



*Summary and Conclusion*

## 6. SUMMARY AND CONCLUSION

Plants play an indispensable part in the environment, especially as a medication to protect man from intrinsic and extrinsic ill effects imparted by stress agents. A complex mixture of phytochemicals present in plants and their products are responsible for the potent antioxidant and anticancer activities, since it has been used to treat several types of cancer.

These properties have accelerated the search for antioxidant principles that lead to the identification of natural resources, isolation of active principles and further modification and refinement of active antioxidant molecules. This search has been further fuelled by the fact that oxidative damage has been identified as the causative event in the onset of several disorders, from diabetes, hypertension, cardiovascular disorders and scurvy, to more life threatening diseases like cancer.

Another defence mechanism that the organisms employ to rid themselves of cells that are damaged beyond repair is apoptosis. Apoptosis is the process of programmed cell death (PCD) that occurs in multicellular organisms. Any modulation in this process leads to various disease conditions. Thus, the concept of pharmacological supplementation, which can influence apoptosis, has become an intensive area of research.

Medicinal plants constitute an ever-expanding goldmine of medicinal preparations, with the added advantage of minimal or no side effects. The identification and scientific validation of rich sources of valuable pharmaco-phytochemicals among the rich biodiversity, as prevailing in India, can provide the cure for many dreaded diseases and disorders.

One such medicinal plant is *Zea mays*. In spite of known uses in traditional medicines, no documented evidence is available on the antioxidant and anticancer effects of the leaves of *Zea mays* or their possible mechanisms of action. This study aimed to probe into the antioxidant effects and the influence of *Zea mays* leaves on events induced by oxidative stress conditions.

In the first phase of the study, activities of enzymic (superoxide dismutase, catalase, peroxidase, glutathione S-transferase and glutathione reductase) and the levels of non-enzymic (ascorbic acid, tocopherol, total carotenoids, lycopene, reduced glutathione,

chlorophyll, total phenols and flavonoids) antioxidants were assessed in the leaves of *Zea mays*. The leaves were studied at six different time points of growth namely 5, 10, 15, 20, 25 and 30 days after sowing, in order to determine if any change occurred in their antioxidant content as the age of the plant increased. The results revealed that the leaves possessed considerable levels of antioxidants at all the time intervals tested, with the maximum levels observed at 10<sup>th</sup> day after sowing. Therefore, further studies were carried out with the 10<sup>th</sup> day plant.

In the second phase of the study, *Zea mays* leaf extracts were tested for their radical scavenging effects and biomolecular protective effects under oxidatively stressed conditions on purified biomolecules, isolated cells and *in vitro* models that simulated the *in vivo* environment. To identify the ideal solvent into which the maximum amount of antioxidants got extracted and the nature of the active components, the leaves were extracted into the solvents of differing polarity (water, methanol and chloroform).

All the extracts were tested for their radical scavenging properties against DPPH, ABTS, hydroxyl radicals, hydrogen peroxide, superoxide and nitric oxide. *Zea mays* leaf extracts exhibited very high scavenging of all the radicals tested, with the exception of nitric oxide, which was scavenged to a lower extent. Among all the extracts tested, the methanolic extract of *Zea mays* leaves was found to be a very effective radical scavenger. Thus, our results showed that the *Zea mays* leaf extracts possessed considerable level of antioxidants and radical scavenging properties.

After ascertaining the free radical scavenging properties of the *Zea mays* leaf extracts (water, methanol and chloroform), their effects on oxidant-exposed lipids and DNA were followed. The effect of the *Zea mays* leaf extracts were analyzed on three different membrane lipid preparations namely plasma membrane, mixture of plasma and internal membranes and intact cells, exposed to oxidant stress *in vitro*. It was observed that a better protection was rendered by the leaf extracts to both plasma membrane as well as intracellular membranes. The methanolic extract provided better protection than the other two extracts.

The potentiality of the *Zea mays* leaf extracts was then analyzed for protection of purified DNA preparations, as well as the DNA in live cells. The protection rendered by the *Zea mays* leaves upon oxidative damage to DNA was followed in commercially

available DNA prepared from sources of varying hierarchies of evolution. They were  $\lambda$  (viral) DNA, herring sperm DNA and calf thymus DNA. The results revealed that the DNA damage was effectively counteracted by *Zea mays* leaf extracts, with the methanolic extract exerting the maximum response, followed by the aqueous extract. This reduction could be due either to the prevention of DNA damage (i.e., scavenging of the DNA damaging agent by the components of leaf extracts) or to the effective repair of the damaged DNA.

In order to ascertain the exact mechanism operating behind the reduction in the extent of DNA damage, Unscheduled DNA Synthesis (UDS) was followed in the Hep2 cell line exposed to  $H_2O_2$ . The results showed that the leaf extracts induced the repair system, as evidenced by the increase in the incorporation of radioactive thymidine. The results showed that the *Zea mays* leaf extracts, in the order of methanol, aqueous and chloroform, were very effective in protecting the primary targets of oxidative assault, namely lipids and DNA, against oxidant induced damage

As the next step, the influence of the leaf extracts was tested on the antioxidant status of cells maintained in their tissue architecture. In order to facilitate the exposure of oxidants and the plant extracts, thin slices of the tissue (goat liver) were made.

Precision-cut slices from goat liver were employed to evaluate the protective effects rendered by *Zea mays* leaves against hydrogen peroxide induced toxicity *in vitro*. Different enzymic (superoxide dismutase, catalase, peroxidase, glutathione S-transferase and glutathione reductase) and non-enzymic (ascorbic acid, tocopherol, vitamin A and reduced glutathione) antioxidants were analysed in the slices in the presence and the absence of *Zea mays* leaf extracts.

$H_2O_2$  induced damage in the goat liver slices, reflected by a marked depletion of all the antioxidants tested, was negated by the concordant treatment with the leaf extracts of *Zea mays*. All the three extracts tested were capable of improving the levels of antioxidants studied to a significant extent. The methanolic extract was the most potent in this regard, followed by aqueous and chloroform extracts. Thus, the study revealed that the extracts of *Zea mays* leaves could improve the antioxidant defense status of the oxidatively stressed ( $H_2O_2$ ) goat liver slices to a considerable extent.

The decrease in the use of laboratory animals in biomedical research over the last two decades can be attributed to a number of factors. Promotion of the Three Rs (Reduction, Refinement and Replacement) approach has demonstrated to scientists that it is possible to work towards a gradual replacement of animal-based studies without compromising on scientific quality.

With this background, in phase III, we focused on checking the antioxidant, antiapoptotic and anticancer properties of *Zea mays* leaf extracts on various alternative models. The alternatives to whole animal testing adopted in our study included both untransformed and transformed cells and *Drosophila melanogaster*.

We assessed the influence of leaf extracts of *Zea mays* on oxidative stress-induced apoptotic events in untransformed (*Saccharomyces cerevisiae* and primary chick embryo fibroblasts) and transformed (Hep2) cells. Oxidative stress was induced in the cells using H<sub>2</sub>O<sub>2</sub>. The parameters analyzed were cytotoxicity assays (MTT and SRB) and observation of morphological and nuclear changes by various staining techniques (Giemsa, PI, EtBr, DAPI and AO/EtBr).

The results revealed that the *Zea mays* leaf extracts can exert a differential response against the oxidative stress-induced apoptosis in different types of cells. H<sub>2</sub>O<sub>2</sub> treated cells (yeast cells and primary cells) showed well-defined apoptotic morphology, which was strongly hindered with by the treatment with the leaf extracts, thus reiterating its antiapoptotic property by reducing the oxidative stress in yeast cells and chick embryo fibroblasts. The protective effect of the methanolic leaf extract was strongly evident, followed by aqueous and chloroform extracts.

H<sub>2</sub>O<sub>2</sub> stress caused a very steep increase in the number of Hep2 cells committed to apoptosis. Surprisingly, the *Zea mays* leaf extracts, by themselves, also caused an increased extent of apoptosis in Hep2 cells, indicating the anticancer potential of the leaves. Thus, it can be inferred that the anticancer potential of the leaf extract is selectively exerted against the cancer cells, while sparing the normal cells.

This observation raised the query of what the effect of the extract would be in the presence of a standard chemotherapeutic agent that induces apoptosis. Hence, the effect of the *Zea mays* leaf extracts on the etoposide-induced effects was studied in primary

cultured chick embryo fibroblasts and Hep2 cells, as representatives of non-cancerous and cancerous cells respectively.

The results revealed a steep rise in the number of apoptotic primary cells in the etoposide treated group. *Zea mays* leaf extracts effectively counteracted etoposide-mediated cytotoxicity, implying that these extracts can be used to protect the non-cancerous cells in the body against etoposide-induced ill-effects during cancer treatment with etoposide. This would help in minimizing the toxic effects of the useful chemotherapeutic agent.

Addition of etoposide resulted in a marked increase in the proportion of apoptosing Hep2 cells. The plant extracts, by themselves, also caused a slight increase in the extent of apoptosis and showed marked increase in the number of apoptotic cells when administered along with etoposide in Hep2 cells. These results showed that the *Zea mays* leaf extract exhibits anticancer property and can enhance the chemotherapeutic action of etoposide in cancer cells, by sensitizing them to the cytotoxic action of etoposide. Thus, our results revealed that the *Zea mays* leaf extracts render the cancer cells susceptible to the action of chemotherapeutic drugs, while protecting the normal cells from the toxic effects.

The alternative models used thus far in the study represent the condition outside the biological system under simulated *in vivo* conditions. In the intact organism, however, several physiological and biochemical events influence the absorption, distribution, metabolism and elimination of the compounds under study, which may, in turn, affect their efficacy. Thus, in order to draw concrete conclusion, we selected *Drosophila melanogaster*, an intact animal, as a model system.

We studied the antioxidant status in the *Drosophila* subjected to oxidative stress induced by H<sub>2</sub>O<sub>2</sub> (direct oxidant) or CCl<sub>4</sub> (which needs metabolic activation) at two different concentrations in the presence and the absence of the *Zea mays* leaf extracts. This study was conducted to analyze the differential response elicited by the two oxidants and leaf extracts under *in vivo* conditions.

The results indicated that the antioxidant levels were found to decrease upon CCl<sub>4</sub> or H<sub>2</sub>O<sub>2</sub> administration in the male and female flies. The results also showed that the depletion was severe as the concentration of the oxidants increased. The

co-administration of the methanolic extract of *Zea mays* leaves significantly increased the antioxidant levels. This observation strongly suggests that the *Zea mays* leaves can be used in medicinal preparations to combat the disorders caused by oxidative stress.

In the fourth and final phase of the study, an attempt was made to identify the active component in the leaf extracts of *Zea mays*. Phytochemical screening of the leaves identified phenolics and flavonoids as the major antioxidant components in the leaves of *Zea mays*. To confirm the results obtained in the phytochemical screening, spectral analyses (UV absorption, HPLC, HPTLC, FT-IR and GC-MS) were carried out, which suggested the presence of polyphenolic and flavonoid type of components. The exact structures of these components could not be derived from the spectra and more in-depth studies are needed in this direction.

The outcome of the study, thus, proves the strong antioxidant effect of *Zea mays* leaves, which is an easily available source of medicinally valuable components.

#### **SUGGESTIONS FOR FUTURE RESEARCH**

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

- The effect of *Zea mays* leaf extracts can be investigated against oxidative stress caused by different types of oxidants, acting by different mechanisms.
- The active components can be isolated and purified, and their effect can be studied further against oxidative stress-induced events.
- *Zea mays* leaves can be tested for anticancer activity by their ability to induce apoptosis in various cancer cell lines
- The other molecular markers of apoptosis, like the affector (initiator) and effector caspases, can be studied after exposure to the leaf extract.
- Novel drugs for alleviating the side effects of chemotherapy can be designed once the active components in the leaves are identified.
- Further bioavailability studies can be investigated after isolation of the pure active compound.