6. SUMMARY AND CONCLUSION

Plants and their products have been used for centuries to cure various ailments. Herbal medicine is still the fulcrum of the primary healthcare in many developing countries. In recent years, utilization of plant-derived bioactive compounds has increased due to the fact that the side effects caused by the synthetic drugs have become more dangerous than the diseases they claim to cure. Plant-derived medicines contain natural substances that enable illness alleviation and health promotion. Additionally, they have been proved to be safe, relatively less expensive, and globally competitive. They also possess better patient tolerance. All these factors have made the use of natural remedies as an absolute requirement at present.

Oxidative stress, an imbalance caused by the excessive production of reactive species and their reduced detoxification is associated with a plethora of pathological phenomena, including infection, inflammation and aging. Additionally, oxygen-derived free radical reactions have been implicated in the etiology of many diseases like atherosclerosis, neurodegenerative disorders, diabetes and cancer. Severe oxidative stress leads to cell death.

Mammalian cells are endowed with a highly complicated defence system against free radical-induced oxidative stress. This defence system has evolved in cells and includes different mechanisms such as physical defences, preventive or repair mechanisms and antioxidant defences. Antioxidants are substances present at lower concentrations that can prevent or quench free radical (oxidant) formation or repair the damage caused due to oxidative stress. Antioxidants can be either enzymes (preventive antioxidants) or small molecules (chain breaking antioxidants). Extensive studies have been carried out to determine the role of oxidative stress and the use of antioxidants in the prevention of many diseases.

The human body is enriched with natural antioxidants that can prevent the onset, as well as treat the oxidative stress-related diseases. Antioxidants can also be supplemented through the diet. Recent researches have shown that the antioxidants, especially those of plant origin, act as potent free-radical scavengers and have gained importance as therapeutic agents for several diseases caused due to oxidative stress. Identification of pharmacologically potent and non-toxic antioxidant compounds from plant sources for use in protective medicine and the food industry has increased globally. Hence in the last few years, using various new technologies such as high-throughput screening and combinatorial synthesis, the natural-product-based drug discovery has gained importance in the field of medicine.

Cancer is a potentially fatal disease that is caused by various genetic and environmental factors. Many studies have proved a well-established role of free radicals in the initiation of cancer through oxidative DNA damage. Recent evidences also have suggested

that ROS-dependent signals are required for tumour growth and the alterations in the levels of ROS with antioxidants or pro-oxidants could modulate tumour growth. Currently, many anticancer drugs are derived from plant sources. They act through different pathways to prevent oxidative DNA damage and induce apoptosis in cancer cells.

The anticancer potential of the plants is attributed to the ability of antioxidants present in them that can scavenge free radicals, prevent DNA damage and the subsequent mutations. Many clinically useful anti-cancer agents, such as vinblastine and vincristine, are derived from plants. In addition, several phytochemicals and their semi-synthetic derivatives have been developed as promising new anticancer agents based on their selective activity against cancer-related molecular targets and are currently under clinically trials. Hence, many research studies focus on identifying the plants with significant antioxidant and anticancer potential, and isolating novel biologically active molecules from these plants that might be useful lead compounds for the development of new conventional anticancer agents.

One such candidate plant, *Caesalpinia pulcherrima*, was chosen for the present study. It blooms in three different colours (orange, pink and yellow). Several medicinal properties are attributed to this plant such as anti-pyretic, purgative, anti-inflammatory and anti-microbial properties. It is also used for the treatment of bronchial asthma and malarial fever. The three different flowers (orange, pink and yellow) of *C. pulcherrima* have already been studied extensively in our laboratory and found that these flowers are rich in both enzymic and non-enzymic antioxidants. They also exhibit both antimutagenic and antioxidant properties. In addition, the flowers of *C. pulcherrima* rendered a significant protection against oxidative DNA damage and reduced the viability of cancer cells, all of which have furthered the present study to determine the molecular mechanisms underlying their antioxidant and anticancer properties.

The present study was carried out in four distinct phases. In phase I, the radical scavenging activity and biomolecule-protective effects of the three different flowers (yellow, pink and orange) of *C. pulcherrima* were determined *in vitro.* In order to understand the active principle rendering the antioxidant activities, the flowers were individually extracted into solvents of increasing polarity under cold conditions. The solvents used were petroleum ether, benzene, chloroform, ethyl acetate and methanol. Apart from the solvent extracts, aqueous extracts of the flowers were also prepared. The radical scavenging activity of the flower extracts were determined against a battery of radicals namely DPPH, ABTS, H_2O_2 and hydroxyl radicals. All the solvent extracts, along with the aqueous extracts, of the three flower extracts showed good radical scavenging activity, among which, the cold methanolic extract exhibited the maximum effect followed by the aqueous extract.

Therefore, the methanolic extract of all the three flowers were chosen for further analyses. The optimum dose of the methanolic extract to be used for the subsequent phases of the present study was determined using free radical scavenging assays (DPPH, ABTS, hydroxyl and hydrogen peroxide), in which different concentrations (0.01 mg to 1.0 mg) of the methanolic extracts were compared and was found that 0.1 mg of the methanolic extract showed the best response. Therefore, further studies were carried out with 0.1 mg concentration of the cold methanolic extract of all the three flowers.

In the second part of phase I, the biomolecule-protective effects of the methanolic extract of the three flowers of *C. pulcherrima* against oxidant-induced damage to lipids, DNA and proteins were analyzed *in vitro*. Lipids are more susceptible to oxidative stress and lipid peroxidation products are the potential biomarkers for oxidative stress status *in vivo* and its related diseases. Hence, the protective effects of the flower extracts against lipid peroxidation were investigated first.

Three different membrane models namely, goat RBC ghosts (plasma membrane lipids), goat liver homogenate (plasma membrane and intracellular lipids) and liver slices (intact cells) were used to assess the extent of lipid peroxidation and the protection rendered by the flower extracts against induced oxidative stress. The results obtained showed that all the three flowers substantially decreased the extent of lipid peroxidation in all the three membrane preparations. A stronger protection to the intracellular lipids (liver homogenate) by the flower extracts was observed. This implies that some bioactive component present in the extract is likely to act synergistically with an endogenous component to render the antioxidant property by inhibiting LPO.

Among the biomolecules susceptible to oxidative modifications, DNA has gained the greatest attention and the number of oxidative stress-mediated DNA lesions identified is still growing. Accumulation of oxidant-induced DNA lesions has been associated with numerous diseases, especially cancer. In the present study, the effect of the flower extracts of the candidate plant on oxidant induced DNA damage was assessed *in vitro* in commercially available preparations of DNA.

DNA from different hierarchies of evolutionary development were selected for the analysis, which included the commercially available preparations of viral DNA (λ DNA), bacterial plasmid (pUC18) and DNA of animal origin (herring sperm DNA). Hydrogen peroxide was used to induce oxidative stress in all the types of DNA, both in the presence and the absence of the flower extract. The results showed that in all the DNA preparations, the

extent of oxidative DNA damage was significantly increased on exposure to H_2O_2 which was effectively decreased by the co-treatment with the flower extracts.

Oxidative stress induces covalent modifications of proteins that leads to the loss of protein functions, alterations in protein activity and their proteolytic breakdown. Protein carbonylation is one of the most common oxidative modifications of proteins and it has been implicated in the pathogenesis of various disease conditions. The results obtained in the present study showed that all the three flowers of *C. pulcherrima* reduced the oxidant-induced protein carbonyl formation, indicating their efficiency in protecting proteins against oxidative damage. This protective effect was further confirmed using SDS-PAGE analysis, wherein the drastic degradation of proteins (as indicated by the diminished protein bands intensity) was remarkably reverted by the *C. pulcherrima* flower extracts in the presence of the oxidant.

The results of phase I showed that the methanolic extract of the three different flowers of *C. pulcherrima* exhibit substantial free radical scavenging activity and antioxidant property, among which, the maximum activity was observed in the orange flower extracts. They also rendered significant biomolecular protection against oxidative stress, both in cell-free systems and in intact cells.

In phase II, the antioxidant potential of the flowers of *C.pulcherrima* was evaluated in an *in vitro* system subjected to oxidative stress. In the present study, the precision-cut goat liver slices were used as an *in vitro* model to study the effect of the flower extracts in intact cells against oxidative stress by analyzing the antioxidant status of the liver slices. The enzymic antioxidants (SOD, CAT, GPx, GST and GR) and the non-enzymic antioxidants (vitamins C, E, A, GSH, total thiols and protein thiols) were analyzed in the presence and the absence of the flower extract and H_2O_2. The treatment with H_2O_2 significantly reduced the activity of enzymic antioxidants in goat liver slices, which improved on co-treatment with the flower extracts. A similar trend was observed in the levels of non-enzymic antioxidants. These observations showed the antioxidant efficacy of all the three flowers.

Dysregulation of apoptosis is one of the major molecular mechanisms of carcinogenesis. The study on free radical-induced DNA damage and dysregulated apoptosis has become a major thrust of carcinogenesis research. Hence, the third phase of the present study was formulated to examine the apoptosis-modulating effects of *C. pulcherrima* flower extracts on oxidative stress-induced apoptotic events in both transformed and non-transformed cells. Two different untransformed cell types (yeast and peripheral blood lymphocytes) and one transformed cell type (KB oral carcinoma cells) were used to examine the oxidative stress-induced apoptosis, and the effect of the flower extracts on them. Oxidative stress was induced by H_2O_2 for yeast cells and by etoposide for peripheral blood

lymphocytes and KB cells. The effect of flower extracts on the cell viability was determined by MTT, SRB and LDH release assays. The characteristic features of apoptosis were analyzed using various staining methods, namely Giemsa (morphological changes), PI, EtBr and AO/EtBr (nuclear changes) and DAPI (apoptotic index) staining. The extent of DNA damage caused by oxidative stress was determined by diphenylamine assay in yeast cells and by single cell gel electrophoresis (comet) in peripheral blood lymphocytes and cancer cells.

In *S. cerevisiae* cells, cell viability assays and various staining methods showed that all the three flower extracts significantly counteracted the apoptosis-inducing activity of the oxidant H_2O_2, indicating their anti-apoptotic effects in non-transformed cells. The flower extracts also significantly reduced the extent of oxidative DNA damage in *S. cerevisiae* cells.

Under normal physiological conditions, a balance between mitosis (where cells divide) and apoptosis (a physiological process of cellular suicide) is maintained. When this balance is disturbed, carcinogenesis can develop, where cells evade apoptosis and proliferate in an uncontrolled manner. Chemotherapeutic agents that can eliminate the cancerous cells via apoptosis without affecting the normal cells have a therapeutic advantage for the elimination of cancer cells. Many anticancer agents exert their effects on cancer cells by inducing apoptosis.

In the present study, the anticancer properties of the flower extracts of *C. pulcherrima* were evaluated by determining their effects on etoposide-induced cell death. The effects were also studied in peripheral blood lymphocytes (untransformed cells) along with the KB cells (cancer cells). In both peripheral blood lymphocytes and KB cells, the treatment with etoposide (oxidant) significantly decreased the cell viability. The flower extracts significantly improved the viability of lymphocytes, whereas in KB cells, they reduced the viability, both in the presence and the absence of etoposide. A similar trend was observed for the staining methods and DNA fragmentation assay.

The flowers of *C. pulcherrima* were, thus, found to exhibit a differential response towards non-cancerous and cancerous cells, wherein the flower extracts protected the normal cells (peripheral blood lymphocytes) from oxidant-induced apoptosis, while selectively increasing the number of cancer cells (KB cell line) undergoing apoptosis. The differential response, therefore, suggests that, by modulating apoptosis, *C. pulcherrima* flowers can affect the steady-state cell population. This targeted action of the flower extracts towards cancer cells make them suitable candidates for supportive chemotherapy in cancer.

The apoptosis-modulating effects of all the three flowers of *C. pulcherrima* on oral carcinoma cells were confirmed by analyzing the expression of proteins that are involved in

the regulation of apoptosis. The expression of pro-apoptotic proteins (TP53 and Bax) and the anti-apoptotic protein (Bcl-2) were analyzed using immucytochemistry. The results showed that all the three flowers of *C. pulcherrima* induce apoptosis in oral carcinoma KB cells by upregulating the expression of pro-apoptotic TP53 and Bax proteins, while downregulating the anti-apoptotic Bcl-2 protein. These findings suggest that the flowers of *C. pulcherrima* are therapeutically effective for malignancies that overexpress Bcl-2 and lack TP53 expression. Further research on the apoptotic pathways need to be conducted to elucidate the complete molecular mechanism underlying the anticancer activity of the flowers of *C. pulcherrima*.

In an attempt to further explore the mechanism of anticancer activity of the flowers of *C. pulcherrima*, cell cycle analysis using flow cytometry was carried out, in which, the apoptosis-modulating effects of *C. pulcherrima* on cancer cells were analyzed in relation to cell cycle position. The results obtained showed that the proportion of KB cells undergoing early stage (G0-G1 phase) or late stage of apoptosis (sub-G0 phase) were significantly increased after treatment with etoposide. A similar increase in the proportion of KB cells undergoing apoptosis (sub-G0 and G0-G1 phases) was also observed after treatment with all the three flower extracts both in the presence and the absence of etoposide. This clearly demonstrate the anticancer potential of all the three flowers of *C. pulcherrima*, among which, the orange flower extract was more effective compared to the other two flowers. Additional studies on cell cycle analysis using non-cancerous cells need to be conducted, so that the differential influence of the flower extracts on cell division and death can be completely comprehended.

The results of the first three phases revealed that the extracts of all the three flowers of *C. pulcherrima* exhibited antioxidant, biomolecule-protective, apoptosis-modulating and anticancer effects against oxidative stress induced under *in vivo*-simulated *in vitro* conditions. Thus, it became clear that further research is required to identify the bioactive compound that renders these beneficial effects. Hence, the final phase of the study was formulated to identify the active principle(s) rendering the antioxidant responses evoked by the flower extracts against oxidative stress.

In phase IV, preliminary screening and qualitative phytochemical analysis revealed the presence of seven major phytoconstituents namely alkaloids, phenols, flavonoids, saponins, steroids, tannins and terpenoids. The isolated fractions of these seven phytochemicals from the flowers of *C. pulcherrima* exhibited significant radical quenching activity, among which, the maximum scavenging potential was observed in the flavonoid fraction, followed by the phenolic fraction. The UV absorption spectrum of the methanolic extracts of the three flowers of *C. pulcherrima* showed many distinct major and minor peaks, which revealed the presence

of multiple components in the flowers of *C. pulcherrima*. HPLC analysis confirmed the presence of multiple components in the flowers of *C. pulcherrima*. The TLC analysis of the three flowers of *C. pulcherrima* showed the presence of alkaloids, phenols, flavonoids, saponins, tannins and terpenoids, which was further confirmed by HPTLC fingerprinting of the individual group of phytochemicals. Spectral analyses using FT-IR and GC-MS showed that the major active constituents present in the flowers may belong to phenolic and flavonoid type of compounds, whose structure is yet to be elucidated.

The research outcome of the present study, thus, accentuates the antioxidant, radical quenching, biomolecule-protective and anticancer properties of the flowers of *C. pulcherrima*. The selective inhibition of cell growth and induction of apoptosis by the flower extracts on cancer cells, with no such effects on normal lymphocytes signifies that the flowers of *C. pulcherrima* can be used as promising candidates for developing new strategies in cancer chemotherapy.

SUGGESTIONS FOR FUTURE RESEARCH

The outcome of the present study has opened several promising propositions for future research. Some of them that can be adopted for an active research are suggested below.

- The anticancer properties of the flowers of *C. pulcherrima* can be tested in various types cancer cell lines to determine whether a tissue specific response exists.
- *In vivo* studies using experimental animals can be carried out to determine the influence of the flower extracts on metastatic tumours.
- The exact mechanism of apoptosis induced by the flowers of *C. pulcherrima* can be predicted by evaluating the expression of various genes/proteins involved in the apoptotic pathways.
- Gene expression profiling can be carried out to explore various biological pathways and networks associated with the antitumour effect of the *C. pulcherrima* flowers.
- The markers that predict responsiveness of cancer cells to the treatment of flower extracts can be identified by genomic and proteomic approaches.
- The biologically active phenolic and flavonoid type of compounds present in the flowers of *C. pulcherrima* can be isolated, purified and their structures can be elucidated.
- *In silico* analysis can be done to characterize the interaction of the active components with the target protein(s), to predict the modes of action, potential adverse drug reactions and the absorption, distribution, metabolism, and excretion (ADME) profiles of the active compounds.