibiting potential.

Among the three different extracts tested for their protective effects, the methanolic extract rendered the maximum protection in cell-free system and intact cells. Hence, in the next part of phase II, the effect of only the methanolic extract of $B. monnieri$ leaves on the extent of cell death was studied under conditions of oxidative stress.

The cells used were $S. cerevisiae$ cells, chick embryo fibroblasts (untransformed cells) and Hep2 cancer cell line (transformed cells). The oxidant used to induce oxidative stress was $H_2O_2$ in $S. cerevisiae$ and etoposide in chick embryo fibroblasts and cancer cell line.

The results obtained clearly indicated that the exposure to $H_2O_2$ resulted in a steep rise in the number of $S. cerevisiae$ (untransformed) cells undergoing apoptosis. $B. monnieri$ leaf extract, by itself, did not cause an increase in the extent of apoptosis. When co-administered along with $H_2O_2$, the plant extract resulted in a markedly decreased number of apoptotic cells. Thus, it is evident that the leaf extract protects the yeast cells from oxidative stress-induced death.

On exposure to $B. monnieri$ leaf extract, in the presence of a standard chemotherapeutic agent, etoposide, a differential response was evoked in the
untransformed and transformed cells. The number of cells undergoing apoptosis was significantly increased on exposure to etoposide in both the types of cells. The leaf extract, by itself, did not induce apoptosis in untransformed cells, but apoptosis was induced in cancer cells. This observation strongly suggested an anticancer effect of the leaves. The administration of the leaf extract effectively counteracted the apoptosis-inducing effect in primary cells, whereas, the apoptosis-inducing effect of etoposide was further augmented in Hep2 cells in the presence of the extract.

It is deducible from our results that the *B. monnieri* leaf extract protects the normal cells from oxidative death, while rendering the cancer cells more susceptible to the cytotoxic action of the chemotherapeutic drug, etoposide. Based on our results, it can be suggested that *B. monnieri* leaves can be administered as a supportive therapy during cancer chemotherapy, to minimize the toxic side effects to non-cancerous cells and to maximize the anticancer drug action.

The DNA fragmentation data followed the same trend as the extent of apoptosis in the different treatment groups in all the cell types studied, except yeast cells. DNA fragmentation was not observable in the *S. cerevisiae* cells, suggesting that yeast cells are unique in their DNA fragmentation pattern. A significant DNA damage was observed in the primary cells and Hep2 cancer cells, which was altered by the methanolic extract of *B. monnieri*.

Thus, from the results obtained, it can be inferred that *B. monnieri* leaves, alone or in combination with chemotherapy drugs, could inhibit the proliferation of cancer cell lines *in vitro*, through the mechanism of apoptosis, which suggests that *B. monnieri* is likely to be a potential drug in the treatment of cancer.

In the first two phases of the study, cell-free systems and intact individual cells were used to assess the various parameters indicating the antioxidant property of *B. monnieri* leaves. It was imperative to assess the effect of the leaf extract on cells maintained in the organ architecture, and also in the intact animal. Hence, in the third phase of the study, an attempt was made to assess the effect of methanolic extract of *B. monnieri* leaves in an *in vivo* simulated *in vitro* model, namely precision cut-liver slices, by exposing them to the oxidant. The counteracting effects of the extract were ascertained by assessing the antioxidant status in the slices.
The enzymic antioxidants analyzed were SOD, CAT, POD and GST and the non-enzymic antioxidants analyzed were ascorbate, tocopherol, vitamin A and reduced GSH. Exposure to the oxidant H$_2$O$_2$ significantly decreased the antioxidant status in the liver slices. The co-administration of the extract of *B. monnieri* improved the antioxidant status. Thus, the results of the study using liver slices revealed that the *B. monnieri* leaves possess antioxidant principles that can fight against free radical mediated disorders.

Having established the antioxidant effects of *B. monnieri* in the *in vitro* models, it was felt imperative to confirm these effects under *in vivo* conditions. Hence, as a next part of the study, the protective effect of the extract was tested in animals (male Wistar rats), by exposing them to alcohol and CCl$_4$ and studying the counteracting effects of the extract. These activities were compared with that of the standard hepatoprotective antioxidant silymarin. The experimental design was approved by the Institutional Animal Ethical Committee (623/02/b/CPCSEA).

The extent of liver damage caused by CCl$_4$ was assessed by estimating serum marker enzymes, namely AST, ALT, ALP and γ-GT and the lipid profile was ascertained. The levels of serum marker enzymes were elevated on treatment with alcohol and CCl$_4$. The administration of *B. monnieri* leaf extract caused a significant decrease in the activities of serum marker enzymes, indicating its protective effect. Similar observations were made in the lipid levels also.

The antioxidant status in the experimental animals was assessed by analyzing the enzymic and non-enzymic antioxidants in the liver and kidney of the rats exposed to ethanol-CCl$_4$ treatment in the presence and the absence of the leaf extract. The biological end point of oxidation manifested as LPO was analyzed in the liver. The total antioxidant status of the liver homogenate was also analyzed as DPPH scavenging.

The results revealed that the extract could effectively counteract the oxidative insult, which manifested as a decrease in the levels / activities of the antioxidants in both the organs under study. A differential response in the antioxidant status was observed in the liver and kidney in the levels of vitamin E. The antioxidant status improved significantly, which also resulted in decreased oxidative stress as evidenced by the decrease in LPO on treatment with the extract. The DPPH scavenging effect did not differ significantly in all the groups. Silymarin treated groups alone showed slightly higher values compared to the controls.
It is clear from the results of phase III of the present study that the treatment with *B. monnieri* leaf extract improves the antioxidant status in the liver and kidney of rats and can render protection against alcohol-CCl₄ induced toxicity.

In the final phase of the study, an attempt was made to determine the active principle present in the leaf extract. The preliminary phytochemical analysis of the leaves of *B. monnieri* revealed the presence of alkaloids, phenolics, flavonoids and saponins. To confirm the nature of the active component present in *B. monnieri* leaves, spectral analysis (UV, HPLC, HPTLC, IR and GC-MS) were carried out, which confirmed the presence of alkaloids, phenolics, flavonoids and saponins.

The outcome of the study, thus, provides strong evidence in establishing the antioxidant properties of *B. monnieri* leaves, which includes not only radical scavenging properties, but also biomolecular protection. This effect is also observed *in vivo*, wherein no toxic effects were observed. The bioactivity of antioxidant and anticancer property of the plant can be attributed to the presence of phytochemicals.

The highly significant observation made in the study is the differential effect that the leaf extract evokes in untransformed and transformed cells. This effect of the leaves has a very important bearing in cancer treatment, as they can minimize the undesirable side effects of cancer chemotherapy, while rendering the cancer cells more susceptible. Thus, based on our results, it can be suggested that *B. monnieri* leaves can be administered as a supportive therapy during cancer chemotherapy, to minimize the toxic side effects to non-cancerous cells and to maximize the anticancer drug action.

**SUGGESTIONS FOR FUTURE RESEARCH**

The outcome of the present study has thrown open a number of avenues for future research. Some of them, which can be pursued for active research, are given below.

- The anticancer potential of *B. monnieri* can be further tested using other cancer cell lines.
- The apoptosis-inducing activity of *B. monnieri* can be analysed further for the mechanism involved.
- The active components in the leaves can be identified, isolated and tested for the apoptosis-modulating effect.
- Novel drugs for alleviating the side effects of chemotherapy can be designed once the active components in the leaves are isolated.