5. DISCUSSION

When molecular oxygen reacts with electrons, it is transformed into reactive oxygen species (ROS). ROS are products of normal cellular metabolism and are derived from various sources in different cellular compartments. At physiological level, the ROS play an important role in redox signaling of various cells. When produced in excess, ROS become harmful and can jeopardize the integrity and physiological functions of cellular macromolecules, thus contributing to the pathogenesis of various diseases such as cancer, atherosclerosis, aging, ischemic heart disease, diabetes, immunosuppression, inflammatory diseases and neurodegenerative disorders such as Alzheimer's disease and Parkinson's disease (Jiménez-Estrada *et al.*, 2013).

ROS are mainly produced from the mitochondrial electron transport chain (ETC). They are also generated by other pathways such as the respiratory burst inside the activated phagocytes, by ionizing radiations and as the byproducts of several cellular enzymes such as NADPH oxidases (Nox), xanthine oxidase (XO) and uncoupled endothelial nitric oxide synthase (eNOS) (Ungvari and Dikalov, 2013).

The cellular redox homeostasis is maintained by a delicate balance between the ROS production and the antioxidant system. When this redox homeostasis is disturbed, an indiscriminate oxidation elicits harmful effects, resulting in "oxidative stress" (Wang *et al.*, 2013b). Altered redox homeostasis (oxidative stress) plays an important role in the onset and the progression of various chronic diseases including cancer, cardiovascular and neurodegenerative diseases (Presnell *et al.*, 2013).

In order to protect the vulnerable targets from free radical-induced damage, the human body has evolved with innate defence mechanisms, collectively known as the antioxidant defences. Antioxidants counteract the imbalance of the cell redox homeostasis and maintain the ROS levels under the cytotoxic threshold (Birben *et al.*, 2012). The antioxidant defence system is comprised of endogenous antioxidant enzymes such as superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx), and low molecular-weight scavengers such as glutathione (GSH), coenzyme Q, uric acid and lipoic acid. It also includes the non-enzymatic scavengers such as vitamins and phytochemicals that are present in diet and medicinal plants (Geronikaki *et al.*, 2013). In recent years, extensive studies have been conducted to study the role of oxidative stress and the use of antioxidants in the prevention of many oxidative stress related diseases especially for cancer (Muslim *et al.*, 2012).

Cancer is a multistage process, in which each stage involves different molecular, biochemical and cellular events that ultimately lead to malignant transformation. Substantial scientific evidence has proved that the ROS-induced cellular damage is the key mechanism underlying various steps involved in the development of the malignant phenotype such as evasion of apoptosis, uncontrolled proliferation, angiogenesis, tissue invasion and metastasis (Ziech *et al.*, 2012).

Many chemotherapeutic reagents that are currently in use are restricted by various factors such as increased drug resistance and undesired side effects. Synthesizing a pure bioactive stereoisomer is highly laborious and time consuming process. Thus, it has become imperative to explore novel drugs or compounds that can act as templates for developing new therapeutic agents for cancer (Kaewpiboon *et al.*, 2012).

Many phytochemicals have been found to possess cancer chemopreventive effects in both preclinical animal models and human epidemiological studies. The phytochemicals prevent the initiation of carcinogenesis either by directly scavenging the reactive oxygen species or by inducing the cellular defence system containing the antioxidant enzymes (Lee *et al.*, 2013). The positive correlation between the antioxidant activity and the anti-proliferative effects of plants has substantiated the potential use of natural antioxidants for inhibiting cancer cell growth (Abrahim *et al.*, 2012).

These observations have encouraged the research efforts from across the globe to identify, characterize and provide scientific basis for the efficacy of various phytochemicals in order to develop effective strategy to control various human malignancies (Kaur *et al.*, 2009). Hence, many research studies are being carried out to explore natural chemopreventive agents from medicinal plants that are enriched with antioxidants and free radical scavenging molecules such as vitamins, terpenoids, phenolic acids, tannins, flavonoids, alkaloids and other metabolites (Omoruyi *et al.*, 2012). The expensive health-care burden, which is a major global concern, has made the chemoprevention strategy using natural products as an inexpensive, readily applicable and easily accessible approach for cancer treatment and currently it has become a major thrust of carcinogenesis research (Acharya *et al.*, 2010).

Plant secondary metabolites and their semi-synthetic derivatives continue to play an important role in anticancer drug therapy. Several new anticancer agents of plant origin have been introduced to the market and a promising pipeline of plant-derived compounds is already under oncology-related clinical trials (Saklani and Kutty, 2008). The five major

plant-derived antineoplastic lead compounds, namely vinblastine, vincristine, podophyllotoxin, paclitaxel and camptothecin, signify the isolation of cancer chemotherapeutic agents from natural sources (Butler, 2008).

Thus, an extensive screening of plants is being carried out to explore the possibility of developing novel, economically viable, anticancer drugs. *Caesalpinia pulcherrima* is one such candidate plant, which blooms in three different colours (orange, pink and yellow) with unique long stamens.

Earlier studies in our laboratory have shown that these flowers are rich in both enzymic and non-enzymic antioxidants (Aparna, 2000). The flowers also exhibit both antimutagenic and antioxidant properties (Nirmala Priyadharshini, 2001). Cell viability studies *in vitro* showed that the aqueous extract of the flowers reduced the survival of cancer cells, suggesting that the flowers may possess anticancer activities. The aqueous extract of the flowers also rendered a significant protection against oxidative DNA damage, especially for eukaryotic DNA (Yamuna, 2004). The protective effect of the flowers of *Caesalpinia pulcherimma* against oxidative DNA damage, which is the main cause for the cancer development and their significant role in reducing cancer cell growth, has furthered the present study on the molecular mechanisms of the antioxidant and anticancer properties of the flowers.

The study was conducted in four distinct phases. In phase I, the free radical scavenging activity and biomolecule-protective effects of the flowers against oxidative stress were determined. In phase II, the antioxidant potential of the flowers was assessed against oxidative stress induced *in vitro*. In the third phase, the influence of the flowers on oxidant-induced apoptotic events was determined in both non-transformed and transformed cells. In the final phase of study, phytochemical analysis using various chromatographic and spectral methods was carried out in order to identify the chemical nature of the active components present in the flowers.

PHASE I

In phase I, the radical scavenging activity and the biomolecular protective effects of the three different flowers (yellow, pink and orange) of *C. pulcherrima* were determined *in vitro.*

5.1. ANTIOXIDANT ACTIVITIES OF *C. pulcherrima* FLOWERS

Plant extracts have been known for years for their significant biological activities especially for their antioxidant and radical quenching properties. A wide range of medicinal

plants have been screened and recognized as a source of natural antioxidants that can protect from oxidative stress, thereby playing an important role in chemoprevention of diseases (Rahzani *et al.*, 2013).

The extent of damage caused by the free radicals can be mitigated through antioxidant supplementation. Antioxidants are the potent scavengers of free radicals and protect the human body from several diseases caused due to free radical reactions (Luqman *et al.*, 2009). Numerous substances have been identified as potent antioxidants. For example, various phenolic antioxidants isolated from plants such as flavonoids, tannins, xanthenes and coumarins, have been reported as efficient free radical scavengers in a dose-dependent manner and thus are identified as promising therapeutic drugs for free radical pathologies (Eissa *et al.*, 2013).

Depending upon the generation of free radicals, various *in vitro* assays have been developed to measure the radical scavenging capacity and total antioxidant activity of plant products. To evaluate accurately the antioxidant potential of a plant extract, it is imperative to select and apply suitable, optimized and high-throughput *in vitro* free radical assays. It is also essential to perform various antioxidant assays to determine the antioxidant capacity of the selected plant material (Ndhlala *et al.*, 2010). Thus, in the present study, the radical scavenging activity of the different solvent extracts of the three flowers of *C. pulcherrima* (yellow, pink and orange) was determined against a battery of radicals namely DPPH, ABTS, H_2O_2 and hydroxyl radicals.

The ability of phytocomponents/ extracts to neutralize or scavenge free radicals and oxidants in cell-free systems reflect their antioxidant potential. It has become routine to test this potential as a preamble to detailed in-depth research on the medicinal properties of such components. In the present study, a stable synthetic radical (DPPH), a radical freshly-generated by reaction (ABTS) and two biologically important oxidants ($OH^{\bullet}$ radicals and H_2O_2) were taken for determining the antioxidant potential of the flower extracts. These oxidants have been the subjects of study reported by several scientists all over the globe.

All the solvent extracts, and the aqueous extracts, of the three flowers showed good radical scavenging activity, among which, the cold methanolic extract exhibited the maximum effect, followed by the aqueous extract.

Risdian *et al.* (2011) showed that the radical scavenging potential of the ethanolic extract and different solvent fractions of the leaves of *Piper betle* varied significantly and this variation was suggested to occur due to the presence of different phytoconstituents in

different solvent extracts. On comparison of different solvent (petroleum ether, benzene, chloroform, ethyl acetate, methanol and water) extracts of the leaves of *Indigofera tinctoria* L. using various *in vitro* assays, the maximum radical scavenging activity was observed in the ethyl acetate extract (Anusuya and Manian, 2013). The antioxidant activity of leaf extracts of Angolan *Cymbopogon citratus* prepared with different solvents (water, methanol and ethanol) showed different levels of antioxidant activity (Soares *et al.*, 2013). Thus, it became essential to compare the antioxidant activity of the plant extracts in solvents of different polarity.

Among six different extracts prepared, the methanolic extract of both the fresh and the dry rhizome of *Curcuma zeodaria* exhibited significant antioxidant activity against a battery of free radicals namely DPPH, ABTS, H_2O_2, hydroxyl, superoxide and nitric oxide (Sumathi *et al.*, 2013a). The crude methanolic extract from the fruits of *Phillyrea latifolia* L., showed a fairly potent DPPH and ABTS radical scavenging activity (Ayranci and Erkan, 2013). Mahdi-Pour *et al.* (2012) showed that the DPPH radical scavenging, xanthine oxidase inhibiting and nitrite scavenging activities of the methanolic extract of the leaves of *Lantana camara* was more effective than those of the other parts of the plant.

Ebrahimzadeh and Bahramian (2009) found that the maximum antioxidant activity was exhibited by the methanolic extract of the fruits of *Crataegus pentaegyna subsp. Elburensis* compared to the aqueous extract. Similarly, a 70% methanol extract of *Spondias pinnata* stem bark showed a significantly higher total antioxidant activity (Hazra *et al.*, 2008). At higher concentrations, the methanolic extract of the guarana powder and its pectic fraction containing the polysaccharides exhibited a strong hydroxyl radical scavenging capacity (Dalonso and Petkowicz, 2012). Policegoudra *et al.* (2012) have reported that the methanolic extracts of the leaf, stem and pulp of *Garcinia lancifolia* showed high DPPH radical scavenging activity than the aqueous extracts and the antioxidant activity was suggested to be attributed to the presence of phenolic compounds in these extracts.

These observations support the results obtained in the present study, in which, the maximum radical scavenging activity was observed in the cold methanolic extract of the three flowers of *C. pulcherrima*, followed by the aqueous extracts and the other solvent extracts.

ROS that are generated by normal physiological processes and various exogenous factors cause oxidative stress, which, in turn, induce damage to a wide range of biological molecules. Such ROS-mediated biomolecule damage has been implicated in the etiology

and physiopathology of various diseases such as coronary artery diseases, diabetes, stroke, rheumatoid arthritis and cancer (Guha *et al.*, 2011).

5.1.1. DPPH Radical Scavenging Activity

DPPH assay is a rapid, simple, inexpensive and widely used method to measure the ability of compounds to act as free radical scavengers or hydrogen donors (Dawidowicz and Olszowy, 2012). In this method, the reaction that takes place between the plant extract and the radical DPPH in methanol facilitates the assessment of antioxidant compounds that are present in the plant extract. The assay also provides sufficient time, so that even the weak antioxidants that react slowly with the DPPH radical can be assessed. It can be applied for both non-polar organic solvent and aqueous extracts and also can be used to examine both hydrophilic and lipophilic antioxidants (Kedare and Singh, 2011).

The antioxidant activity of the different solvent extracts of the flowers of *C. pulcherrima*, along with the aqueous extract, was determined using DPPH method, in which, both the dot blot screening and spectrophotometric assay showed that the DPPH scavenging activity increased with extracts in solvents of increasing polarity. The maximum scavenging activity was exhibited by the cold methanolic extract of all the three flowers, followed closely by the ethyl acetate and aqueous extracts.

Nariya *et al.* (2013) showed that the methanolic extract of the *Cordia dichotoma* bark exhibited significant DPPH radical scavenging activity when compared to that of standard ascorbic acid. The antioxidant potential of methanolic and aqueous extracts of *Galium odoratum* (L.) Scop. was assessed using DPPH assay, in which both the extracts displayed significant antioxidant activity with IC50 values of 148 μg/ml and 83 μg/ml (Kahkeshani *et al.*, 2013).

Islam *et al.* (2013a) have reported that the methanolic extract of the fruits of *Spondias dulcis* exhibited the higher DPPH radical scavenging activity than that of the leaves. The DPPH radical scavenging potential of the methanol and aqueous extracts of *Punica granatum* peel was assessed and found that the methanolic extract was the most effective (Middha *et al.*, 2013). A similar trend was observed in other studies where the methanolic extract of the star fruits and *Annona squamosa* fruit pulp showed higher percentage of DPPH inhibition than the aqueous extracts (Annegowda *et al.*, 2012; Nandhakumar and Indumathi, 2013).

El-Haci *et al.* (2013) tested the crude methanolic extracts of aerial parts of *Polygonum maritimum* for their ability to neutralize the free radical (DPPH) and a

significantly higher percent of DPPH inhibition was observed. The methanolic extracts of *Dendropanax morbifera* branches, debarked stems, bark and two different stages of leaves were evaluated for antioxidant activity using DPPH assay and a strong DPPH scavenging effect was observed in the debarked stem extract than the other samples (Hyun *et al.*, 2013).

Islam *et al.* (2013b) have reported that among the five extracts (petroleum ether, chloroform, ethyl acetate, methanol and water) of *Sygygium fruticosum (Roxb.)* seeds tested for antioxidant activity using DPPH radical, the ethyl acetate extract showed the maximum antioxidant activity. Two new resveratrol trimer derivatives, namely rheumlhasol A and rheumlhasol B, together with four known dimers isolated from the methanolic extract of roots of *Rheum lhasaense* showed strong DPPH radical scavenging effect (Liu *et al.*, 2013b).

The results obtained in the present study also showed that, among the six solvent extracts, the DPPH radical scavenging activity was higher in extracts prepared in solvents with higher polarity (ethyl acetate, methanol and aqueous) of all the three flowers of *C. pulcherrima*. Such increase in DPPH radical scavenging activity may be due to the polarity of the solvents that can extract the antioxidative compounds in *C. pulcherrima* flowers more effectively than the non-polar solvents. Among these three extracts, the methanolic extract showed the maximum activity.

5.1.2. ABTS Radical Scavenging Activity

The ABTS decolourization method can be used to determine both hydrophilic (in buffered media) and lipophilic (in organic media) antioxidant properties in complex samples (Gulaboski *et al.*, 2013). ABTS radical is highly stable in both buffered and organic media and can be easily generated in a rapid controlled fashion (Guedes *et al.*, 2013). The six different extracts of the three flowers of *C. pulcherrima* were assessed for their ability to scavenge the ABTS radical cation. The maximum scavenging of ABTS was observed in the cold methanolic extract of all three flowers, followed by the ethyl acetate and aqueous extracts.

It was observed by Shabbir *et al.* (2013) that the n-butanol fraction of the methanolic extract of the leaves of *Maytenus royleanus* showed the highest scavenging activity for ABTS than the ethyl acetate fraction. Salem *et al.* (2013) reported that the antioxidant potential of parathion-pretreated leaves of *Salvia officinalis* was significantly decreased by the γ-irradiation, as determined by ABTS scavenging activity.

A crude methanolic extract and its derived fractions (n-hexane, chloroform, ethyl acetate and n-butanol) of the aerial parts of *Dicliptera roxburghiana* exhibited a promising antioxidant potential for ABTS (Ahmed *et al.*, 2013). Ayesha *et al.* (2013) observed that the gemmomodification process improved the antioxidant potential of *Silybum marianum*, which was evident from the better ABTS radical scavenging activity exhibited by of the gemmo modified extract than the native extract (dry leaves).

The antioxidant potential of the ethyl acetate extracts of three plants namely *Syzygium gratum*, *Justicia gangetica* and *Limnocharis flava* was compared using ABTS assay, among which the *S. gratum* extract showed a 10 fold greater response (Stewart *et al.*, 2013). Tayade *et al.* (2013) observed that the ABTS cation scavenging capacity of the methanolic and aqueous extracts of the roots of *Rhodiola imbricata* Edgew increased in a dose dependent manner.

Among different solvent extracts (hexane, dichloromethane, ethyl acetate, ethanol and methanol) of the leaves of *Punica granatum*, the methanolic extract exhibited the maximum ABTS radical quenching effect compared to the other extracts (Bekir *et al.*, 2013). Similarly, the methanolic extract isolated from the different parts of *D. suffruticosa* (root, flower, fruit and leaf) showed better ABTS radical scavenging activity than that of the aqueous extracts (Armania *et al.*, 2013). The crude methanolic extracts from the fruits of *Phillyrea latifolia* showed significant antioxidant potential against ABTS radical (Ayranci and Erkan, 2013).

A significant correlation between the total phenolic content and the antioxidant potential of the commercial tinctures obtained from three *Lamiaceae* plants namely *Salvia officinalis*, *Mentha piperita* and *Melissa officinalis* was confirmed using ABTS scavenging assay (Kowalczyk *et al.*, 2012). Among five new quinic acid derivatives isolated from the roots of *Scorzonera divaricata* Turcz., three quinic acid derivatives exhibited strong antioxidant activity against ABTS cation (Yang *et al.*, 2013b).

All these reports show that ABTS radical scavenging property is a very reliable measure of the antioxidant potential of the plant extract. The results of the present study showed that all the three flowers of *C. pulcherrima* also exhibited significant scavenging against ABTS, reflecting their strong antioxidant activity.

5.1.3. Hydrogen Peroxide Radical Scavenging Activity

Hydrogen peroxide is a highly reactive ubiquitous by-product of aerobic metabolism and plays an important role in immune response and various physiological processes.

Hydrogen peroxide in the presence of released iron, generates the highly reactive hydroxyl radical through Fenton reaction (Kalyanaraman, 2013).

In the present study, the ability of *C. pulcherrima* flower extracts to scavenge hydrogen peroxide was assessed *in vitro*. Among the six different solvent extracts examined, the methanolic extract of the flowers showed higher scavenging effect against hydrogen peroxide followed by the aqueous extracts.

While DPPH and ABTS radical scavenging is indicative of the antioxidant activity of herbal extracts/components, it should be borne in mind that they are synthetic antioxidants. H_2O_2, on the other hand, is a biological metabolite and by-product of the aerobic metabolism. Thus, the ability of a plant extract or its component to neutralize H_2O_2 *in vitro* gains importance, in that, it is likely to happen *in vivo* also. This parameter has, thus, been a central aspect in many studies as discussed below.

Nishida *et al.* (2013) reported that the homoisoflavones isolated from the ethyl acetate extract prepared from the bulbs of *Scilla scilloides* Druce exhibited strong H_2O_2 scavenging activity. The n-hexane extract of *P. hexandrum* also exhibited good neutralization of H_2O_2 radicals in a dose dependent manner (Ganie *et al.*, 2013).

Other studies have reported a potent antioxidant activity against H_2O_2 in the aqueous extract of *Artemisia absinthium* (Saxena and Shukla, 2012), n-hexane and acetone extracts of *Carpobrotus edulis* leaves (Omoruyi *et al.*, 2012) and acetone extracts of two medicinal plants namely *Gasteria bicolor* and *Pittosporum viridiflorum* (Otong *et al.*, 2012). Among various solvent extracts of different polarity (hexane, ethyl acetate, chloroform and methanol) prepared from the whole plant *Sonchus asper*, the methanolic extract showed the maximum H_2O_2 scavenging effect (Khan *et al.*, 2012a). The methanolic extracts of flower, seed and leaves of *Alcea hyrcana* Grossh (Zakizadeh *et al.*, 2011) and the aqueous extract of *Withania somnifera* roots (Pawar *et al.*, 2011) exhibited better antioxidant activity against free radicals, including both the hydrogen peroxide and DPPH radicals.

Several bioactive compounds isolated from plants have been identified as potent H_2O_2 scavengers. For example, glyceollins from soya bean (Kim *et al.*, 2010), penta-O-galloyl-β-D-glucose derived from *Oenothera paradoxa* defatted seeds (Kis *et al.*, 2010) and five phenolic compounds (tyrosyl acetate, (+)-phillygenin, (8E)-ligustroside, rutin and verbascoside) isolated from the chloroform extract of the flowers of *Osmanthus fragrans* (Hung *et al.*, 2012) have been reported to have good H_2O_2 scavenging properties.

Thus, the observations from the above studies, strongly support the antioxidant activity of the flowers of *C. pulcherrima*, as reflected by their ability to scavenge H_2O_2.

5.1.4. Hydroxyl Radical Scavenging Activity

The hydroxyl radical is a highly reactive, short lived moiety that causes devastating effects within the body (Taraborrelli *et al.*, 2012). It is a third generation radical species derived through the reduction of hydrogen peroxide by the enzymes glutathione peroxidase and catalase in the presence of transition metals such as iron or copper. It can react virtually with all types of macromolecules including DNA, membrane lipids, proteins and carbohydrates (Ganea *et al.*, 2011).

The antioxidant properties of many plant-derived bioactive compounds, phytochemicals and plants extracts are quantified from their hydroxyl scavenging potential. The antioxidant properties of polyphenols are based on their ability to scavenge hydroxyl radical by forming a corresponding hydroxyl derivative (Lipinski, 2011). The antioxidant potency of a polyphenol extract of *Nyctanthes arbor-tristis* leaves was analyzed based on their hydroxyl scavenging capacity (Meghashri and Gopal, 2012).

In the present study, the six different extracts of the three flowers of *C. pulcherrima* were tested for $OH^{\bullet}$ scavenging activity. The results showed that all the extracts of *C. pulcherrima* leaves exhibited strong hydroxyl scavenging effect, among which, the methanolic extract of all the three flowers was found to be better than the other extracts.

Among various solvent extracts of *Cassia singueana* parts, the ethyl acetate extract of the stem bark exhibited more powerful hydroxyl radical scavenging activity than other extracts (Ibrahim *et al.*, 2013). A similar trend was observed in other studies, in which ethyl acetate fraction of the methanolic extract of *Sygygium fruticosum* (Roxb.) seeds (Islam *et al.*, 2013b) and *Anthocephalus cadamba* (Roxb.) leaves (Chandel *et al.*, 2012) showed strong hydroxyl scavenging activity compared to the other solvent fractions.

In another study, Bokhari *et al.* (2013) showed that the aqueous fraction of the methanolic extract of *Galium aparine* L. strongly scavenged the DPPH, ABTS, hydroxyl, hydrogen peroxide and superoxide radicals compared to the other solvent fractions (n-hexane, ethyl acetate and butanol). Senthilkumar *et al.* (2013) reported that very high hydroxyl scavenging effect was observed in the acetone and methanol extracts of the rhizome of *Rhodiola imbricate*.

Meng *et al.* (2012) compared the antioxidant potential of four different varieties (three red and one white) of Spine grape (*Vitis davidii* Foex.) using DPPH and hydroxyl

scavenging assays. Similarly, Khan *et al.* (2013a) compared the hydroxyl scavenging ability of the methanolic extracts of different parts of *Morus alba* (stem barks, root bark, leaves and fruits). The methanolic and ethanolic extracts of *Rumex dentatus* exhibited their radical scavenging effect in a dose-dependent manner on superoxide anion radicals and hydroxyl radicals (Humeera *et al.*, 2013).

Thus, it is clear that the three flowers of *C. pulcherrima* possess strong antioxidant potential as evident from their ability to scavenge hydroxyl radical. The maximum hydroxyl scavenging effect was observed in the methanolic extract of all the three flowers. Since a similar trend was also observed in the DPPH, ABTS and H_2O_2 scavenging assays, the methanolic extract of all the three flowers were chosen for further analyses.

Khan *et al.* (2013b) reported that the free radical scavenging activity of the ethanolic extract of *Triticum aestivum* seeds varied in a dose dependent manner. A similar concentration-dependent free radical scavenging activity was observed in the aqueous extracts of *Potentilla* L. (*Rosaceae*) species against DPPH (Tomczyk *et al.*, 2013) and in the methanolic extract of *Madhuca indica* bark against DPPH, superoxide anion radical, nitric oxide radical and hydroxyl radical (Chaudhary *et al.*, 2012).

In the present study, the optimum dose of the methanolic extract to be used for the subsequent phases was determined. The dose was optimized 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. Thus, further studies were carried out with 0.1 mg concentration of the cold methanolic extracts of all the three flowers.

5.2 BIOMOLECULAR PROTECTIVE EFFECTS OF *C. pulcherrima* FLOWER EXTRACTS AGAINST OXIDATIVE STRESS

At pathological levels, free radicals and oxidants generate a deleterious process called oxidative stress, which, in turn, causes damage to structures like cell membranes and other macromolecules including lipids, proteins, lipoproteins and DNA (Dato *et al.*, 2013). Overproduced reactive species (ROS and RNS) react with cell membrane fatty acids and proteins, thereby impairing their function permanently and triggering a number of human diseases. Free radicals also induce DNA damage, resulting in mutations that will predispose to cancer and age-related disorders (Bocci and Valacchi, 2013).

Apart from the radical scavenging activity, the antioxidant potential of a test compound or herbal preparation is also based on their protective effect against oxidant-

induced damage to cellular biomolecules. Hence, in the present study, the effect of the methanolic extract of the three flowers of *C. pulcherrima* against oxidant-induced damage to lipids, DNA and proteins were analyzed *in vitro*.

5.2.1 Inhibition of Lipid Peroxidation

Oxygen radicals cause damage to cellular membranes through the initiation of a process known as lipid peroxidation. Lipid peroxidation occurs as a radical chain reaction that spreads rapidly, affecting a large number of lipid molecules, which leads to altered membrane integrity and permeability (Yoshida *et al.*, 2013). A positive correlation between high levels of lipid peroxidation and the etiology of various diseases such as neurological disorders and atherosclerosis have been reported (Niki, 2009).

In recent years, many studies have focused on measuring lipid peroxidation products that can be used as potential biomarkers to assess the oxidative stress status *in vivo* and to evaluate the effectiveness of antioxidants (Niki, 2013). In the present study, the biomolecule-protective effects of *C. pulcherrima* flower extracts against lipid peroxidation were investigated using three different membrane models. In all the three membrane preparations, the extent of lipid peroxidation was substantially decreased by the three flower extracts. A better protection by the flower extracts against lipid peroxidation was observed in the goat liver homogenate, followed by the liver slices (intact cells) and RBC ghosts (plasma membrane preparation).

A vast literature assessing the inhibitory effects of plants and herbs on LPO in different membrane lipid sources is available to support our findings.

Sreelatha and Padma (2009a) reported a significant inhibition of malondialdehyde formation by the aqueous extract of *Moringa oleifera* leaves in liver homogenate. The same extract also significantly reduced the levels of lipid peroxides in goat liver slices under CCl_4-induced oxidative stress (Sreelatha and Padma, 2010).

A high degree of inhibition of LPO was shown by the aqueous and ethanolic extract of *Phyllanthus fraternus* callus (Upadhyay *et al.*, 2013). Crude extract and fractions of *Harpagophytum procumbens* inhibited LPO in brain homogenates in a concentration-dependent manner (Schaffer *et al.*, 2013). A dose-dependent reduction in LPO was observed in experimental rats treated with amukkara choornam ethanolic extract (Patra *et al.*, 2013).

Sarkar *et al.* (2013) reported that administration of 70% methanolic leaf extract of *Cajanus cajan* significantly reduced the hepatic LPO levels in mice induced with liver damage. Pre-treatment with *Ginkgo biloba* extract reduced the cardiac malondialdehyde

levels in rats induced with acute cardiotoxicity (El-Boghdady, 2013). Co-treatment with *Emblica officinalis* fruit extract decreased the level of lipid peroxidation by 36% in thymocytes of mice exposed to arsenic-induced oxidative damage (Singh *et al.*, 2013).

Six-week supplementation of *Chlorella vulgaris* extract was associated with marked reduction of serum MDA levels in smokers (Panahi *et al.*, 2013). Inhibition of pancreatic LPO by *Evolvulus alsinoides* extract in streptozotocin induced diabetic rats was reported by Gomathi *et al.* (2013). Safranal reduced the levels of serum malondialdehyde in diabetic rats (Samarghandian *et al.*, 2013).

These reports support the findings of the present study, wherein, the three flower extracts were effective in inhibiting LPO in the three different membrane lipid systems. All the three flower extracts rendered a strong protection to intracellular lipids. The flower extracts also rendered a better protection to liver slices, which implies that some bioactive component present in the extract is capable of penetrating through the cell membrane that renders the antioxidant property for inhibiting LPO.

Thus, our results clearly demonstrate that the flower extracts of *C. pulcherrima* protect the membrane lipids effectively against oxidative damage.

5.2.2. Inhibition of Oxidative DNA Damage

Oxidative damage arises from endogenous and exogenous sources and affects both nuclear and mitochondrial DNA as well as RNA and proteins. DNA is constantly damaged by ROS and RNS directly. The lipid peroxidation (LPO) products also affect DNA, forming exocyclic adducts to DNA bases (Pascucci *et al.*, 2011). A wide variety of oxidatively-generated DNA lesions such as single strand breaks to complex lesions like double strand breaks and other oxidatively generated clustered DNA lesions are present in living cells (Tudek *et al.*, 2010). Accumulation of oxidative DNA lesions due to misrepair or incomplete repair causes mutagenesis, which consequently leads to carcinogenesis (Kryston *et al.*, 2011).

The protective effects of the flowers of *C. pulcherrima* on oxidant-induced DNA damage were determined using DNA from different hierarchies of evolutionary development that included the commercially available preparations of viral DNA (λ DNA), bacterial plasmid (pUC18) and DNA of animal origin (herring sperm DNA). The results showed that all the three flower extracts of *C. pulcherrima* were able to protect the DNA from oxidant-induced damage, at all the hierarchies.

Many research studies have reported the protective effects of plant extracts and their isolated bioactive compounds against oxidative DNA damage. Arriaga-Alba *et al.* (2013) reported the protective effect of the natural product affinin isolated from the ethanol extract of *H. longipes* against norfloxacin-induced DNA damage. Kumar *et al.* (2012) showed the genoprotective activity of the methanolic extract of *Koelreuteria paniculata* leaves against H_2O_2-induced DNA damage using pUC18 and calf thymus DNA.

The ethanolic extract of *Caesalpinia sappan* rendered a strong protection against H_2O_2-induced damage to pUC18 plasmid DNA (Saenjum *et al.*, 2010). The aqueous extract of various parts of *Moringa oleifera* (leaf, fruit and seed) significantly inhibited the hydroxyl radical-induced damage of pUC18 plasmid DNA (Singh *et al.*, 2009b).

Many other plant extracts such as *Polyalthia longifolia* leaf extract (Jothy *et al.*, 2013), methanolic extract of *Phyllanthus virgatus* (Hashim *et al.*, 2013) and heartwood extract of *Acacia catechu* (Hazra *et al.*, 2010) have been found to inhibit H_2O_2-induced damage to pUC18 plasmid DNA.

Aqueous extract of *Curcuma amada* (Roxb) showed a concentration-dependent protecting effect against H_2O_2-induced damage in herring sperm DNA (Vishnupriya *et al.*, 2012). Sumathi *et al.* (2010) reported that the leaf and herbal extracts of *Withania somnifera* against rendered a significant protection against H_2O_2-induced oxidative damage to pUC18, lambda and herring sperm DNA. A dose-dependent protection by black tea extract against radiation-induced damage in pBR322 DNA and calf thymus DNA has also been reported (Ghosh *et al.*, 2012). The DNA protective effect of the aqueous extract of *Schleichera oleosa* against H_2O_2-induced damage was shown using pBR322 DNA (Thind *et al.*, 2010).

The results of the present study are in agreement with the above reports that the flowers of *C. pulcherrima* are very effective in protecting the DNA from oxidative damage. The variation found in the extent of protection with different hierarchical levels of DNA signifies that the biomolecular protective efficiency of a substance is quantified by analyzing its effect on different forms and sources of DNA exposed to oxidative stress.

5.2.3. Inhibition of Protein Oxidation

Oxidative modifications of protein by ROS/RNS include the formation of protein carbonyls, dityrosine, nitrated and chlorinated tyrosines, resulting in diverse functional consequences. Accumulation of oxidized proteins have been found in diseased tissues of patients with various diseases like inflammatory diseases, atherosclerosis, rheumatoid arthritis and cataractogenesis (Yan, 2009).

A major form of protein oxidation is protein carbonylation. Protein carbonyls can be used as markers for oxidative stress (Yan and Forster, 2011). In the present study, the reduction in the oxidant-induced protein carbonyl formation observed in the flower extract treated groups indicated the protective effect of the flowers of *C. pulcherrima* against protein oxidation.

These results corroborated with a study, in which the aqueous extracts of *Rosa roxburghii* significantly inhibited the accumulation of protein carbonyls of bovine serum albumin (BSA) induced by $Cu^{2+/}H_2O_2$ and 2,2-azobis (2-amidinopropane) dihydrochloride in a dose-dependent manner (Xiang *et al.*, 2012). In a similar study by Esmaeili *et al.* (2010), using BSA, a dose-dependent inhibitory effect of the methanolic extract of *Salvia reuterana* on glycation-induced protein oxidation was reported by measuring the protein carbonyl formation.

The aqueous extract of *Aphanes arvensis* leaves possessed a strong inhibitory effect on Cu^{2+}/H_2O_2-induced protein oxidation of BSA in a dose dependent manner (Hamad *et al.*, 2010). Using BSA for protein carbonyl formation, Njayou *et al.* (2008) reported that, among 23 medicinal plants used in Bamun folk medicine, the methanol-methylene chloride extracts of fifteen plants were active in preventing the protein oxidation.

It was observed that a mixture of aqueous extract of *Allium sativum* and methanolic extract of *Lagerstroemia speciosa* decreased the hepatic protein carbonyl levels significantly in the type-II diabetic rats (Kesavanarayanan *et al.*, 2013). A dose dependent decrease in the serum protein carbonyl was observed in Swiss albino mice treated with 70% methanol extract of *Spondias pinnata* (Hazra *et al.*, 2008).

Administration of polyphenolic-rich extract prepared from *Sorghum bicolor* grains decreased the level of protein carbonyl in DEN-treated rat microsomes (Ajiboye *et al.*, 2013). The chloroform fraction of *Solanum guaraniticum* leaves completely inhibited the formation of protein carbonyls in serum of male Wistar rats (Zadra *et al.*, 2012).

Kędzierska *et al.* (2013) suggested that the protein carbonyls can be used as markers for hemostasis changes in breast cancer patients. They also showed that in an *in vitro* system, treatment with a commercial extract prepared from *A. melanocarpa* berries reduced the levels of protein carbonyls in plasma from breast cancer patients, after surgery and different phases of chemotherapy. Co-treatment with *Etlingera elatior* extract reduced the protein carbonyl content in the testis of experimental rats with lead acetate-induced testicular injury (Haw *et al.*, 2012).

Inhibition of protein carbonyl formation in sham-operated rats after treatment with polyphenols isolated from *Euterpe oleracea* Mart. extract was reported in a study by da Costa *et al.* (2012). Another phenolic compound called phloretin isolated from apple attenuated the acrolein-induced incorporation of carbonyl groups into BSA (Zhu *et al.*, 2012).

The protective effect of the *C. pulcherrima* flower extracts was further confirmed using SDS-PAGE analysis, in which, a drastic degradation of proteins (as indicated by the diminished protein bands intensity) was observed on exposure to H_2O_2. Treatment with the *C. pulcherrima* flower extracts caused a remarkable reversal in the band intensities in the presence of the oxidant, indicating the significant protection rendered by the flower extracts against protein oxidation.

Makri *et al.* (2013) confirmed the protective effect *Crocus sativus* stigmas (saffron) extract against selenium-induced crystalline proteolysis of rat lens proteins using SDS-PAGE. Park *et al.* (2010) showed a decreased multimerization pattern of apolipoprotein (apo) A-I on SDS-PAGE after treatment with the purple sweet potato extract. Among the aqueous extracts of *Phaseolus vulgaris* pods, *Olea europaea* leaves, unripe bitter melon fruits and *Morus nigra* leaves, only the extracts of the *Phaseolus vulgaris* and bitter melon fruits reduced the oxidative fragmentation of BSA as evaluated by SDS-PAGE technique (El-Khawaga and Abou-Seif, 2010).

Inhibitory effect of epicatechin conjugates obtained from grape against oxidation of erythrocyte membrane proteins was confirmed using SDS-PAGE (Martínez *et al.*, 2012). Gugliucci *et al.* (2009) found that chlorogenic acid and caffeic acid are the main bioactive compounds that are responsible for anti-glycation effect of yerba maté tea (*Ilex paraguariensis*) using SDS-PAGE analysis. Our results also showed that the methanolic extracts of the flowers of *C. pulcherrima*, blooming in all the three different colours, were very efficient in protecting proteins against oxidative damage.

The results of phase I of the present study showed that the extracts of all the three different flowers of *C. pulcherrima* exhibit substantial free radical scavenging activity and antioxidant property. The flowers also rendered a significant biomolecular protection against oxidative stress, both in cell-free systems and in intact cells.

PHASE II

5.3 INFLUENCE OF THE *C. pulcherrima* FLOWER EXTRACTS ON THE ANTIOXIDANT STATUS OF LIVER SLICES SUBJECTED TO OXIDATIVE STRESS

The results of the phase I confirmed the strong antioxidant potential and biomolecular protective effects flowers of *C. pulcherrima* against oxidative stress. In phase II, the protective effects of the flowers of *C. pulcherrima* against H_2O_2 induced oxidative stress was evaluated using precision cut goat liver slices.

Precision-cut liver slices are an organotypic liver model that is widely used in physiological, pharmacological and toxicology studies (Soldatow *et al.*, 2013). The major advantage of using the liver slices is that the interactions between Kupffer cells and hepatocytes are preserved. The extracellular matrix and its associated hormones, growth factors and cytokines enable the maintenance of the tissue coherence and regulation of the cell functions (Dragoni *et al.*, 2012). Moreover, in precision cut liver slices, the tissue integrity and the viable cell populations in cell culture can be maintained for several days, which enables the evaluation of global tissue response (LeCluyse *et al.*, 2012).

Indeed, a number of studies are being carried out using precision-cut liver slices to investigate the cytotoxicity and carcinogenicity of various drugs and novel substances under standardized conditions. Using microarrays, Guan *et al.* (2013) have shown that precision cut liver slices mimic the *in vivo* toxicity more closely than the isolated hepatocytes or cell line cultures, making it a valuable tool for gene expression studies. Precision-cut liver slices from humans and other species have been used to study the pathways of phase I and II of xenobiotics metabolism (Lake and Price, 2013). van Midwoud *et al.* (2011) have developed a HPLC method with UV detection, which enables the on-line monitoring of drug metabolism in continuously perfused, precision-cut liver slices in a microfluidic system.

In the present study, the goat liver slices were selected as an *in vitro* model to determine the antioxidant potential of the methanolic extract of the three different flowers of *Caesalpinia pulcherrima* (yellow, pink and orange) against H_2O_2-induced oxidative stress. Both the enzymic and non-enzymic antioxidants were assessed in the liver slices subjected to oxidative stress in the presence and the absence of the leaf extract.

5.3.1. Enzymic Antioxidant Activities

The enzymic antioxidants analyzed were superoxide dismutase, catalase, peroxidase, glutathione S-transferase and glutathione reductase.

i) Superoxide dismutase

There are three forms of SOD depending upon the intracellular location. They are CuZn-SOD, which is present in the cytoplasm, Mn-SOD, which is mainly found in the mitochondria and extracellular SOD (EC-SOD) which is initially present in the intracellular spaces in neonates, but found in the extracellular space in later stages. The main function of SOD is to convert superoxide radical to H_2O_2 and O_2 (Shim and Kim, 2013).

In precision-cut goat liver slices, exposure to H_2O_2 caused a significant decrease in SOD activity compared to that of untreated control, which was significantly improved on co-treatment with the methanolic extract of the three flowers (yellow, pink and orange) of *C. pulcherrima*.

Several studies have shown that the assault with an oxidant causes a depletion in the antioxidant status of the tissues, which is effectively restored by the administration of an antioxidant-rich plant extract. The improved activity of liver superoxide dismutase (SOD) after treatment with the aqueous extracts of the freeze-dried, germinated and fermented mung bean confirmed their hepatoprotective effects against ethanol-mediated liver injury (Ali *et al.*, 2013). Mahmud *et al.* (2012) reported a significant increase in the reduced levels of superoxide dismutase (SOD), catalase and peroxidase after administration of ethanolic extract of *Premna esculenta* Roxb. leaves against CCl_4-induced liver damage in rats.

A similar observation was reported by Ganie *et al.* (2013) in which the n-hexane extract of *Podophyllum hexandrum* rhizome significantly increased the activity of GR, GPx, GST and SOD in the rat liver tissue against CCl_4 induced oxidative stress. The methanolic extract of the bark of *Bacopa monnieri* significantly increased the reduced activity of SOD, CAT and GST in the liver and kidney of mice against CCl_4-induced oxidative stress (Radha and Padma, 2013).

The flavonoid fraction extracted from the flowers of *Abelmoschus manihot* (L.) improved the activities of GR, GPx, GST and SOD in the mice liver (Ai *et al.*, 2013). Pretreatment with curcumin significantly improved the activity of SOD, CAT and GPx in liver tissues of mice exposed to gamma radiation (Tawfik *et al.*, 2013). A similar trend was observed in a study where lycopene increased the activity of SOD, CAT, GST and GPx in rat liver tissues against oxidative damage occurred during experimental hepatitis induced by a lipopolysaccharide (Abdulazeez and Thiruvengadam, 2013).

In corroboration with these studies, our results showed a depletion of SOD activity upon exposure to H_2O_2, which was restored upon co-exposure to the methanolic extract of

all the three flowers of *C. pulcherrima*. These observations indicate the protection rendered by the flowers to the liver tissue.

ii) Catalase

Catalase is an oxidoreductase that catalyzes the conversion of hydrogen peroxide into oxygen and water. It is an important protein in the cellular metabolism, which scavenges both intracellular and extracellular superoxide radicals and prevents lipid peroxidation of plasma membrane (Wang *et al.*, 2011b).

In the present study, the catalase activity in the liver slices exposed to H_2O_2 reduced significantly compared to that of untreated group. The methanolic extract of all the three flowers of *C. pulcherrima* significantly elevated the catalase activity in the presence of the oxidant, proving that the flower extracts can restore the antioxidant status of oxidant-assaulted tissues. Similar findings were reported in many *in vitro* and *in vivo* studies, wherein plant extracts and their isolated phytoconstituents protect the liver tissues from oxidative damage by restoring their antioxidant activity to normalcy.

Yang *et al.* (2013c) reported that the aqueous extract formula derived from a mixture of *Artemisia capillaris*, *Lonicera japonica* and *Silybum marianum* attenuated the carbon tetrachloride CCl_4-induced liver damage in rats by restoring the GSH level and improving the activity of SOD, CAT and GPx in a dose dependent manner. The leaf extracts of *Clitoria ternatea* significantly increased the activity of enzymic antioxidants SOD, CAT, GST and GPx in goat liver slices in the presence of the oxidant H_2O_2 (Jayachitra and Padma, 2012).

The hepatoprotective effect of the ethanolic extract of *Mollugo nudicaulis* against percholoroethylene-induced acute liver injury was confirmed from the increased activity of SOD, CAT, GPx, GST and increased levels of GSH and vitamin C in rat liver (Rajamanikandan *et al.*, 2012). The aqueous extract of stem bark of *Pterocarpus marsupium* restored the activity of antioxidant enzymes SOD, CAT, GPx and GR in rat liver slice culture exposed to ethanol-mediated oxidative stress (Mohammadi *et al.*, 2009).

The red grape seed extracts restored the activity of CAT and GPX in mice liver slice culture against ethanol-induced cytotoxicity (Hassan, 2012). A similar observation was reported by Sinha *et al.* (2011), where the *Picrorhiza kurroa* extract restored the activities of SOD, CAT, GST, GPx and GR and also increased the GSH levels in mice liver slice culture against ethanol-induced cytotoxicity.

In the present study, co-treatment with the flower extracts of *C. pulcherrima* increased the catalase activity, which implies that it can stimulate the antioxidative bioactive

molecule expression during oxidative stress and can attenuate the H_2O_2-induced damage in the liver.

ii) Peroxidase

Hydrogen peroxide is detoxified to water by catalases and peroxidases. The peroxidase enzymes also detoxifies lipid peroxides (LOOH) by converting them into their corresponding alcohol (Kalyanaraman, 2013).

In the present study, the presence of the oxidant, H_2O_2, caused a marked decrease in the peroxidase activity, which was effectively restored after treatment with *C. pulcherrima* flower extracts. Many studies have reported an increase in the reduced peroxidase activity when plant extracts exert their hepatoprotective effect against oxidant-induced damage.

The *Schisandra chinensis* pollen extract showed strong hepatoprotective effect against CCl_4-induced acute liver damage by lowering the MDA level and elevating the activities of SOD and GPx in rat liver (Cheng *et al.*, 2013). Pretreatment with chloroform and ethanolic extract of *Vitis vinifera* L. stem bark showed significant antidiabetic activity by improving the SOD, catalase and peroxidase levels in diabetes induced rats compared to that of standard drug treated animals (Ahmed *et al.*, 2012).

Treatment with *Moringa oleifera* leaf extract increased the levels of enzymic antioxidants (CAT, SOD, GPx, GST and GR) and glutathione content significantly in CCl_4-treated goat liver slices (Sreelatha and Padma, 2010). A similar observation was reported by Khan *et al.* (2012b) in which the chloroform extract of *Launaea procumbens* exhibited significant hepatoprotective effect against CCl_4 by increasing the levels of enzymic antioxidants and the glutathione content in rat liver slices.

A significant decrease in the activities of serum aspartate aminotransferase, alanine aminotransferase and alkaline phosphatase and an increase in the activities of hepatic SOD and GPx activities indicated the protective effects of the polysaccharide isolated from *Tarphochlamys affinis* against CCl_4-induced hepatotoxicity in rats (Lin *et al.*, 2012b). Polydatin, a bioactive compound isolated from *Polygonum cuspidatum* also rendered hepatoprotection against CCl_4-induced hepatotoxicity in mice by stimulating the antioxidant response in the liver (Zhang *et al.*, 2012).

Rutin increased the activity of endogenous hepatic antioxidant enzymes CAT, SOD, GPx, GST, GR and GSH levels in a dose dependent manner against CCl_4-induced liver injury in rats (Khan *et al.*, 2012c). These observations correlated with our findings, where

the methanolic extract of the three flowers of *C. pulcherrima* improved the activity of GPX in the liver slices undergoing oxidative stress.

iv) Glutathione S-transferase

Regulation and function of GSTs influence cell growth, oxidative stress, disease progression and prevention (Raza, 2011). GST catalyzes the conjugation of GSH with a variety of endogenic and exogenic electrophilic compounds that includes carcinogens and antineoplastics (Gulubova *et al.*, 2011). GSTs play an important role in the detoxification and metabolism of many xenobiotic and endobiotic compounds (Mourad and Noor, 2011).

GST is a sensitive and specific biomarker of cell permeability in various etiologies that leads to inflammation of hepatocytes. A higher concentration of GST is found in the human liver, which is released in response to hepatocellular damage (Muttigi *et al.*, 2009).

In the present study, H_2O_2 significantly reduced the GST activity compared to untreated control liver slices, which was counteracted upon co-treatment with the three flower extracts.

Several studies have reported the hepatoprotective effect of many plant extracts and their isolated bioactive compounds against CCl_4-induced hepatotoxicity are mediated through elevation of antioxidant enzymes (SOD, CAT, GPx, GST and GR) in liver. Such plant extracts and their compounds included aqueous extract of *Origanum vulgare* leaves (Sikander *et al.*, 2013), ethanolic extract of *Vitis thunbergii* var. (Deng *et al.*, 2012), ethanolic extract of cassia seeds (Xie *et al*., 2012) and polyprenols from *Taxus chinensis* var. (Yu *et al.*, 2012).

The methanolic extract of *Hyptis suaveolens* exhibited potent hepatoprotective activity against CCl_4-induced induced hepatotoxicity elevating the activities of SOD, GST and GR and also increased the levels of GSH in rat liver (Ghaffari *et al.*, 2012). Wang *et al.* (2012) showed that the ethanolic extract of *L. christinae* and its isolated compounds, quercetin and bifendate, significantly elevated the levels of hepatic enzymic antioxidants (CAT, GPx, SOD, GST) in mice with alcohol-induced acute liver injury.

In corroboration with the above studies, in the present study, the ability of the flower extracts of *C. pulcherrima* to restore GST activity that are by H_2O_2 confirms that the *C. pulcherrima* flowers can protect the liver slices from oxidative stress by enhancing their antioxidant capacity.

v) Glutathione reductase

The main function of glutathione reductase (GR) is to maintain high levels of reduced glutathione in the cytosol. It reduces oxidized glutathione (GSSG) to reduced glutathione (GSH) (Yadav *et al.*, 2013). Glutathione reductase is a flavoprotein that reduces glutathione disulfid to its thiol form GSH with concomitant oxidation of NADPH. Alterations in the activity of GR affect the GSH/GSSG ratio, which leads to oxidative stress and the pathogenesis of many diseases (Tandogan *et al.*, 2011b).

In the present study, a significant decline in the glutathione reductase activity was observed in the liver slices exposed to the oxidant H_2O_2. This reduction was normalized upon treatment with the flower extracts.

The above findings also correlated with another study where the co-treatment with methanolic extract of *Carissa opaca* leaves and silymarin exhibited strong hepatoprotective effect by restoring the activities of hepatic CAT, GPx, SOD, GST, GPx, GR and quinone reductase in rat liver homogenates (Sahreen *et al.*, 2011). Pretreatment with *Fumaria parviflora* Lam. extract modulated both the mRNA expression and activity of hepatic antioxidant enzymes (SOD, GPx and GR) in rats during nimesulide toxicity (Tripathi *et al.*, 2011).

Palaniswamy and Padma (2011a) showed that the activities of antioxidant enzymes (SOD, CAT, GPx, GST and GR) in the goat liver slices exposed to oxidative stress were significantly improved on treatment with the methanolic and chloroform extracts of *Majorana hortensis* leaves. Aly *et al.* (2013) showed that lycopene exerts its protective effect against rat liver microsomal toxicity by restoring the levels of microsomal antioxidant enzymes (CAT, SOD, GST, GPx and GR) towards normalcy. A recent study on the management of nephrolithiasis using natural products have reported that the supplementation with ethanolic extract of *Saccharum spontaneum* restored the levels of GST, GR, SOD, CAT and GPx in liver and kidney homogenate thereby exhibited significant antiurolithiatic activity against ethylene glycol induced nephrolithiasis in male wistar albino rats (Sathya and Kokilavani, 2012).

These observations correlated with our results where the flower extracts restored the depleted GR activity by counteracting the effects of H_2O_2. Thus, our findings signify that the flowers of *C. pulcherrima* can effectively restore the antioxidant-defence systems in liver slices during H_2O_2-induced oxidative stress.

5.3.2. Non-enzymic Antioxidant Levels

Apart from enzymic antioxidants, non-enzymic antioxidants are also found in biological systems and are found to play an important role in defence mechanisms against oxidative stress. Non-enzymic antioxidants are of two types, namely water-soluble (vitamin C and phenolic compounds) or lipid-soluble (vitamin E and carotenoids) antioxidants (Podsędek, 2007). The non-enzymic antioxidants analyzed in the present study were vitamin C, vitamin E, vitamin A, reduced glutathione, total thiols and protein thiols.

i) Vitamin C

Ascorbic acid acts as an enzyme cofactor, a radical scavenger and as a donor or acceptor in electrontransport at the plasma membrane. It also scavenges the superoxide and hydroxyl radicals and regenerates α-tocopherol (Cronje *et al.*, 2012).

H_2O_2 exposure caused a significant decrease in the vitamin C levels in the goat liver slices. Co-treatment with the methanolic extract of the *C. pulcherrima* flowers increased the vitamin C levels compared to the oxidant treated groups.

Our results correlated with another study, which reported that the methanolic extract of leaves and rhizomes of *Curcuma amada* significantly improved the levels of viamin C in precision-cut liver slices exposed to oxidative stress (Sivaprabha *et al.*, 2012). In a study conducted by Muthappa *et al.* (2013), dietary lipotropes (betaine and lecithin) supplementation improved the antioxidant status (activities of SOD, CAT and levels of vitamin C) in muscle, liver, brain and kidney of *Labeo rohita* fish fingerlings exposed to endosulfan-induced stress.

Pretreatment with lycopene restored the levels of non-enzymatic antioxidant markers such as GSH, vitamin C and E in rat liver during lipopolysaccharide-induced toxic injury (Abdulazeez and Thiruvengadam, 2013). A similar observation was reported in a study by Shakya *et al.* (2013), in which, kaempferol exerted its hepatoprotective action against oxidative stress induced by alcohol and thermally oxidized polyunsaturated fatty acid in rat liver by increasing the enzymatic antioxidants activities (CAT, SOD and GPx) and non-enzymatic antioxidant levels (GSH, vitamin E and vitamin C).

The above observations correlated with those of the present study, in which, the increase in the vitamin C levels after treatment with the flower extracts of *C. pulcherrima* proved their ability to improve the antioxidant status in liver tissues.

ii) Vitamin E

Tocopherols are powerful lipid-soluble antioxidants, which constitute a series of related benzopyranols or methyl tocols and are abundant in plant tissues and vegetable oils (Seppanen, 2010). The antioxidant activity of tocopherol mainly involves the donation of a hydrogen atom, followed by the formation of a tocopheroxyl radical (Wong and Radhakrishnan, 2012). Due to their lipophilic nature and membrane localization along with storage lipids, tocopherols can rapidly react with lipid hydroperoxides in a non-enzymic manner thereby inhibiting the propagation of lipid peroxidation (Colombo, 2010).

In the present study, H_2O_2 exposure caused a significant depletion in the levels of tocopherol compared to untreated controls. This depleting effect was reversed by the co-treatment with the flower extracts of *C. pulcherrima.*

These findings correlated with the study in which, the supplementation of the protein deficient diet (PDD) with the methanolic extracts of six locally consumed plants in Nigeria for nutritionally stressed male albino rats resulted in significantly higher levels of vitamin E and vitamin C in liver and kidney tissues compared to that of PDD group (Hemalatha *et al.*, 2013).

Our results also correlated with another study in which a significant increase in the levels of vitamin C, tocopherol, reduced glutathione and vitamin A was observed in goat liver slices exposed to H_2O_2 after treatment with *Zea mays* leaf extract (Balasubramanian and Padma, 2012). Supplementation of vitamin E effectively ameliorated the oxidative stress status by increasing the hepatic antioxidant enzyme activity (SOD, CAT, GPx) and GSH levels in mice (Zhang *et al.*, 2013).

Ohta *et al.* (2013) reported that vitamin E supplementation is essential for hepatoprotection against water-immersion restraint stress in rats. Combination of carnosine with vitamin E increased the hepatic antioxidative status (GSH and vitamin E levels) in rats against age-related oxidative stress (Coban *et al.*, 2013).

In the light of these reports, our results indicate that the flowers of *C. pulcherrima* may act as a good source of vitamin E. This suggestion is also supported by our findings reported earlier by Aparna (2000).

Vitamin E is the only antioxidant that is primarily located within the phospholipid bilayer of cell membranes. It plays an important role in preventing peroxidation of polyunsaturated fatty acids and other components of cell membranes. (http://www.fao.org/docrep/004/y2809e/y2809e0f.htm). These facts thus support our

findings that the flowers of *C. pulcherrima* can render a strong protection for membrane lipids by increasing the levels of the membrane antioxidant vitamin E.

iii) Vitamin A

Maintenance of normal retinoid (vitamin A and its derivatives) homeostasis is essential for many biological functions. Retinoids are derived from diet, as preformed vitamin A from animal products or as β-carotene from vegetables and fruits (Ortiz *et al.*, 2009). Vitamin A protects cells from radical-induced damage, regulates immune function by acting as the first defence barrier and promotes epithelial cell differentiation. These beneficial properties of vitamin A are attributable to its antioxidant activity (Yang *et al.*, 2010).

In the present study, the flowers of *C. pulcherrima* restored the reduced levels of vitamin A in liver slices subjected to H_2O_2-induced oxidative stress.

These results correlated with a study in which the methanolic extract of the leaves of *Nyctanthes arbortristis* improved the levels of non-enzymic antioxidants (vitamins C, E, A and GSH) in goat liver slices exposed to oxidative stress (Sumathi *et al.*, 2011a). A similar trend was observed in another study, where the non-enzymic antioxidant levels were elevated after treatment with *Alternanthera sessilis* in rat liver (Sumathi and Padma, 2010).

Supplementation of antioxidant mixture containing vitamin A, coenzyme Q10, vitamin C and vitamin E were found to reduce the formation of 7-DHC oxysterols in fibroblasts of Smith-Lemli-Opitz syndrome patients (Korade *et al.*, 2013). Pipaliya and Vaghasiya (2012) demonstrated that vitamin A markedly attenuated the isoproterenol-induced myocardial injury in rats by significant decreasing the MDA levels and by increasing the antioxidant status (SOD, CAT and GSH).

In a study conducted by Cemek *et al.* (2010), a significant increase in the GSH levels of whole blood and tissues together with the increased levels of ceruloplasmin, sialic acid, vitamin C and retinol in serum proved the hepatoprotective and antioxidant activities of the Royal Jelly against CCl_4-induced acute liver damage.

In view of these reports, an increase in the vitamin A levels by the flower extracts of *C. pulcherrima* signifies the antioxidant defence rendered by the flowers against oxidative stress.

iv) Reduced Glutathione

GSH is one of the major intracellular defence against free radical induced damage. Free radicals oxidize glutathione, but the reduced form of GSH is regenerated in a redox cycle that involves by glutathione reductase and NADPH (Pereira *et al.*, 2013). Glutathione and thiols are the only endogenous antioxidants that we have studied.

In the present study, the liver slices treated with H_2O_2 decreased the level of GSH, which was reverted by the presence of *C. pulcherrima* flower extracts indicating the antioxidant potential of the flower extracts. Many research studies have emphasized a similar antioxidant effect of various plant extracts and the compounds derived from them on GSH levels using both *in vitro* and *in vivo* models.

Haniya and Padma (2013) have reported that the methanolic extract of the leaves of *Artemisia vulgaris* significantly counteracted the depleting effect of H_2O_2 on GSH levels in goat liver slices. An aqueous extract of *Picorrhiza kurroa* effectively inhibited the alcohol-induced oxidative stress in mouse liver slice culture by restoring the reduced levels of GSH (Sinha *et al.*, 2011). Linardaki *et al.* (2013) demonstrated the neuroprotective effect of *Crocus sativus* L. against established aluminum toxicity in adult mice, in which the levels of MDA and GSH were increased by *Crocus sativus*.

The concentrations of SOD, CAT and GSH were significantly decreased in the liver of Wistar rats after treatment with doxorubicin which was reversed on co-treatment with *Punica granatum* Linn (*Punicaceae*) extract (Nirwane and Patil, 2012). A significant restoration of the non-enzymatic antioxidants (GSH and vitamin C) coupled with a significant decrease in the MDA content in liver tissues was observed following consumption of diets containing garlic (*Allium sativum*) in rats with gentamycin-induced hepatotoxicity (Ademiluyi *et al.*, 2013).

Lycopene significantly recovered the liver and kidney tissue injuries in rats with obstructive jaundice by enhancing the levels of GSH, and activities of CAT, SOD and GPx (Aydın *et al.*, 2013). This observation correlated with another study, in which, the oral administration of myelophil attenuated the cold-stress-induced brain oxidative damage by recovering the depleted GSH and SOD activity and by upregulating the catalase gene and protein expression (Kim *et al.*, 2013).

Thus, it can be inferred from the increased levels of GSH in liver slices by the flower extracts that the *C. pulcherrima* flowers exhibit strong antioxidant effect against oxidative stress.

iv) Total thiols and Protein thiols

Thiols constitute a group of aliphatic and aromatic organic compounds that contain a sulphydryl group. Thiols constitute the major portion of the total body antioxidants playing an important role in defence against oxidative stress (Peng *et al.*, 2012).

Total thiols are composed of both intracellular and extracellular thiols. They occur either in free form, as oxidized or reduced glutathione, or as protein bound thiols. Reduced levels of thiols have been implicated in the etiology of various diseases, including chronic renal failure, cardiovascular diseases, stroke, diabetes, alcohol cirrhosis and neurological disorders (Prakash *et al.*, 2009).

In the present study, hydrogen peroxide decreased the levels of both total thiols and protein thiols. This depleting effect was significantly counteracted by the flower extracts of *C. pulcherrima*. These findings corroborated with a study, in which aqueous leaf extract of *Passiflora manicata* effectively restored the levels of both total and protein thiols in rat liver slices subjected to H_2O_2-induced oxidative stress (Morrone *et al.*, 2013).

The hepatoprotective effect of *Apium graveolens* leaves against acetaminophen-induced liver damage in fresh water fish was associated with the enhancement of both enzymatic (SOD, CAT, GPx and GST) and non-enzymatic (GSH) antioxidants along with the cellular thiol levels (T-SH, P-SH and NP-SH) in liver tissues (Shivashri *et al.*, 2013).

Pretreatment with the alcoholic extract of *Bacopa monnieri* leaf powder prevented the depletion of GSH and thiol levels in brain of mice subjected to 3-nitropropionic acid-induced oxidative stress (Shinomol *et al.*, 2012). This observation correlated with another study, in which, the hydroethanolic extract of the leaves and flowers of *Moringa oleifera* effectively alleviated the N-acetylcysteine-induced hepatotoxicity in rats by significantly elevating the GSH levels and activities of CAT and SOD (Fakurazi *et al.*, 2012). Similarly in another study, Das *et al.* (2012) reported that the methanolic extract of *Moringa oleifera* leaves prevented the depletion of hepatic GSH levels induced by high-fat diet in mice.

An aqueous extract of *Centella asiatica* rendered neuroprotective effect against 3-nitropropionic acid-induced oxidative stress in the brain of prepubertal mice by restoring the reduced levels of GSH and total thiols to normalcy (Shinomol *et al.*, 2010). In a clinical trial conducted by Agarwal *et al.* (2010), a significant correlation between the total thiol content and the ameliorating effect of catechins from green tea extract on oxidative stress in patients with acid fast bacilli-positive pulmonary tuberculosis was observed.

Thus, it is perceivable that the increase in the levels of both total and protein thiols by the flower extracts of *C. pulcherrima* proves their ability to improve the antioxidant status in the liver tissues.

The results of the phase II, thus, confirm that the methanolic extract of all the three flowers of *C. pulcherrima* can improve the antioxidant status of the tissue by increasing both the activities of antioxidant enzymes and the levels of non-enzymic antioxidants. The methanolic extract of the orange flower of *C. pulcherrima* exhibited better antioxidant activity, followed by the pink and the yellow flower extracts.

PHASE III

The results of the earlier two phases of the study, confirmed that the methanolic extract of all three flowers of *C. pulcherrima* are good sources of antioxidants with strong free radical scavenging potential and also render maximum protection to cellular biomolecules against oxidative stress-induced damage and improve the antioxidant status of the liver tissue.

Oxidative stress causes irreversible damage to cell membrane, DNA and other cellular structures by oxidizing lipids, proteins, and nucleic acids, resulting in the dysfunction of biomolecules within cells and, finally, cell death (Mohsenzadegan and Mirshafiey, 2012). Hence, the third phase of this study was formulated to analyze the apoptosis-modulating effects of *C. pulcherrrima* flowers in order to understand their protective mechanism. The influence of the flowers of *C. pulcherrrima* on oxidant-induced apoptosis was studied in both untransformed and 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.

5.4. EFFECTS OF *C. pulcherrima* FLOWER EXTRACTS ON H_2O_2-INDUCED APOPTOSIS IN *Saccharomyces cerevisiae* CELLS

Apoptosis, the intrinsic cell suicide program is essential for maintaining tissue homeostasis by regulating many physiological and pathophysiological processes such as the embryonic body differentiation, formation of the immune system and the homeostasis of the number of cells in proliferating tissue (Fulda, 2009; Samm *et al.*, 2011). Unlike necrosis, apoptosis occurs without inflammatory reaction and is characterized by cell shrinkage,

chromatin condensation and plasma membrane ruffling and finally breaking-up of the cell into apoptotic bodies surrounded by intact plasma membrane (Fulda, 2009).

Over the last decades, the budding *Saccharomyces cerevisiae* has progressively evolved into a preferred research tool in cell biology research. Easy handling and tractable genetics, together with the functional advantage of being a eukaryote, have made it the most widely used *in vitro* model (Carmona-Gutierrez *et al.*, 2010).

Even though apoptosis is an inevitable process implicated in many diseases like cancer, neurodegenerative disorders and AIDS, the factors such as lack of details about its regulation and apoptotic phenotypes, have led many researches to conduct an intense research for a better understanding of apoptosis. In addition, an easier *in vitro* model system is desired to study apoptosis as a result of the complex regulatory network and the contradictory results obtained using human cell lines (Kazemzadeh *et al.*, 2012).

The conservation of apoptotic machinery in yeast makes it a suitable model organism to conduct research on human apoptosis. Thus, in the past few years, scientists have identified new apoptosis regulators of humans, plants and fungi using *S. cerevisiae* cells (Carmona-Gutierrez *et al.*, 2010). For example, Ocampo and Barrientos (2011) have developed *in vitro* yeast models to study neurodegenerative disorders.

In the present study, *S. cerevisiae* was chosen as an *in vitro* model to study the protective effect of *C. pulcherrima* flowers on oxidant-induced apoptosis in untransformed cells. Our observations showed that H_2O_2 exposure caused a significant increase in the number of *S. cerevisiae* cells undergoing apoptosis, which was effectively counteracted by the flower extracts of *C. pulcherrima*, as evident from the reduced apoptotic ratio.

5.4.1. Assessment of Cell Viability in *S. cerevisiae* Cells Using MTT and SRB Assays

The per cent cell viability was quantified using MTT and SRB assays. It was evident from the results that H_2O_2 treatment significantly decreased the viability of *S. cerevisiae* cells. The co-administration of the flower extracts showed marked improvement in the cell survival. This shows that the flowers of *C. pulcherrima* render a significant protection to non-transformed yeast cells against oxidative stress.

A similar observation was reported by Sivaprabha *et al.*, (2013) wherein, the methanolic extract of both the leaves and the rhizomes of *Curcuma amada* Roxb. significantly increased the viability of yeast cells exposed to H_2O_2-induced oxidative stress as determined by MTT and SRB assays. The three different extracts of *Majorana hortensis* leaves (aqueous, methanol and chloroform) rendered good protection against H_2O_2-induced

apoptosis in *S. cerevisiae* cells, among which the methanolic extract exhibited the maximum protective effect (Palaniswamy and Padma, 2011b).

As determined by MTT and SRB assays, the cytotoxicity of the methanolic extract of latex of *Euphorbia antiquorum* in *S. cerevisiae* cells increased in a dose dependent manner (Sumathi *et al.*, 2011b). Using MTT and SRB assays, Balasubramanian and Padma (2012) showed that the chloroform, methanol and aqueous extracts of *Zea mays* leaves can effectively alleviate the toxicity induced by hydrogen peroxide in yeast cells. Similarly, the methanolic extract of *Artemisia vulgaris* improved the viability of yeast cells subjected to oxidative stress as evident from the MTT and SRB assays (Sathya, 2006).

The above studies substantiate the reliability of the MTT and SRB assays to determine the cell viability. In the present study, the MTT and SRB assays showed that a significant protection was rendered by all the three *C. pulcherrima* flower extracts to *S. cerevisiae* cells, against H_2O_2-induced cell death, wherein the orange flower extract exhibited a higher protective effect.

5.4.2. Assessment of Cytotoxicity in *S. cerevisiae* Cells Using LDH Assay

Lactate dehydrogenase (LDH) assay is a simple, fast and very reliable method used commonly for measuring cell viability. It is a non-destructive method based on the measurement of the lactate dehydrogenase activity, which is released from the cytosol of the lysed or damaged cells in the extracellular medium. Thus, in LDH assay, the cytotoxicity measure is based on the integrity of the plasma membrane (Bopp and Lettieri, 2008).

Our results showed that flower extracts of *C. pulcherrima* significantly reduced the LDH release from the *S. cerevisiae* cells, which indicated the protection rendered by the flower extracts. Due to its reliability and highly predictive validity, LDH release assay has been widely used in various pharmacological and other studies to evaluate the cytotoxicity of various compounds and herbal preparations (Atif *et al.*, 2009).

Using LDH release assay, Yamashoji and Matsuda (2013) confirmed the synergistic cytotoxic effects of two alkaloids namely α-solanine and α-chaconine in C6 rat glioma cells. The cytotoxicity of the aqueous extract of *Erythroxylum cuneatum* dry leaves in human hepatocellular carcinoma (HepG2) cells and human hepatic fetal epithelial (WRL68) cells was evident from the LDH leakage (Wesam *et al.*, 2013). Orjuela-Sánchez *et al.* (2012) developed an *in vitro* ELISA-based LDH release assay to determine the drug sensitivity of *Plasmodium bergei* for different concentrations of anti-malarial drugs using two monoclonal antibodies against the parasite lactate dehydrogenase (pLDH).

The viability of yeast cells treated with methanolic extract of *Majorana hortensis* leaves was higher than that of those cells treated with H_2O_2 as determined by a decrease in the LDH release (Palaniswamy, 2012). Sahreen *et al.* (2011) confirmed the hepatoprotective effect of the methanol extract of *Carissa opaca* leaves on CCl_4-induced damage in rats from the reduced activity of serum LDH.

At lower concentrations, the methanolic extract of the latex of *Euphorbia antiquorum* exhibited less cytotoxicity towards yeast cells as determined by the LDH release assay (Sumathi *et al.*, 2011b). The protective effects of *Moringa oleifera* leaf extract (Sreelatha and Padma, 2010) and *Coriandrum sativum* extract (Sreelatha *et al.*, 2009b) against CCl_4-induced hepatotoxicity in rats was inferred from their ability to reduce the LDH release.

These above findings support the results obtained in the present study that the methanolic extract of all the three flowers of *C. pulcherrima* are non-cytotoxic to untransformed *Saccharomyces cerevisiae* cells. Additionally, the extracts were also very effective in protecting the cells against H_2O_2-induced death.

5.4.3. Morphological Changes Associated with Apoptosis in *S. cerevisiae* Cells subjected to Oxidative Stress

Characterization of apoptosis derives mainly from morphological and ultrastructural observations. Both intracellular and plasma membrane structural modifications are recognized as crucial factors involved in cell injury and death. Changes that occur in nuclear morphology and in organelle structure, together with other phenomena such as cell surface smoothing and blebbing, are considered as markers associated with apoptosis (Christenson *et al.*, 2012). Among a wide variety of imaging and cytochemical techniques available for the specific identification of apoptotic cells in culture and tissues, Giemsa staining enables the recognition of the main morphological characteristics of apoptosis (Baskić *et al.*, 2006).

The observations obtained through Giemsa staining in the present study clearly indicated that the methanolic extract of all the three flowers of *C. pulcherrima* rendered protection to *S. cerevisiae* cells from H_2O_2-induced stress. The extent of protection was higher in the cells treated with the orange flower extract than the other two.

Giemsa staining has been applied in many research studies involving analysis of apoptotic cell death. The osteogenetic influence of the alcoholic extract of *Tinospora cordifolia* on proliferation, differentiation and mineralization of bone like matrix on osteoblast model systems was evident from an increase in cell numbers and absence of adverse changes in the cell morphology as observed by Giemsa staining (Abiramasundari

et al., 2012). The influence of *Larrea divaricata* Cav. fractions on apoptosis in peritoneal macrophages isolated from mice was evaluated using Giemsa and AO/EtBr staining methods (Martino *et al.*, 2011). The morphological changes of normal-transformed human liver (L02) cells undergoing apoptosis after treatment with *Polygonum multiflorum* extract was analyzed using Giemsa staining (Zhang *et al.*, 2010).

The effect of various plant extracts such as *Rhinacanthus nasutus* (Nirmaladevi, 2008), *Triticum aestivum* (Malathy, 2008), *Clitoria ternatea* (Vijayachandran, 2007) and *Artemisia vulgaris* (Sathya, 2006) on oxidative stress-induced apoptosis were studied in *S. cerevisiae* cells. These research findings validate the use of Giemsa staining for evaluating the protective effect of *C. pulcherrima* flowers on morphological changes that occur during oxidant-induced apoptosis in untransformed *S. cerevisiae* cells.

5.4.4. Nuclear Changes Associated with Apoptosis in *S. cerevisiae* Cells Subjected to Oxidative Stress

Nuclear condensation and DNA fragmentation are the most reliable features used to distinguish cells undergoing apoptosis. Fluorescent DNA-intercalating dyes bind with apoptotic nuclei and produce small, fragmented, highly textured nuclear images.

In the present study, the nuclear changes like nuclear fragmentation and cornering of the nucleus that occur in *S. cerevisiae cells* undergoing H_2O_2-induced apoptosis were quantified using four different staining methods namely EtBr, PI, DAPI and AO/EtBr staining. The observations obtained from these staining methods showed that the methanolic extract of all the three flowers of *C. pulcherrima* can effectively reduce the number of oxidatively stressed *S. cerevisiae* cells. The maximum protection was rendered by the orange flower extract.

Balasubramanian and Padma (2013a) showed that among three extracts (chloroform, methanol and aqueous) of *Zea mays* leaves, the methanolic extract exhibited maximum protection for yeast cells subjected to oxidative stress, which was evident from the reduced apoptotic events determined by EtBr, PI and DAPI staining. The different nuclear staining methods (EtBr, PI and DAPI) showed that among three extracts of *Majorana hortensis* leaves (aqueous, methanol and chloroform), the methanolic extract effectively reduced the number apoptotic *S. cerevisiae* cells (Palaniswamy and Padma, 2011b).

The anticancer activity of water and acetone extracts of *Sclerocarya birrea* was studied in HT-29, HeLa and MCF-7 cells, in which AO/EtBr and PI staining methods were used to evaluate apoptosis (Tanih and Ndip, 2013). Based on the observations of DAPI

staining, Lin *et al.* (2013) suggested that the cell death induced by ethanolic *Solanum lyratum* Thunb. extract in human osteosacroma U-2 OS cells is closely related to apoptosis.

Liu *et al.* (2012) demonstrated the protective effect of curcumin and demethoxycurcumin acridine on AGEs-induced oxidative stress and apoptosis in mesangial cells using AO/EtBr staining method. The anticancer activity of *Polyalthia evecta* was examined on HepG2 cells by evaluating the nuclear morphological changes using DAPI staining (Machana *et al.*, 2012).

An increase in the number cells with chromatin condensation and inter-nucleosomal DNA fragmentation, as revealed by EtBr staining, showed that lycopodine isolated from the ethanolic extract of the plant *Lycopodium clavatum* inhibited the proliferation of HeLa cells by inducing apoptosis (Mandal *et al.*, 2010). In our laboratory, the protective effect of leaf extracts of *Artemisia vulgaris* (Haniya, 2010) and *Rhinacanthus nasutus* (Nirmaladevi, 2008) against oxidant induced stress was evaluated in *S. cerevisiae* cells, wherein the apoptotic events were analyzed using EtBr, PI and DAPI staining methods.

The literature quoted above support our findings in the present study where EtBr, PI, DAPI and AO/EtBr staining proved the protective effect of the flower extracts of *C. pulcherrima* against oxidant induced apoptotsis in *S. cerevisiae* cells.

5.4.5. Effect of *C. pulcherrima* Flower Extracts on Oxidative DNA Damage in *S. cerevisiae* Cells

In many cells, the formation of distinct DNA fragments is a biochemical hallmark of apoptotic cell death (Elmore, 2007). In the present study, the extent of DNA damage induced by oxidative stress in *S. cerevisiae* cells in the presence or the absence of the flower extracts was assessed using spectrophotometric DPA method. In *S.cerevisiae* cells, severe DNA damage was caused by H_2O_2 and the extent of DNA damage decreased on co-treatment with the flower extracts of *C. pulcherrima.*

The yeast cells lack internucleosomal DNA laddering or other apoptotic DNA fragmentation due to the presence of short linker DNA between nucleosomes (Madeo *et al.*, 1997; Lowary and Widom, 1989). All these factors make the DNA fragmentation analysis by DNA agarose gel electrophoresis imprecise in yeast cells (Ribeiro *et al.*, 2006). Hence, in the present study, DPA spectrophotometric method was used to determine the extent of oxidative DNA fragmentation in yeast cells.

Numerous studies have been carried out using DPA method to determine DNA fragmentation. Using DPA assay, it was found that polyphenol-rich fraction of *Tinospora*

cordifolia leaves rendered good protection against pro-oxidant induced DNA fragmentation in isolated human peripheral lymphocytes (Chandrashekar *et al.*, 2010).

Rejiya *et al.* (2009) showed the antiproliferative activity of the methanolic extract of *Cassia tora* leaves in HeLa cells by quantifying the extent of DNA damage using a modified DPA method. The association of the toxic effects of zearalenone and its two major metabolites with DNA damage was confirmed in human Caco-2 cells by means of the comet and the diphenylamine assays (Abid-Essefi *et al.*, 2009). Balachandar *et al.*, (2013) showed that the leaf extracts of *Clitoria ternatea* and *Alternanthra sessilis* effectively decreased the extent of oxidative DNA damage in *S. cerevisiae* cells.

Earlier studies conducted in our laboratory have proved the reliability of DPA assay to determine DNA fragmentation in *S.cerevisiae* cells. This assay has confirmed the protective effect of *Bacopa monnieri* leaves (Sreeja, 2006; Radha, 2010), *Artemisia vulgaris* leaves (Sathya, 2006) and *Clitorea ternatea* leaves (Vijayachandran, 2007) against oxidative DNA damage in *S.cerevisiae* cells.

In accordance with these studies, the results obtained from the DPA assay reiterated that all three flower extracts of *C. plucherrima* are excellent antiapoptotic agents, as evident from their ability to reduce H_2O_2-induced oxidative DNA damage in *S. cerevisiae* cells.

5.5. MODULATORY EFFECTS OF *C. pulcherrima* FLOWER EXTRACTS ON ETOPOSIDE-INDUCED APOPTOSIS IN HUMAN PERIPHERAL BLOOD LYMPHOCYTES AND ORAL CARCINOMA (KB) CELLS

In the present study, the influence of the flower extracts of *C. pulcherrima* on etoposide-induced cell death in peripheral blood lymphocytes (untransformed cells) and KB cells (transformed cells) were evaluated. The cell viability (by MTT and SRB), morphological changes (by Giemsa staining), nuclear changes (by PI, EtBr, DAPI and AO/EtBr staining) and DNA fragmentation (comet assay) were analyzed in order to understand the apoptotic events in the cells.

The major advantage of peripheral blood lymphocytes is that they are primary cells, which can be easily cultured in suspension. It is a simple, inexpensive *in vitro* model system widely used for cytogenetic analysis, immunotoxicity and genotoxicity studies (Kirsch-Volders *et al.*, 2011). The peripheral blood lymphocytes have been applied in many research studies. Primary culture of human lymphocytes is one of the commonly used cell types in *in vitro* regulatory genotoxicity assays that evaluate the genotoxic potential of various nanoparticels (Moche *et al.*, 2013).

The ability of sulforaphane to ameliorate γ-radiation-induced genotoxicity and to reduce micronucleus induction by other DNA-damaging anticancer agents, such as bleomycin and doxorubicin was confirmed using cultured peripheral blood lymphocytes of both healthy subjects and those exposed to mixed γ and β-radiation (Katoch *et al.*, 2013).

Hence we have chosen the cultured peripheral blood lymphocytes as an *in vitro* model for the present study to evaluate the effects of the *C. pulcherrima* flower extracts on oxidative stress-induced apoptosis in untransformed cells. KB cells were used as a representative of transformed cells.

5.5.1. Evaluation of Cell Viability in Peripheral Blood Lymphocytes and KB cells Using MTT and SRB assays

The results of the MTT and SRB assays showed that the etoposide treatment caused a significant reduction in the viability of both peripheral blood lymphocytes and KB cells. In the case of peripheral blood lymphocytes, this apoptotic effect was counteracted by the flower extracts in the presence of etoposide, indicating their anti-apoptotic effect in non-transformed cells. On the other hand, the proportion of apoptotic KB cells increased when treated with the flower extracts, which indicated their anticancer activity.

These observations clearly indicate that the flower extracts of *C. pulcherrima* not only possess anticancer potential against KB oral carcinoma cells, but also make the cancer cells more susceptible to the chemotherapeutic agent. These apoptosis modulating effects of the flowers of *C. pulcherrima* showed that they exhibit a differential response towards non-cancerous and cancerous cells, protecting the non-cancerous cells against oxidant-induced cell death, and rendering the cancer cells more susceptible.

A similar differential response was reported in a study, in which the grape seed extract significant reduced the viability of oral squamous cell carcinoma (KB cells) in a dose- and time-dependent manner without inducing damage to non-cancerous human umbilical vein endothelial cells (HUVEC) cells, as determined by MTT assay (Aghbali *et al.*, 2013). The methanolic extract of *Ammannia baccifera* L. was cytotoxic to the HeLa cancer cells but relatively non-toxic to the normal mouse embryonic fibroblast (NIH 3T3) cells, as determined using MTT (Loganayaki and Manian, 2012). Huabprasert *et al.* (2012) observed a similar differential effect, where the *Phyllanthus emblica* infusion was cytotoxic to mouse lymphoma (YAC-1) cells, but had no effect on human umbilical endothelial cells and fibroblasts.

The methanolic extract of *Xanthium strumarium* showed potent cytotoxicity against Dalton's lymphoma ascitic (DLA) cells as determined by MTT and SRB assay (Aranjani *et al.*, 2013). A concentration dependent inhibition of MCF-7 breast cancer cell viability by *Carissa opaca* and *Toona ciliate* extracts was determined using MTT assay (Nisa *et al.*, 2013).

The cytotoxicity of many herbal extracts has been tested using MTT and SRB cell viability assays. The root extracts of *Eryngium kotschyi* and *Eryngium maritimum* significantly reduced the viability of both human laryngeal squamous cancer (Hep2) cells and hepatocellular carcinoma (HepG2) cells as determined by MTT (Yurdakök and Baydan, 2013). Using MTT assay, it was shown that pretreatment with *Scrophularia striata* extract increased the survival of rat pheochromocytoma (PC12) cells in a dose dependent manner against H_2O_2-induced oxidative stress (Azadmehr *et al.*, 2013).

Methanol and 70% ethanolic extracts of the rhizome of *Podophyllum hexandrum* exerted maximum cytotoxic effect on human breast carcinoma (MCF-7) and colon carcinoma (Colo-25) cells, as determined by the SRB assay (Ganie *et al.*, 2011). Using MTT and SRB assays, Samarakoon *et al.* (2010) demonstrated that the standardized aqueous and ethanolic extracts of a polyherbal mixture comprised of *Nigella sativa* (seeds), *Hemidesmus indicus* (roots) and *Smilax glabra* (rhizome) exerted strong dose-dependent *in vitro* cytotoxicity to HepG2 cells.

Yu and Chen (2013) showed that when β-carboline alkaloids-enriched extract from *Pao pereira* combined with chemotherapeutic drug carboplatin, tumor inhibition of ovarian cancer cells was increased to 97%, which indicated that Pao extract could enhance the carboplatin activity. Using MTT and SRB assays, He *et al.* (2012) reported that 6'-hydroxy justicidin A, a new compound isolated from *Justicia procumbens* remarkably reduced the viability in human bladder cancer (EJ) cells. Using MTT assay, it was found that the aqueous and 50% ethanolic extracts of *Rhus parviflora* Roxb. leaves were non-toxic to peripheral blood lymphocytes up to a concentration of 100 µg/ml (Modi *et al.*, 2013). The cold aqueous extract of *Baccharis articulate* showed no cytotoxic effects on human lymphocytes as evaluated by the MTT assay (Cariddi *et al.*, 2010).

A phloroglucinol derivative (2,4-bis(4-fluorophenylacetyl)phloroglucinol; BFP) induced cell death in three glioma (U251, U87 and C6) cells in a concentration-dependent manner, but had no effect on primary human astrocytes, as determined by MTT and SRB assay (Lu *et al.*, 2012). Arecoline, areca alkaloid in betel quid, inhibited the growth of EAhy

926 endothelial cells in a dose- and time-dependent manner, as determined by MTT assay (Tseng *et al.*, 2012).

In the present study, the results obtained from the cell viability assays (MTT and SRB) in cultured transformed and untransformed cells show that all the three flowers of *C. pulcherrima* render cytoprotection in peripheral blood lymphocytes, but exhibit potent antitumor activity towards KB cells. The extracts, therefore, hold a strong therapeutic potential in the treatment of cancer.

5.5.2. Evaluation of Cytotoxicity in Peripheral Blood Lymphocytes and KB cells Using LDH assay

In the present study, the peripheral blood lymphocytes and KB cells were subjected to LDH release assay. Upon etoposide treatment, a marked increase in the LDH release was observed. On co-administration with flower extracts of *C. pulcherrima*, the LDH release decreased in the lymphocytes. In KB cells, the LDH release increased in the presence of flower extracts, indicating the antitumor activity of the flower extracts.

Many studies have applied LDH release assay to determine cytotoxicity. Gengan *et al.* (2013) reported that the silver nanoparticles synthesized using *Albizia adianthifolia* leaf significantly decreased the viability of human lung cancer A549 cells without affecting the normal healthy peripheral lymphocytes, as determined by MTT, ATP and lactate dehydrogenase assays. A similar observation was reported in another study, in which the cytotoxic effect of the ethanol extract of *Pleurotus ostreatus* was significantly higher in Jurkat cells than in healthy primary lymphocytes, as seen by LDH release and MTT assays (Bassil *et al.*, 2012).

William *et al.* (2012) examined the apoptosis inducing effect of stilbenoids (isolated from several species of the *Orchidaceae* family) in human non-small cell lung cancer (NCI-H460) cells using LDH release assay. The hydro-alcoholic extract of *Nyctanthes arbor-tristis* Linn. flowers protected the normal lymphocytes from H_2O_2-induced oxidative stress, as evident from the decreased LDH release (Hussain and Ramteke, 2012). Similarly, pretreatment of normal avian lymphocytes with the root extract of *Phlogacanthus tubiflorus* resulted in a dose- dependent decline in the LDH release that increased the viability of lymphocytes against H_2O_2-induced oxidative stress (Ramteke *et al.*, 2012).

The presence of defatted seed extract of *Oenothera paradoxa*, pentagalloylglucose and procyanidins caused a marked reduction in viability (MTT assay) and rise in mortality (LDH release assay) of human melanoma (HTB-140) cells (Jaszewska *et al.*, 2010). The

antiproliferative and cytotoxic activities of two pyranocoumarins isolated from methanolic extract of *Peucedanum praeruptorum* Dunn. on gastric cancer SGC7901 cells was confirmed using MTT and LDH release assays (Liang *et al.*, 2010).

On treatment with bog bilberry anthocyanin extract, the LDH release was significantly increased in Hep-G2 cells and Caco-2 cells in a time-dependent manner, whereas in non-cancerous murine embryonic fibroblast (3T3-L1) cells, an unexpectedly decreased LDH release was observed (Liu *et al.*, 2010). The cytotoxic effect of the aqueous extract of *Psidium guajava* L. budding leaves in prostate cancer (LNCaP) cells was shown by enhanced LDH release (Chen *et al.*, 2010). The inhibitory effect of essential oils from the aerial parts of *Salvia bracteata* and *Salvia rubifolia* on cell vitality, cell membrane integrity and genomic DNA fragmentation in M14 human melanoma cells was determined using MTT, LDH release and comet assays (Cardile *et al.*, 2009).

In our study, the results of the LDH release assay showed that the flower extracts of *C. pulcherrima* protect the non-cancerous peripheral blood lymphocytes by effectively counteracting the etoposide-induced apoptosis, whereas, they make the cancerous KB cells more prone to etoposide-induced apoptosis. These findings suggest that the differential action of the flower extracts of *C. pulcherrima* might find application in supportive therapy for cancer, by decreasing the side effects of chemotherapy by sparing the normal cells and increasing the efficiency of the drug action on cancer cells.

5.5.3. Assessment of Morphological Changes Associated with Apoptosis in Peripheral Blood Lymphocytes and KB cells

On treatment with etoposide alone, the proportion of cells undergoing apoptosis increased in both peripheral blood lymphocytes and KB cells. In the presence of the flower extracts, the apoptotic ratios in peripheral blood lymphocytes significantly reduced, indicating the protection rendered by the *C. pulcherrima* flowers to non-cancerous cells. In the case of cancer cells, the flower extracts enhanced the etoposide activity, which is evident from the augmented apoptotic ratio of KB cells. This differential effect was more prominent in the orange flower extract treated group, compared to that of pink and yellow flower extract treated groups.

A similar observation on apoptotic morphological changes was reported in a study, in which, all the three different extracts (methanol, hexane and ethyl acetate) of *Phyllanthus watsonii* leaves were cytotoxic and selectively induced cell death in MCF-7 cells in a dose-dependent manner, but were non-toxic to normal human lung fibroblast MRC-5 cells, as

characterized by Giemsa staining (Ramasamy *et al.*, 2013). Using Giemsa staining, it was found that *Helichrysum zivojinii* extracts selectively increased the number of apoptotic cells in three cancerous cells lines (HeLa, Fem-x and K562) in a dose-dependent manner, while exerting no effect on unstimulated healthy peripheral blood mononuclear cells (Matić *et al.*, 2013a).

A similar differential response was found in another study using MTT assay, May-Grünwald-Giemsa's staining, BrdU incorporation test and flow cytometry analysis, where neosergeolide, the biologically active secondary metabolite strongly inhibited the proliferation human promyelocytic leukemia (HL-60) cells, but had no antiproliferative effect on human peripheral blood mononuclear cells (Cavalcanti *et al.*, 2012). Giemsa stain has also been used in many other studies involving cytotoxicity analysis. Sharmila and Padma (2013) have shown that the methanolic extract of the leaves of *Artemisia vulgaris* act synergistically by enhancing the etoposide-induced apoptosis in HepG2 cells, as determined by Giemsa staining.

The cytotoxicity of the aqueous extracts of five medicinal plants (*Phyllanthus niruri*, *Coleus aromaticus*, *Azadirachta indica*, *Camellia sinensis* and *Garcinia indica*) were evaluated in human peripheral lymphocytes using MTT and Giemsa staining. The results showed that among the five plants, *C. sinensis* alone exerted cytostimulatory effect on lymphocytes while the rest were found to be cytotoxic (Varalakshmi *et al.*, 2011).

The extracts of three plants namely *Brucea* sp., *Typhonium* sp. and *Azadirachta* sp., exerted significant cytotoxicity towards two cancerous oral mucosal cell lines (KB and ORL48). The extracts did not produce any toxic effect on normal oral mucosal cells, as revealed by neutral red and Giemsa staining methods (Razak *et al.*, 2011).

By using Giemsa stain for chromosome aberrations and a modified fluorescence plus Giemsa stain for sister chromatid exchange, Kayraldiz *et al.* (2010) found that *Aloe vera* leaf extract augmented the genotoxicity of urethane in rat bone marrow cells and mitomycin-C in cultured human lymphocytes. Similarly, by using Giemsa stain for micronucleus analysis, Eroğlu *et al.* (2010) reported the genotoxic effect of *Helichrysum arenarium* in cultured human lymphocytes.

The above literature render support to our findings using Giemsa stain, that the flower extracts of *C. pulcherrima* selectively induce apoptosis in cancer cells without affecting the non-cancerous cells. Additionally, they also render significant protection to the non-cancerous cells against etoposide-induced cytotoxicity.

5.5.4. Assessment of Nuclear Changes Associated with Apoptosis in Peripheral Blood Lymphocytes and KB cells

The methanolic extract of all three flowers of *C. pulcherrima* effectively counteracted oxidant-induced apoptosis in peripheral blood lymphocytes and, in contrast, they increased the apoptotic ratio of KB cells. This observation reiterates their anti-apoptotic property against normal cells and their anticancer property.

A similar apoptosis-influencing activity was observed in a study using nuclear staining methods (EtBr, PI, DAPI and AO/EtBr), in which, the methanolic extract of *Zea mays* leaves enhanced the H_2O_2-induced apoptotic cell death in Hep2 (laryngeal carcinoma) cells, but protected the non-cancerous primary chick embryo fibroblasts from H_2O_2-induced apoptosis (Balasubramanian and Padma, 2013b; Kiruthika *et al.*, 2013). The nuclear apoptotic changes observed using various staining methods (EtBr, PI, DAPI, Hoechst 33342 and AO/EtBr) showed that the methanolic extract of *Prosopis cineraria* significantly increased the number of cells undergoing apoptosis in MCF-7 breast cancer cell line, while no such toxic effect was seen in non-cancerous HBL100 breast epithelial cells (Sumathi *et al.*, 2013b).

The nuclear morphological changes observed using DAPI staining showed that three different extracts (water, ethanol and oil) of clove (*Syzygium aromaticum* L) induced apoptotic cell demise in five different cancer cell lines namely HeLa, MCF-7, MDA-MB-231, DU-145 and TE-13, whereas no significant apoptotic effect was found in human peripheral blood mononuclear cells (Dwivedi *et al.*, 2011). The silver nanoparticles prepared from amla extract inhibited the growth of Hep2 cells by inducing apoptosis, which was evident from the nuclear changes observed using AO/EtBr staining (Rosarin *et al.*, 2013).

Using AO/EtBr staining, Manosroi *et al.* (2012) found that among 48 extracts tested, the hot and cold chloroform extracts of young Longkong fruits exhibited the highest apoptotic activity against KB cells. The AO/EtBr staining confirmed the apoptotic inducing effect of *Acacia nilotica* leaf extract and γ-sitosterol on breast cancer (MCF-7) and lung cancer (A549) cells (Sundarraj *et al.*, 2012). The *Centella asiatica* extract showed strong *in vitro* growth inhibitory activity against MCF-7 cells by inducing apoptosis, as indicated by nuclear condensation (AO/EtBr and PI staining) of cancer cells (Babykutty *et al.*, 2009). The cytotoxicity of a fusicoccane-type diterpenoid isolated from the Chinese liverwort *Plagiochila pulcherrima* towards HeLa cells was related to apoptotis as confirmed by DAPI nuclear staining and flow cytometry (Wang *et al.*, 2013c).

Thus, in the present study, the nuclear morphological changes observed using EtBr, PI, DAPI and AO/EtBr staining methods confirmed that the flower extracts of *C. pulcherrima* exhibit antiapoptotic activity towards non-cancerous cells, but are cytotoxic to cancerous cells. This differential effect also extended to the protection rendered against the toxicity induced by etoposide.

5.5.5. Effect of *C. pulcherrima* Flower Extracts on Etoposide-induced Oxidative DNA damage in Peripheral Blood Lymphocytes and KB cells

In recent years, the study on oxygen radical dependent damage to DNA has become a major thrust in carcinogenesis research. The extent of oxidative DNA damage induced by etoposide in peripheral blood lymphocytes and KB cells were analyzed both in the presence and the absence of the flower extracts of *C. pulcherrima* by single cell gel electrophoresis (Comet assay).

The comet assay is a fast, highly sensitive and inexpensive method widely used for measuring DNA strand breaks. When compared to other DNA damage detection methods, the comet assay has a major advantage in that the various types of DNA lesions including single-strand breaks (SSBs), double strand breaks (DSBs) and oxidized bases can be detected in individual cells (Georgakilas *et al.*, 2010).

The results of the present study using Comet assay showed that, in the presence of flower extracts of *C. pulcherrima*, the number of comet bearing cells was markedly reduced in peripheral blood lymphocytes, whereas a significant increase in the number of cells bearing comets was observed in KB cells.

Many studies have reported both the protective and cytotoxic effects of herbal extracts and formulations on oxidative DNA damage using comet assay. The ethanolic leaf extract of *Melia azedarach* rendered a strong protection against H_2O_2-induced DNA damage in cultured lymphocytes, as revealed by comet assay (Marimuthu *et al.*, 2013). The antigenotoxic effects of the chloroform fraction of *Arrabidaea chica* was confirmed from the decreased H_2O_2-induced oxidative DNA damage in blood and liver of rats, as evaluated by comet assay (dos Santos *et al.*, 2013).

In the comet assay, the methanolic extract of *Euphorbia hirta* exhibited a time-dependent genotoxicity against breast adenocarcinoma MCF-7 DNA by increasing the mean percentage of DNA damage (Ping *et al.*, 2013). Sultan and Tülay (2013) reported that *Euphorbia platyphyllos* extracts exerted their genotoxicity on MCF-7 cells in a dose-

dependent manner, as revealed by the increased migration of DNA fragments in the comet assay.

The dichloromethane extracts of *Pteridium aquilinem* was found to exert proapoptotic effect on TCC cancer cells, but had no effect on normal cells (HDF1 and HFF3), as determined by DAPI staining and comet assays (Roudsari *et al.*, 2012). Using comet assay, Percival *et al.* (2012) found that the consumption of herbs and spices, such as paprika, rosemary, ginger, heat-treated turmeric, sage and cumin, effectively reduced the H_2O_2-induced oxidative DNA strand breaks in peripheral blood mononuclear cells of healthy subjects.

The efficient radioprotection rendered by the black tea extract against γ-irradiation-induced oxidative DNA damage in normal lymphocytes was observed using comet assay (Ghosh *et al.*, 2012). The aqueous extract of the edible red seaweed *Gracilaria tenuistipitata* was found to suppress H_2O_2-induced oxidative DNA damage in H1299 cells in a dose-dependent manner, as measured by a modified comet assay (Yang *et al.*, 2012).

Behravan *et al.* (2011) evaluated the protective effects of ethanolic and aqueous extracts of *Portulaca oleracea* on human lymphocyte DNA lesions induced by H_2O_2 by comet assay and found that only the aqueous extract showed significant protection against oxidative DNA damage. A polysaccharide fraction extracted from *C. lachryma-jobi* L. induced apoptosis in A549 cells in a dose-dependent manner (as revealed by MTT assay) and also extended the comet tail length on single cell gel electrophoresis together with the disruption of the mitochondrial membrane potential (Lu *et al.*, 2013). The polyphenols-rich fraction of *Tinospora cordifolia* leaves significantly prevented the pro-oxidant induced DNA fragmentation of isolated human peripheral lymphocytes (Chandrashekar *et al.*, 2010).

In accordance with these studies, our findings showed that, in the presence of the oxidant etoposide, all the three flowers of *C. pulcherrima* exert protective effect in non-cancerous lymphocyte DNA. On the other hand, they exhibit proapoptotic effect towards KB cells by increasing the percentage of damaged DNA.

A significant observation found in this phase of the study was that the flower extracts protected the normal cells (yeast and peripheral blood lymphocytes) from oxidant-induced apoptosis, but increased the susceptibility of cancer cells (KB cell line) to oxidant-induced apoptosis. This differential response is noteworthy, as the extracts possess the ability to protect the non-cancerous cells against the toxicity of standard chemotherapeutic agent (etoposide), while rendering the cancer cells more susceptible to its action. This targeted

action of the flower extracts can be exploited as an effective supportive therapy during cancer chemotherapy.

5.6. EXPRESSION OF PROTEINS INVOLVED IN APOPTOSIS (TP53, Bax AND Bcl-2) IN KB CELLS

Apoptosis plays a vital role in cancer therapy and is an inevitable target of many treatment strategies. Tumourigenesis involves the upregulation of anti-apoptotic proteins such as Bcl-2 and/or the downregulation of pro-apoptotic proteins like Bax and TP53. Such dysregulation of apoptotic proteins facilitate the cancer cells to evade apoptosis, which, in turn, leads to therapeutic resistance (Giménez-Bonafé *et al.*, 2009).

The development of resistance in tumor cells towards anticancer therapy is very much related to their resistance to apoptosis. Therefore, elucidation of the genes that constitute the core machinery of apoptotic pathways is required, which, in turn, provide new perspectives in tumor biology by developing novel strategies for combating cancer (Wong, 2011).

Immunocytochemistry is a vastly diverse and essential method used for the localization of proteins in both suspension and adherent cell cultures. The key advantage of using immunocytochemistry over traditionally used special and enzyme staining techniques is that it involves specific antigen-antibody reaction and therefore a large number of proteins and enzymes can be studied using specific antibodies (Goldstein and Watkins, 2008).

In the present study, using immunocytochemistry, the molecular changes that have the potential to induce apoptosis in cancer cells, such as the expression of pro-apoptotic proteins (TP53 and Bax) and anti-apoptotic protein (Bcl-2) were analyzed in oral carcinoma KB cells after treatment with the flower extracts of *C. pulcherrima* and/or etoposide. The results obtained revealed that, while the pro-apoptotic proteins (TP53 and Bax) expression was increased, the anti-apoptotic protein Bcl-2 expression was decreased in the presence of flower extracts compared to that of untreated cells.

In recent years, many research findings have proved the significance of inducing apoptosis in cancer cells during therapy through the regulation of proteins involved in apoptotic pathways (Li *et al.*, 2010). Neohesperidin, a flavonoid compound found in *Poncirus trifoliata* induced apoptosis in human breast adenocarcinoma MDA-MB-231 cells by upregulating TP53 and Bax proteins and by downregulating the expression of Bcl-2 protein (Xu *et al.*, 2012). *Paris polyphylla* Smith extract exerted its antitumour effects against human oesophageal cancer ECA109 cells by inhibiting cell proliferation and by inducing cell apoptosis through upregulation of both connexin26 mRNA and protein.

Conversely, it decreased the expression of Bcl-2 protein and increased Bad protein expression as revelaed by immunostaining (Li *et al.*, 2012c).

Immunocytochemical analysis showed that oleanolic acid isolated from the ethanolic extract of *Prunella vulgaris* induced apoptosis in non-small cell lung cancer SPC-A-1 cells by up-regulating the expressions of Bax and Bad proteins, while downregulating the expression of Bcl-2 protein (Feng *et al.*, 2011). Using immunocytochemistry and Western blot analysis, Zheng *et al.* (2010) confirmed that the Bax protein expression was significantly increased with no changes in the Bcl-2 expression in human gastric cancer SGC7901 cells undergoing arsenic trioxide-induced apoptosis.

Upregulation of TP53 (as determined by Western blot analysis) and Bax (as determined by both Western blot analysis and immunostaining) together with the downregulation of Bcl-2 (as determined by Western blot analysis) were observed in human cervical carcinoma HeLa cells undergoing apoptosis induced by eurycomanone isolated from the root of *Eurycoma longifolia* (Mahfudh and Pihie, 2008).

The above literature supports the present study, in which the methanolic extract of all the three flowers of *C. pulcherrima* induced apoptosis in oral carcinoma KB cells via upregulation of TP53 and Bax proteins, while downregulating the anti-apoptotic Bcl-2 protein. These observations suggest that the flowers of *C. pulcherrima* may be a promising anticancer agent to treat malignancies that overexpress Bcl-2 and lack TP53 expression. A substantial research on apoptotic pathways needs to be carried out to elucidate the complete molecular mechanism of anticancer activity of the flowers of *C. pulcherrima*.

5.7. CELL CYCLE DISTRIBUTION AND APOPTOSIS IN KB CELLS

Flow cytometry is a widely used technology that has significant impact on both cell biology and clinical medicine. The key advantage of flow cytometry is that it allows simultaneous multi-parameter analysis of single cells and enables the rapid evaluation of a very large number of cells in a very short period of time. Hence, flow cytometry is the most preferred technology to detect, identify and record the incidence of apoptosis in large group of cell populations (Wlodkowic *et al.*, 2012). It also has become essential to conduct a multi-parameter analysis of apoptosis in relation to cell cycle position, in order to explore the mechanism of action of anticancer drugs that target specific molecular targets of the cell cycle (Skommer *et al.*, 2013).

The observations of the present study showed that all the three flowers of *C. pulcherrima* exhibit cytotoxic effect towards cancer (KB) cells by inducing apoptosis,

which is evident from the decreased cell viability, together with morphological and nuclear changes of apoptotic cell death. The apoptosis-inducing effect of the flower extracts were further analyzed using flow cytometry, wherein the cell cycle distribution of KB cells were determined. The results obtained showed that the treatment with etoposide significantly increased the proportion of KB cells in the sub-G0 phase, indicating an increased induction of apoptosis. A further increase in the proportion of KB cells in the sub-G0 phase was also observed after treatment with all the three flower extracts both in the presence and absence of etoposide. The effect of the orange flower extract was strikingly more than the other two flowers in this effect.

In the analysis of the cell cycle events using flow cytometry, the intensity of DNA-binding fluorochromes is measured. The cells in G0-G1 phase are characterized by the intensity corresponding to 1X DNA content and that of G2-M phase by 2X DNA. When the DNA content is between 1X and 2X, S-phase is indicated. The intensity less than 1X DNA characterizes sub-G0 phase, which is typical of cells undergoing apoptosis, wherein the DNA is fragmented systematically and packed into apoptotic bodies (Krishan, 1975).

Thus, the effect observed in the present study, clearly indicate that the flower extracts can cause the KB oral carcinoma cells to commit to apoptosis. This, in turn, proves the anticancer properties of the flower extracts. The technique of flow cytometry also quantifies the proportion of cells in each phase of the cell cycle, which showed that, in the presence of the flower extracts, a majority of the cells committed to apoptosis, either in the early stage of death (G0-G1 arrest) or in the late stage (sub G0). This effect was observed in the flower extract treated groups, both in the absence and the presence of etoposide, clearly demonstrating the anticancer potential of the flower extracts.

Using flow cytometric analysis, many studies have reported the cell cycle modulatory effects of herbal extracts on cancer cells. Chen *et al.* (2013) observed a significant increase in the apoptotic cell population of human lung A549 cancer cells in the sub-G0 and G0/G1 phases after treatment with the ethanolic extract of *Houttuynia cordata*. Similarly, Maliyakkal *et al.* (2013) reported a significant increase in the proportion of breast cancer (MCF-7 and MDA MB 231) cells in G2/M phase and sub-G0 regions after treatment with the ethanolic extract of *Withania somnifera* roots.

The methanolic extract of three Cameroonian medicinal plants (*Gladiolus quartinianus*, *Vepris soyauxii* and *Anonidium mannii*) induced a time-dependent increase in the proportion of multi-drug-resistant CEM/MDR5000 leukemia cells in sub-G0/G1 phase,

which confirmed their apoptosis-inducing potential on cancer cells (Kuete *et al.*, 2013). An increase in the percentage of cells in the sub-G0/G1 phase confirmed the antimetastatic potential of the ethanolic extract of *Aerva lanata* against highly metastatic B16F-10 melanoma cells (Siveen and Kuttan, 2013).

Treatment with the acetone extract of *Ficus religosa* leaf significantly increased the accumulation of human breast cancer (MCF-7) cells in the sub-G0/G1 phase in a dose- and time-dependent manner (Haneef *et al.*, 2012). Similarly, the apoptosis-inducing potential of n-hexane and chloroform extracts of the whole plant of *Anisomeles malabarica* on human cervical cancer cell lines (SiHa and ME 180) was confirmed from the dramatic increase in the proportion of sub-G0/G1 phase cells (Preethy *et al.*, 2012).

A flavonol isolated from the ethanolic extract of *Thuja occidentalis* leaves exerted its anticancer effect on lung adenocarcinoma (A549) cells through apoptosis induction as indicated by the progressive increase in the proportion of sub-G0 phase cells (Mukherjee *et al.*, 2013). A benzothiazole linked phenylpyridopyrimidinone compound caused a drastic cell cycle perturbation in human cervical cancer ME-180 cells by increasing the sub-G0 phase cells in a concentration dependent manner (Kamal *et al.*, 2014).

These observations, thus, corroborate with the present study, wherein the proportion of oral carcinoma KB cells arrested in sub-G0 phase increased after treatment with the methanolic extract of the *C. pulcherrima* flowers. This shows that the cell cycle arrest induced by *C. pulcherrima* flowers is associated with apoptosis. Further studies on the cell cycle events associated with the flower extracts and/or etoposide need to be conducted on various non-cancerous cells to have more insight into the influence on cell division and death.

From the research findings of Phase III, it is evident that the methanolic extract of all the three flowers of *C. pulcherrima* can be used as a potent anticancer agent, which would facilitate the new prospects of anticancer therapy using natural products.

PHASE IV

Plants, herbs and ethnobotanicals have been used since the early days of humankind throughout the world to promote health and to treat a wide spectrum of diseases (Pan *et al.*, 2013). Natural plant products are perceived to be healthier than modern medicine. The concern over the adverse side effects of modern drugs, the desire for non-toxic, affordable therapy using herbal drugs and the availability of broad scientific evidence about the effectiveness of herbs has increased the focus on exploring natural plant-derived substances

as a potential and promising source of medicines for the prevention and treatment of several diseases, including cancer (Salimifar *et al.*, 2013).

Phytochemicals, the plant-derived bioactive compounds, not only hold historical significance in various traditional medicines, but also form the basis of many modern-day drugs. Many conventional drugs that are widely used in mainstream medicine are derived from phytochemicals. They also help in developing the primary therapeutic strategies for numerous diseases, including cancer (Ulbricht and Chao, 2010). Accumulating evidences have demonstrated that a variety of phytochemicals, especially the phenolic compounds, possess anticancer properties and also reduce the risk of degenerative diseases by inhibiting oxidative stress and by protecting macromolecules from oxidative damage (Wahle *et al.*, 2010; Jayasena *et al.*, 2013). Therefore, it has become essential to conduct a qualitative and quantitative assessment of the phytochemicals like alkaloids, phenolics, flavonoids, sterols and tannins that serve as important indices for the medicinal property of a plant.

Hence, in the final phase of the present study, qualitative phytochemical analysis, followed by various chromatographic (TLC, HPTLC and HPLC) and spectral (absorption FT-IR and GC-MS) analyses were carried out. The results showed that the three flowers of *C. pulcherrima* contain major bioactive phytochemicals with proven radical scavenging potential among which, the major activity was observed in the flavonoid and phenolic fractions.

5.8. QUALITATIVE PHYTOCHEMICAL SCREENING OF *C. pulcherrima* FLOWERS

The qualitative phytochemical screening of the methanolic extract of all the three flowers of *C. pulcherrima* showed the presence alkaloids, phenols, flavonoids, saponins, steroids, tannins and terpenoids.

Qualitative phytochemical analysis is being carried out in many studies in order to confirm the presence of biologically active phytochemicals and their association with various properties of the plant tested, such as antioxidant, antimicrobial, detoxification, anticancer, anti-diabetic and cyto/genotoxic activities. Qualitative phytochemical screening of the root extracts of *Morella serata*, a traditionally used medicinal plant for the treatment of microbial infections, revealed the presence of tannins, saponins, flavonoids, terpenoids and steroids (Ashafa, 2013).

Dahiya and Purkayastha (2013) studied the *in vitro* antibacterial activity of various solvents and water extracts of aloe vera, neem, bryophyllum, lemongrass, tulsi, oregano,

rosemary and thyme, together with phytochemical screening, which showed the presence of tannins and saponins in all the plants. The cytotoxic effects exhibited by the various extracts of *D. suffruticosa* against cancer cells were suggested to be contributed by the saponins, triterpenes, sterols and polyphenolic compounds present in the extracts (Armania *et al.*, 2013).

Both qualitative and quantitative phytochemical analysis of the aqueous and methanolic extracts of the various parts (root, stem and flower) of *Taraxacum officinale* showed that saponins, flavonoids, alkaloids, phenols and steroids were highly concentrated in all the three parts, while a higher concentration of flavonoids were found in the flower extracts (Mir *et al.*, 2013). Qualitative phytochemical screening of three different extracts (chloroform, n-butanol and water) of *Bauhinia tomentosa* flowers showed that different extracts contained different phytochemicals. All the three extracts contained saponins, whereas the chloroform extract contained saponins, sterols and phenols. The n-butanol extract was positive for alkaloids and sterols, whereas the aqueous extract showed the presence of saponins and phenols (Sathya *et al.*, 2013). Benarba *et al.* (2012) suggested that the flavonoids, triterpenes and sterols present in the aqueous extract of *Bryonia dioica* could be responsible for its cytotoxic and apoptogenic activities against Burkitt's lymphoma BL41 cells.

In corroboration with the above literature, our findings indicate that there might be an association between the presence of the phytochemicals and the radical quenching, antioxidant, biomoleculer protective and apoptosis-modulating activities of the flowers of *C. pulcherrrima*.

5.9. RADICAL SCAVENGING ACTIVITY OF THE PHYTOCHEMICAL FRACTIONS OF *C. pulcherrima* FLOWERS

Phytochemicals play a vital role in the protection of human health. There have been more than thousand phytochemicals isolated, but many are yet to be identified. These compounds are secondary plant metabolites and have been found to possess various biological properties such as antioxidant, antimicrobial, immune stimulatory, detoxifying, enzyme modulating, hormone metabolism modulating and anticancer properties (Obrenovich, 2011).

Based on their ring structure and the number of carbon atoms substituting the ring, phytochemicals are classified into many groups. In our study, the seven major phytochemical fractions namely alkaloids, phenols, flavonoids, saponins, steroids, tannins

and terpenoids, isolated from the flowers of *C. pulcherrima* showed significant radical quenching activity. The maximum scavenging potential was observed in the flavonoid fraction, followed by the phenolic fraction.

In accordance with our results, many studies have reported the free radical scavenging activity of phytochemicals isolated from plants and some of the salient ones are discussed below.

The ethanol extract and the isolated flavonoid fraction of the aerial parts of *Cissus quadrangularis* showed good free radical scavenging property against DPPH, hydroxyl and nitric oxide in a dose-dependent manner (Vijayalakshmi *et al.*, 2013). Tiong *et al.* (2013) reported that four major alkaloids isolated from the leaf extract of *Catharanthus roseus* (L.) exhibited strong antioxidant activity (as determined by *in vitro* DPPH and ORAC assays) and hypoglycemic activity in β-TC6 mouse pancreatic cells. In a study conducted by Nasrullah *et al.* (2013), four known alkaloids isolated and characterized (by high field 1D- and 2D-NMR, IR, UV and LC-MS spectral analysis) from the stem bark of *Cryptocarya nigra* showed strong *in vitro* antiplasmodial and antioxidant activities (as determined by DPPH and FRAP assays).

Eight flavonoid compounds isolated from the leaves of *Rosa davurica* and their structurally related compounds (quercetin 3-O-glycosides and gallic acid derivatives) showed significant superoxide anion-scavenging and DPPH free radical-scavenging activities (Ma *et al.*, 2013).

Fourteen lignoids were isolated from the leaves of *Ribes nigrum*, among which ribesin D and ribesin G were found to exhibit maximum radical quenching activity as evaluated by DPPH and superoxide anion scavenging assays (Sasaki *et al.*, 2013). A dose-dependent increase in the DPPH radical scavenging activity of the phenolic extracts isolated from the seeds of African yam bean has been reported (Enujiugha *et al.*, 2012).

Erol *et al.* (2010) compared the quality of seven grades of Turkish black tea by investigating the antioxidant activities of polyphenols and alkaloids present in them using DPPH assay and found that some grades of teas showed higher antioxidant activity, which was attributed to the higher concentration of flavonol glycosides and theaflavins present in these tea extracts.

The phytochemical studies mentioned above corroborate with the present study wherein, all the three flowers of *C. pulcherrima* were identified as rich sources of the seven major phytoconstituents (alkaloids, phenols, flavonoids, saponins, steroids, tannins and

terpenoids), all of which exhibited strong antioxidant potential. The flavonoid fraction of all the three flowers exhibited the maximum radical quenching effect, followed by the phenolic fraction.

5.10. CHROMATOGRAPHIC AND SPECTRAL ANALYSIS OF THE FLOWERS OF *C. pulcherrima*

The curative properties of medicinal plants are attributed to the presence of various classes of secondary metabolites such as alkaloids, flavonoids, phenolics, tannins and sterols (Lalnundanga *et al.*, 2012). The traditional approach towards the standardization is insufficient for herbal medicine and, hence, there is an increased need for more advanced analytical techniques to standardize the herbal formulations, such as TLC, HPTLC, HPLC and GC-MS analysis (Nikam *et al.*, 2012).

The methanolic extract of the three flowers of *C. pulcherrima* were subjected to various chromatographic (TLC, HPTLC and HPLC) and spectral (absorption, FT-IR and GC-MS) analyses.

5.10.1. UV Absorption Spectral Analysis

Ultraviolet-visible absorption spectroscopy is an important analytical instrument in the modern day laboratory. It has been extensively used in many phytochemical studies for its speed, simplicity, accuracy, versatility and cost-effectiveness (http://www.plant.uoguelph.ca/research/homepages/raizada/Equipment/RaizadaWeb%20Equipment%20PDFs/5B.%20UV%20VIS%20theory%20ThermoSpectric.pdf). The UV absorption spectrum of the methanolic extracts of the three flowers of *C. pulcherrima* showed many distinct major and minor peaks between 190 to 420 nm. These peaks revealed the presence of multiple components in the flowers of *C. pulcherrima*.

UV-visible spectral analysis together with other spectral analysis has been used in many studies to identify the major phytochemicals present in the plant extracts. The FT-IR and UV-visible spectral analysis of the leaf extract of *Acorus Calamus* revealed the presence of phenolic and flavonoid compounds (Mamta and Jyoti, 2012). Omodara *et al.* (2012) observed many absorption peaks in the UV range of 230 to 320nm in the three different crystals isolated from the methanolic extract of *Phyllanthus muellerianus* roots. The UV-visible profile of the ethanolic extract of *Vitex altissima* L. showed different peaks ranging from 400-700 nm with different absorptions (Sathish *et al.*, 2012).

Based on the UV absorption spectra, Jiménez-Ferrer *et al.* (2010) found that the major compounds present in the different extract fractions of *Salvia elegans* Vahl. were of

flavonoid and phenyl propanoid types. Similarly, Inamake *et al.* (2010) confirmed the presence of the compound stevioside in the dried leaves of *Stevia rebaundiana* from the peak obtained at the UV spectrum 333 nm. Weidner *et al.* (1999) and Mabry *et al.* (1970) reported that the UV spectra of phenolic and flavonoid compounds are characterized by the maxima at wavelengths of 320-330 nm and 340-350 nm.

In addition, many recent studies have also reported that the UV absorption spectra of phenolic and flavonoid compounds have peaks in the range 230-350 nm. For example; Miri *et al.* (2011) showed that the two flavonoid compounds isolated from the methanolic extract of the aerial parts of *T. persicum* revealed distinct absorption peaks in the ranges 238 to 340 nm and 277 to 332 nm. In another study, Rispail *et al.* (2010) studied the secondary metabolic profile of *Lotus japonicus* roots and reported the quantitative changes in the level of fourteen phenolic peaks in the UV range 260 to 380 nm.

Thus, in the present study, the absorption peaks obtained for the all the three flowers that were in the wavelengths between 190 and 350 nm, indicate that the major components present in the flowers might be of phenolic and flavonoid type of compounds and their derivatives.

5.10.2. TLC and HPTLC Analyses

Thin-layer Chromatography (TLC) serves as a significant tool for the initial characterization of the crude plant extracts and herbal preparations. TLC has been widely used for phytochemical analysis (Cieśla and Waksmundzka-Hajnos, 2010). The chromatographic fingerprinting using HPTLC is an advanced technique based on the chromatographic separation and identification of marker compound from other constituents. It serves as a significant tool for the initial characterization of the crude plant extract (Sampathkumar and Ramakrishnan, 2011).

The TLC analysis of the three flowers of *C. pulcherrima* showed the presence of alkaloids, phenols, flavonoids, saponins, tannins and terpenoids. This was confirmed by HPTLC fingerprinting of the individual group of phytochemicals.

TLC analysis of all the three flower extracts showed six bands for alkaloids and five peaks were found on confirmation by HPLTC. In TLC analysis, for both phenolics and flavonoids, five bands were found for all the three flowers, whereas HPTLC fingerprinting showed eight peaks for phenolics and four peaks for flavonoids. The TLC profile of saponins for all the three flower extracts showed four bands, which on confirmation with

HPTLC showed six peaks for yellow and pink flower extracts, and seven peaks for the orange flower extract.

The TLC profile of steroids showed eight bands for the yellow flower and seven bands for the pink and orange flower extracts. But the HPTLC profile of steroids showed seven peaks for yellow flower, five for pink flower and six for orange flower. Similar variation was observed for tannins, wherein ten bands were found in TLC analysis and only five visible peaks were observed in HPTLC fingerprinting for all the three flowers. TLC revealed fourteen bands for terpenoids for all the three flowers, but in HPTLC fingerprinting, eight peaks for yellow flower, six peaks for pink flower and nine peaks for orange flower were observed.

TLC and HPTLC analyses have been applied in many studies for the identification and quantification of the active compounds present in the plant extracts that attribute to their medicinal properties. The TLC analysis of the flavonoid fraction of the methanolic extract of *W. tinctoria* bark showed the presence of quercetin and ferulic acid, which was confirmed by HPTLC fingerprinting with matching R_f values (Bigoniya *et al.*, 2013). Similarly, in another study, TLC, followed by HPTLC fingerprinting, revealed the presence of carbohydrates, phenolics, proteins, resins, lipids and flavonoids in the extracts of *Triticum aestivum* L, seeds (Khan *et al.*, 2013b).

Three different chemical profiles (alkaloid, steroid and flavonoid) of the methanolic extract of the whole plant of *Leucas aspera* L. was analyzed using HPTLC (Karthikeyan *et al.*, 2013). A densitometric HPTLC analysis of the ethanolic extract of *M. arundinacea* L. rhizome revealed the presence of phenolics, flavonoids, steroids, tannins and glycosides (Nishaa *et al.*, 2013).

TLC and HPTLC profile of the methanolic extract of *C. neilgherrensis* showed the presence of flavonoids, tannins, organic acids and saponin glycosides with similar R_f values after visualization under UV radiation of different wavelengths (Shyam *et al.*, 2012). Kulkarni *et al.* (2010) detected the presence of camptothecin, an important anticancer alkaloid, in the ethanolic extracts of stem with bark and callus cultures of *C. grandiflora* using TLC and HPTLC analysis.

The results obtained from the present study showed that TLC and HPTLC profiling serve as a significant tool for the initial characterization of the crude plant extracts. HPTLC profiling of the flower extracts suggested that HPTLC is more accurate, reliable and sensitive compared to TLC.

5.10.3. HPLC Analysis

HPLC has been established as a premier technique for phytochemical analysis and purification of a wide range of molecules. The inherent features of HPLC, such as high recovery, reproducibility and ease of selectivity manipulation, have made this technique extensively applied in many studies (Aguilar, 2003).

The HPLC spectrum of the methanolic extract of all the three flowers showed fifteen peaks, including a major peak with retention time (R_t) 25 minutes, all of which indicated the presence of major phytochemical components.

Many researchers have used HPLC to confirm the presence of biologically active phytoconstituents in the crude plant extracts. Reversed-phase HPLC was applied to confirm the presence of flavonoid and glycoside in the methanolic extract of *Zanthoxylum armatum* fruits by comparing their R_t with that of the standard compounds (Mehta *et al.*, 2013). Using quantitative HPLC analysis, myricetin was identified as the major component present in the methanol extract of *Cotinus coggygria* stem (Matić *et al.*, 2013b).

HPLC analysis of the polyphenol-rich hydro alcohol extract of *Lactuca sativa* revealed the presence of various phenolic compounds, such as rutin hydrate, chlorogenic acid, epicatechin, vanillin, caffeic acid, sinapic acid, p-coumeric acid, quercetin-3-rhamnoside and quercetin (Harsha and Anilakumar, 2013). In a similar study by Gökbulut *et al.* (2013), various phenolic components such as rutin, chlorogenic acid, caffeic acid, quercetin, myricetin, kaempferol and luteolin were quantified in the methanol extracts of the Inula species using HPLC-DAD system.

Using four HPLC methods, each with different mobile phases, Mradu *et al.* (2012) compared the R_t of twelve prominent phenolic compounds found in medicinal plants and constructed their standard HPLC chromatograms. Nyembo *et al.* (2012) screened the presence of phenolic compounds in the crude extracts of the leaves and root barks of *Jatropha curcas* using HPLC.

Vasudev *et al.* (2012) developed and validated a RP-HPLC method, by which two major active components namely ellagic acid and paclitaxel, were quantified in the *Punica granatum* fruit extract. The presence of ten different phenolic compounds in the ethanolic extract of leaves and roots of spice plants were identified and quantitatively assessed using HPLC by comparing their R_t and the UV absorbance maxima (λ_{max}) with that of the standards (Stankevičius *et al.*, 2010).

In accordance with the above literature, the peaks found in the HPLC spectrum of all the three flowers of *C. pulcherrima* in the UV range may represent the active components present in the flowers.

5.10.4. Fourier Transform Infrared (FT-IR) Spectral Analysis

Every compound exhibits a characteristic absorption or emission pattern in the IR spectral region that can be analyzed both quantitatively and qualitatively by FT-IR absorption spectroscopy. The Fourier Transform Infrared (FT-IR) absorption spectroscopy is more sensitive and selective than colorimetric methods that can be performed both on pure compounds and complex mixtures, without separation into individual components. It is used for the analysis of a relevant amount of compositional and structural information in plants (Janakiraman *et al.*, 2011).

In the present study, the FT-IR spectral analysis was carried out to identify the functional groups present in the plant extract based on the peak value obtained in the infrared radiation region. The results showed the presence of carboxyl and hydroxyl groups, which imply that the major bioactive compounds present in the three flowers of *C. pulcherrima* belong to phenolic and flavonoid group of compounds.

Many researchers have coupled FT-IR absorption spectroscopy with other spectroscopic techniques to identify the nature of the bioactive compounds in plant extracts and their phytochemical fractions. Neha and Jyoti (2013) confirmed the presence of phenolics and flavonoids in the flower extracts of *Bougainvillea glabra* by FT-IR analysis, followed by quantitative UV-visible spectrophotometry. Rajan and Muthukrishnan (2013) collected three phenolic fractions (gallic acid, rutin and quercetin) from the root extracts of *Pseudarthria viscida* using HPLC at standard retention time and subjected the fractions to FT-IR analysis, thereby confirming the presence of polyphenols.

The IR spectrum of *Larrea tridentata* extract was similar to that of a saponin and contained bands corresponding to phenolic hydroxyl groups and carbonyl groups, suggesting that saponins are the major group of compounds present in the *L. tridentata* leaves (Inzunza *et al.*, 2013). The FT-IR spectrum of the different leaf extracts of *Vitex altissima* L confirmed the presence of alcohols, phenols, alkanes, alkynes, alkyl halides, aldehydes, carboxylic acids, aromatics, nitro compounds and amines (Sathish *et al.*, 2012). The FT-IR analysis of the dry powder and ethanolic extract of *Albizia lebbeck* leaves confirmed the presence of amide, alcohols, phenols, alkynes, alkanes, carboxylic acids, alkenes, ketones, aromatics, aliphatic amines and alkyl halides compounds (Nazneen *et al.*, 2012).

The FT-IR absorption spectrum of all the three flowers of *C. pulcherrima* added support to the observation that the phenolics and flavonoids are the major bioactive compounds present in them.

5.10.5. Gas Chromatography–Mass Spectrometry (GC-MS) Analysis

GC-MS chromatogram analysis of the methanolic extracts of the three flowers of *C. pulcherrima* showed five major peaks for yellow flower, seven major peaks for pink and eight major peaks for orange flowers, all of which indicated the presence of carbonyl, hydroxyl, alkanes and ketone groups.

GC-MS analysis have been applied by many researchers to identify the possible bioactive components present in the plant extracts and herbal preparations, which might be useful lead compounds for the development of new pharmaceutical drugs.

Geetha *et al.* (2013) have identified thirty bioactive components in the ethanolic extract of leaves of *Elaeocarpus serratus* by GC-MS analysis. In another study conducted by Yang *et al.* (2013d), five monoterpenes and eleven sesquiterpenes were detected from the extracts of *Gossypium hirsutum* cv. using GC-MS.

Hexadecanoic acid, 1,2-benzenedicarboxylic acid and squalene were the three major components identified in the methanolic extract of *Vernonia cinerea* using GC-MS analysis (Abirami and Rajendran, 2012). Using GC-MS analysis, ten compounds were identified in the methanolic extract of the whole plant *Eupatorium triplinerve*, among which hexadecanoic acid, tetradecanoic acid and octadecanoic acid were the most prevailing compounds in the extract (Selvamangai and Bhaskar, 2012). The GC-MS spectrum of the ethanolic extract of *P. chinense* showed five peaks, indicating the presence of five phytochemical constituents, among which squalene and 1,2-benzenedicarboxylic acid were identified as the two major compounds (Ezhilan and Neelamegam, 2012).

In the present study, the presence of carbonyl and hydroxyl groups in the flower extracts, as analyzed by GC-MS, suggests that the phenolics and the flavonoids constitute the major bioactive components present in the flowers of *C. pulcherrima.*

The research outcome of the present study, thus, highlights the antioxidant, radical quenching, biomolecule-protective and anticancer properties of the flowers of *C. pulcherrima*. In addition, their protective effect towards non-cancerous cells against oxidant induced apoptosis and their apoptosis-inducing effect in case of cancerous cells implies that the flowers of *C. pulcherrima* exhibit a differential response towards non-cancerous and cancerous cells. The study also showed that all the three flowers of

C. pulcherrima are rich in seven major types of biologically active phytoconstituents namely alkaloids, phenols, flavonoids, saponins, steroids, tannins and terpenoids. Phenols and flavonoids were identified as the major active components present in the flowers that might be responsible for the protective effects rendered by the flower extracts.

Further analysis needs to be carried out to elucidate the structure of the phenol and flavonoid compounds that are present in all the three flowers of *C. pulcherrima*. All these findings of the present study provide strong evidence to substantiate the use of the flowers of *C. pulcherrima* in medicinal preparations for oxidative stress related diseases. The findings of the present study are summarized and the conclusions drawn from the observations are presented in the next chapter.