3. METHODOLOGY

The present study focussed mainly on determining the antioxidant and anticancer properties of the three different flowers (yellow, pink and orange) of *Caesalpinia pulcherrima* under conditions of oxidative stress. The study was conducted using *in vitro* models simulating the *in vivo* systems. Fresh flowers were collected from the local areas of Coimbatore. The plant was identified and certified by the Botanical Survey of India, Tamil Nadu Agricultural University, Coimbatore. The voucher of the specimen was collected and maintained.

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

The work was carried out in four distinct phases. In the first phase, the free radical scavenging activity and biomolecule-protective effect of the flowers against oxidative stress induced *in vitro* were studied. In the second phase, the antioxidant potential of the flower extracts against oxidative stress was analysed in an *in vivo*-simulated *in vitro* system. In the third phase, the anti-apoptotic and anticancer effect of the flower extract was determined in transformed and non-transformed cells. In the fourth phase, phytochemical analysis using various spectral techniques was applied to identify the major bioactive components in the flowers.

The experimental conditions and the procedures of the various parameters analysed in each phase of the present study are explained in detail in this chapter.

CHEMICALS

All chemicals used were of analytical grade and were used as received without any further purification.

PHASE I

3.1. FREE RADICAL SCAVENGING ACTIVITY OF *C. pulcherrima* FLOWERS

In Phase I, the radical scavenging activities of the three different flowers (yellow, pink and orange) of *C. pulcherrima* were determined *in vitro* against a battery of radicals namely DPPH, ABTS, H_2O_2 and hydroxyl radicals.

3.1.1. Solvent Extraction

In order to understand the active principle rendering the antioxidant activities, the flowers were individually extracted into solvents of increasing polarity under cold conditions. The solvents used were petroleum ether, benzene, chloroform, ethyl acetate and methanol. For this, fresh flowers of *C. pulcherrima* (5g) were collected. The petals were collected, washed with tap water to remove the surface contaminants, dried by gently blotting between folds of tissue paper and cut into fine strips using a knife. These pieces were taken in a flask covered with aluminium foil, and covered with the respective solvents. The flasks were stoppered and the contents were extracted for 72 hours at 4°C with mild shaking. After 72 hours, the extracts in different solvents were filtered by passing through Whatmann filter paper using a Büchner funnel connected with a vacuum pump. The filtrate was then concentrated at low temperature (40 - 50°C) and reduced pressure. The yields of the extracts were calculated and the residues were re-dissolved in a known volume of the same solvents in which they were extracted.

Apart from the solvent extracts, aqueous extracts were also prepared as mentioned above. The aqueous extracts were frozen at -80°C overnight and lyophilised. The lyophilised powder was weighed and dissolved in autoclaved double distilled water.

The different solvent extracts and the aqueous extract were tested for their DPPH, ABTS, hydroxyl and hydrogen peroxide scavenging effects, based on the procedures mentioned below.

3.1.2. Determination of Free Radical-Scavenging Activity of *C. pulcherrima* Flower Extracts

The antioxidant effects of the flower extracts were assessed by the ability to scavenge free radicals.

i) DPPH Scavenging Activity

The ability of the flower extracts to scavenge the DPPH radical was tested in a rapid dot-blot screening and quantified spectrophotometrically.

Principle

DPPH (1,1'-diphenyl-2-picryl hydrazyl), a stable free radical with purple colour, reacts with antioxidant and is converted into a yellow coloured product 1,1'-diphenyl-2-picryl hydrazine by donating its OH group which can be visualized or quantified at 518nm.

a) Dot-Blot Rapid Screening Assay

The rapid screening assay was performed by the method proposed by Soler-Rivas *et al*. (2000).

Reagents

1. TLC plates (silica gel 60 F_{254} - Merck)
2. DPPH (0.3mM in methanol)

Procedure

In this assay, a TLC plate spotted with the six different extracts (3µl each) of all the three types of flowers was placed upside down in DPPH solution for 10 seconds. The spots exhibiting radical scavenging antioxidant activity appeared as yellow spots in a purple background.

b) DPPH spectrophotometric Assay

Spectrophotometric quantification of the radical scavenging ability of the flowers towards DPPH free radical was carried out by the method of Mensor *et al*. (2001).

Reagents

1. DPPH – 1, 1'-diphenyl-2-picryl hydrazyl hydrate (0.3 mM in methanol)
2. Methanol

Procedure

In a reaction mixture of 1 ml, 20µl corresponding to 1mg of *C. pulcherrima* flower extracts were mixed with 0.5 ml of DPPH in methanol and 0.48 ml of methanol. After incubating at room temperature for 30 minutes, the decolourization of the purple colour was measured at 518nm. Methanol alone was used as the blank and DPPH in methanol, without the flower extracts, was used as positive control. The radical scavenging activity was calculated as;

$$\text{Percent scavenging activity} = 100 - \frac{A_{518}\ (\text{Sample}) - A_{518}\ (\text{Blank})}{A_{518}(\text{Control})} \times 100$$

ii) ABTS Scavenging Effects

The procedure for ABTS (2,2'-azino-bis-3-ethyl benzthiazoline-6-sulphonic acid) radical cation de-colourisation assay was based on the method proposed by Shirwaikar *et al*. (2006).

Reagent

ABTS Solution (7mM with 2.45 mM ammonium persulfate)

Procedure

ABTS radical cation ($ABTS^+$) was produced by reacting 7mM ABTS solution with 2.45mM ammonium persulphate. The mixture was allowed to stand in the dark at room temperature for 12-16 hours before use. For the study, the different flower extracts of concentration 1mg (volume of 20µl) were mixed with 0.3 ml of ABTS solution and the final volume was made up to 1ml with ethanol. The absorbance was read at 745nm and the per cent inhibition was calculated using the formula;

$$\text{Per cent inhibition} = \frac{\text{A (Control)} - \text{A (Sample)}}{\text{A (Control)}} \times 100$$

iii) Hydrogen Peroxide Scavenging Activity

The ability of the flowers of *C. pulcherrima* to scavenge the oxidant hydrogen peroxide was determined by the method proposed by Ruch *et al.* (1989).

Principle

Upon oxidation, there is a decrease in the absorbance of H_2O_2, which was measured at 230nm spectrophotometrically.

Reagents

1. Phosphate buffer (0.1M, pH 7.4)
2. H_2O_2 in phosphate buffer (40mM)

Procedure

The H_2O_2 solution (40mM) was prepared in 0.1M phosphate buffer (pH 7.4). The flower extract at 1mg/20 µl concentration in 2.4 ml of phosphate buffer was added to 0.6ml of H_2O_2 solution and incubated at room temperature for 10 minutes. The blank solution containing phosphate buffer without H_2O_2 was prepared. After 10 minutes, the absorbance was recorded at 230nm.

The percent inhibition was calculated using the formula,

$$\text{Per cent Scavenging} = \frac{\text{A (Control)} - \text{A (Sample)}}{\text{A (Control)}} \times 100$$

iv) Hydroxyl Radical Scavenging Activity

The extent of hydroxyl radical scavenging was determined by the method proposed by Elizabeth and Rao (1990).

Principle

Hydroxyl radicals are generated from Fe^{2+} -ascorbate-EDTA-H_2O_2 system (Fenton's reaction), which attacks the deoxyribose followed by a series of reactions, resulting in the formation of malondialdehyde, a pink coloured chromogen, which is measured at 532nm.

Reagents

1. Deoxyribose (2.8 mM)
2. Ferric chloride (0.1mM)
3. EDTA (0.1mM)
4. H_2O_2 (1mM)
5. Ascorbate (0.1mM)
6. KH_2PO_4-KOH (20mM, pH 7.4)
7. Thiobarbituric acid (1%)

Procedure

The reaction mixture was prepared with 0.1 ml of deoxyribose, 0.1 ml of ferric chloride, 0.1 ml of EDTA and 0.1 ml of H_2O_2. To this reaction mixture, 0.1 ml of ascorbate, 0.1 ml of KH_2PO_4-KOH buffer and 20µl of plant extract (concentration of 1 mg) were added to a final volume of 1.0 ml and was incubated at 37°C for 1 hour. After incubation, 1.0ml of TBA was added and the solution was heated at 95°C for 20 minutes to develop the colour. After cooling, TBARS formation was measured spectrophotometrically at 532nm against an appropriate blank. The hydroxyl radical scavenging activity was determined by comparing the absorbance of the control with that of the samples. The extent of TBARS formation for the positive control (H_2O_2) was fixed at 100% and the relative per cent TBARS formation was calculated for the other treatment groups.

$$\text{Per cent TBARS formation} = \frac{\text{A (Sample)}}{\text{A (Control)}} \times 100$$

In the free radical scavenging assays mentioned above (DPPH, ABTS, H_2O_2 and hydroxyl), the six different extracts of the flowers (petroleum ether, benzene, chloroform, ethyl acetate, methanol and aqueous) were compared, among which, the methanolic extract of the three flowers showed the maximum activity (results presented in the next chapter). Therefore, further studies were carried out using the methanolic extract of all the three flowers.

The optimum dose of the methanolic extract to be used for the subsequent phases of the present study was determined. The dose was optimised using free radical scavenging assays (DPPH, ABTS, H_2O_2 and hydroxyl) in which different concentrations (0.01 mg to 1.0 mg) of the methanolic extracts were compared and found that 0.1 mg of methanolic extract showed the best response. Therefore, further studies were carried out with 0.1 mg concentration of the three flower extracts.

3.2. EFFECT OF *C. pulcherrima* FLOWER EXTRACTS ON OXIDATIVE DAMAGE TO BIOMOLECULES

Free radicals and ROS production above basal levels cause irreversible damage to cell membrane, DNA and other cellular structures by oxidizing lipids, proteins and nucleic acids resulting in dysfunction of bio molecules within cells and, finally, cell death (Mohsenzadegan and Mirshafiey, 2012). Free radicals produce oxidized lipids from polyunsaturated lipids through the lipid peroxidation process and thereby causes cell membrane damage. Malondialdehyde (MDA) is one of the final products of peroxidation of unsaturated fatty acids in phospholipids and is responsible for cell membrane damage (Jarerattanachat *et al.*, 2013).

Protein oxidation is one of covalent modification of protein induced by ROS or other products of oxidative stress. The most common oxidative modification of proteins is carbonylation of particular amino acid residues (arginine, lysine, threonine or proline), which leads to alteration in protein activity and its proteolytic breakdown (Debska *et al.*, 2012). The oxidative cleavage of proteins, that occurs through the α-amidation pathway or by the oxidation of glutamyl side chains, also results in generating protein carbonyl derivatives (Jaganjac *et al.*, 2012).

Protein carbonylation leads to the loss or gain of function of target proteins related to age-related conditions and various diseases (Grimsrud *et al.*, 2008). Modifications of metabolic and structural proteins cause alterations in the processing and trafficking of proteins and also cause protein dysfunction that leads to regenerating tissue damage. Protein carbonylation has been to found to play a vital role in the pathogenesis of numerous diseases (Suzuki *et al.*, 2009)

Reactive species can modify DNA bases, induce inter- and intra-strand crosslinks, promote DNA-protein crosslinks, produce sugar moiety modifications and create strand break (Jena, 2012). Accumulation of DNA damage induces mutagenesis that result in carcinogenesis (Majsterek *et al.*, 2012).

The protective effect of the flowers of *C. pulcherrima* against oxidative stress-induced damage to biomolecules like lipids, DNA and proteins was analysed in cell-free systems and intact cells. The effect of the flower extracts on different types of cells subjected to oxidative stress was also studied. The methodology adopted is given below.

3.2.1. Evaluation of the Effects of *C. pulcherrima* Flower Extracts on Membrane Lipids

Lipids are more susceptible to oxidative stress and lipid peroxidation products are potential biomarkers for oxidative stress status *in vivo* and its related diseases. Hence, the biomolecule-protective effects of *C. pulcherrima* flower extracts against lipid peroxidation were investigated first. Three different membrane models namely, goat RBC ghosts (plasma membrane lipids), goat liver homogenate (plasma membrane and intracellular lipids) and liver slices (intact cells) were used to assess the extent of lipid peroxidation and the protection rendered by the flower extracts against induced oxidative stress.

i) Evaluation of LPO in RBC Ghosts

Principle

Oxidative damage of lipids by reactive species can be measured from the extent of formation of thiobarbituric acid reactive substance (TBARS) from the damaged lipids.

Reagents

1. Isotonic KCl (1.15%)
2. Hypotonic KCl (0.5%)
3. Tris buffered saline (TBS) (10mM Tris, 0.15M NaCl, pH 7.4)
4. Ferrous sulphate ($FeSO_4$) (10μM)
5. Thiobarbituric acid (TBA) (1%)
6. Ascorbic acid (0.06mM)
7. Ethanol (70%)
8. Acetone

Preparation of Goat RBC Ghosts

Erythrocyte ghost membranes were prepared by osmotic lysis using the method of Dodge *et al*. (1963). Fresh goat blood (50ml) was collected from the slaughterhouse in a sterile container. Using acid-washed stones, the blood was defibrinated immediately. After defibrination, the blood was transferred to another sterile container and diluted with sterile isotonic KCl in 1:1 ratio. The RBCs were pelleted by centrifuging at 3000xg for 10 minutes at 4°C. The supernatant was discarded and the pellet was washed thrice with isotonic KCl. The cells were then lysed at 37°C for one hour in hypotonic (0.5%) KCl. After lysis, the lysate was centrifuged at 5000 x g for 10 minutes at 4°C. The pellet obtained was washed repeatedly with hypotonic KCl to remove the haemoglobin. The pale pink pellet obtained was suspended in 1.5ml of TBS and aliquots (50µl) were used for the assay.

Procedure

For each treatment group, 50µl of RBC ghosts, 20µl of *C. pulcherrima* flower extract, 50µl $FeSO_4$ and 100µl of ascorbate were added and made up to a total volume of 500µl with TBS. A blank was prepared which contained only $FeSO_4$, ascorbate and TBS in a final volume of 0.5ml. The positive control corresponding to 100% oxidation was prepared, which contained all the constituents except the flower extract. The experimental medium corresponding to auto-oxidation contained only RBC ghosts. All the tubes were incubated at 37°C for one hour. After incubation, 0.5ml of 70% alcohol was added to all the tubes to arrest the reaction, followed by 1.0ml of TBA. The tubes were then kept in a boiling water bath for 20 minutes. The tubes were cooled to room temperature, centrifuged and 0.5ml of acetone was added to the supernatant. The pink colour developed was measured at 535nm spectrophotometrically (Genesys 10-S, USA).

ii) Estimation of LPO in Goat Liver Homogenate

LPO assay in goat liver homogenate was performed according to the method of Okhawa *et al.* (1979). Fresh goat liver was procured from a slaughterhouse and washed thoroughly with Tris HCl buffer (40mM, pH 7.0). The liver was cut into thin pieces and a 20% liver homogenate was prepared in Tris HCl buffer using a motorized Teflon homogenizer, followed by low speed centrifugation and the supernatant was used as the lipid source for the assay.

Reagents

1. KCl (30mM)
2. $FeSO_4$ (0.16mM)
3. TBA (1%)
4. Glacial acetic acid
5. n-Propanol : Pyridine (15:1 v/v)

Procedure

To 0.1ml of liver homogenate, 0.1 ml of KCl, 0.1ml of $FeSO_4$ and 0.1ml of ascorbate were added and mixed well. The reaction mixture was incubated at 37°C for one hour in the presence and the absence of extracts of *C. pulcherrima* flowers (20µl corresponding to 0.1mg). After incubation, 1.5 ml each of TBA and acetic acid were added to the reaction mixture, mixed well and the contents were heated in a boiling water bath for 20 minutes. After cooling, 1.0ml of distilled water and 5.0ml of n-propanol and pyridine mixture were added. The final reaction mixture was centrifuged and the absorbance of the pink coloured chromophore obtained was recorded at 532nm in a spectrophotometer (Genesys 10-S, USA). The per cent inhibition of LPO was determined by comparing the results of the control and the test samples.

iii) Estimation of LPO in Goat Liver Slices

The extent of inhibition of LPO in goat liver slices was estimated by the method proposed by Niehaus and Samuelsson (1968).

Reagents

1. Phosphate buffered saline (PBS)
2. H_2O_2
3. TBA-TCA-HCl reagent (0.375% TBA, 15% TCA, 0.25N HCl)

Preparation of Goat Liver Slices

Fresh goat liver was collected from a slaughterhouse, plunged in cold sterile PBS and maintained at 4°C till use. Thin slices of 1mm thickness were precisely cut using a sterile scalpel.

Procedure

For each treatment group, 1.0g of precision-cut goat liver slices was taken in 4.0ml of sterile PBS in a flat-bottomed flask and treated with 5µl of 30% H_2O_2 and /or the extracts of *C. pulcherrima* flowers. Appropriate controls were also set up. The assay mixture was incubated at 37ºC with mild shaking for one hour. After incubation, the goat liver slices were homogenized using a Teflon homogenizer, centrifuged and the supernatant was used for the assay. To 1.0ml of homogenate, 2ml of TBA-TCA-HCl reagent was added to terminate the reaction. The mixture was heated in a boiling water bath for 15 minutes and the pink colour developed was read at 535nm against a reagent blank.

3.2.2. Effect of *C. pulcherrima* Flower Extracts on Oxidant Induced DNA Damage

In the present study, the effect of the flowers of the candidate plant on oxidant induced DNA damage was assessed *in vitro* in commercially available preparations of DNA. DNA from different hierarchies of evolutionary development were selected for the analysis, which included the commercially available preparations of viral DNA (λ DNA), bacterial plasmid (pUC18) and DNA of animal origin (herring sperm DNA).

i) Estimation of the DNA Damage in λ DNA and pUC18 DNA

The extent of DNA damage in λ DNA and pUC18 DNA was determined by the method proposed by Chang *et al.* (2002).

Reagents

1. λ DNA/pUC18 DNA (2µg/5µl)
2. Tris buffer (30mM, pH 7.4)
3. H_2O_2 (30%)
4. $FeCl_3$ (500µM)
5. Agarose (1%) in 1X TAE buffer
6. EtBr (10mg/ml)
7. Gel loading dye (0.25% bromophenol blue, 0.25% xylene cyanol, 50% glycerol)
8. 50X TAE buffer (24.2g Tris base, 18.612g EDTA, 5.71 ml glacial acetic acid in a total volume of 100ml, pH 8.0)

Procedure

To the reaction mixture, 5µl of tris buffer or pUC18 DNA/ λDNA (2µg/5µl) and 20 µl of tris buffer or flower extracts, followed by the addition of 10µl H_2O_2 and 5µl of $FeCl_3$ were added, mixed well and incubated at 37ºC. The incubation period was 30 minutes for λ DNA and 15 minutes for pUC18 DNA. After incubation, 6µl of loading dye was added to the reaction mixture and electrophoresed in 1% agarose gel containing 3µg/ml EtBr, at 100V for 15 minutes, using 1X TAE as running buffer. DNA bands were viewed under transilluminating UV light and photographed using an Alpha Digidoc digital gel documentation system (Alpha Innotech, USA). The intensities of the bands were quantified using the software of the system.

ii) Estimation of Damage in Herring Sperm DNA

The extent of DNA damage in herring sperm DNA and the effects of *C. pulcherrima* flower extracts were studied according to the method proposed by Aeschlach *et al*. (1994).

Reagents

1. Herring sperm DNA (0.5mg/ml in 500mM tris buffer, pH 7.4)
2. H_2O_2 (30%)
3. $MgCl_2$ (5mM)
4. $FeCl_3$ (50µm)
5. EDTA (0.1M)
6. TBA (1% w/v)
7. HCl (25%)
8. Tris buffer (10mM, pH 7.4)

Procedure

The assay mixture of total volume 0.5 ml was prepared with 0.05ml of herring sperm DNA, 0.167ml of H_2O_2, 0.05ml of $MgCl_2$, 0.05ml of $FeCl_3$ (50 µM) and flower extract (20 µl containing 0.1 mg) in 10 mM Tris buffer (pH 7.4) and incubated at 37ºC for 1 hour. After incubation, the reaction was terminated by adding 0.05ml of 0.1M EDTA. To the reaction mixture, 0.5 ml of thiobarbituric acid and HCl were added and incubated at 37ºC for 15 minutes. At the end of incubation, the colour developed was measured as the extent of DNA damage at 532nm.

3.2.3. Effect of *C. pulcherrima* Flowers on Protein Oxidation

i) Protein Carbonyl Assay

The method outlined by Jean *et al*. (2010) was used to analyse the protein carbonyl.

Principle

Protein carbonyls react with 2,4-dinitrophenylhyrdazine (DNPH) and produce hydrazones, which can be detected spectrophotometrically at an absorbance of 375 nm.

Reagents

1. BSA (2mg)
2. Ferric chloride (50µM)
3. Hydrogen peroxide (1mM)
4. Ascorbic acid (100mM)
5. DNPH (10mM)
6. Hydrochloric acid (2M)
7. TCA (10% w/v)

8. Ethanol/ethylacetate (1:1, v/v)
9. Guanidine hydrochloride (6M)

Assay:

BSA was oxidized by a Fenton-type reaction. The reaction mixture containing 2 mg BSA, $FeCl_3$ (5µl), H_2O_2 (10µl), ascorbic acid (10µl) and sample extract (20µl) was made up to 1ml with distilled water and incubated for 30 minutes at 37^oC. After incubation, 250µl of the reaction mixture was taken, to which 1ml of 10mM (DNPH) in 2M HCl was added and incubated for 30 minutes at room temperature in the dark with occasional shaking. The protein was precipitated by adding 1ml of cold TCA (10%, w/v) followed by centrifugation at 8000 rpm for 10 minutes. The protein pellet obtained was washed three times with 1ml of ethanol/ethyl acetate (1:1, v/v) and finally dissolved in 250µl of guanidine hydrochloride (6M). The solubilized protein sample was diluted 2 fold with distilled water. The supernatant was transferred to a minicuvette and read at 375 nm spectrophotometrically. The absorbance was read against the blank of each sample.

ii) Analysis of Protein Oxidation by 1-D Gel Electrophoresis

The oxidative modifications of proteins play a major role in a number of human diseases. The ability to identify specific proteins that are most susceptible to oxidative modifications facilitates the development of methods for early diagnosis, assessment of new potential therapies and understanding the overall disease mechanisms. However, the ability to identify specific proteins that are most susceptible to oxidative modifications is difficult. In the present study, the differences in the mobility of the protein subjected to oxidative stress *in vitro* and the influence of the flower extracts on the electrophoretic mobility, was visualized using polyacrylamide gel electrophoresis (PAGE).

Reagents

1. Tris (25mM, pH 8.3)
2. Tris (1.5M, pH 8.8)
3. Tris (1M, pH 6.8) (From this, other buffer concentrations like 50 mM and 100 mM tris of pH 6.8 were prepared respectively and used)
4. DTT (100mM)
5. Glycine (250mM)
6. Glycerol
7. SDS (20%)
8. Bromophenol blue (0.1%)
9. Ammonium per sulphate (10%)
10. SDS (10%)

11. Acrylamide : Bisacrylamide (29:1)
12. Composition of gels

Component	Separating gel (12%)	Stacking gel (5%)
Acrylamide stock	12.0 ml	1.75 ml
Tris buffer	7.5 ml (pH 8.8)	1.25 ml (pH 6.8)
Water	9.9 ml	6.8 ml
SDS	0.3 ml	0.1 ml
APS	0.3 ml	0.1 ml
TEMED	12µl	10µl

13. Gel loading dye: 5ml of 100mM tris (pH 6.8), 1ml of 20% SDS,10mg of bromophenol blue (tracking dye), and 1ml of glycerol were mixed and made up to 9ml with distilled water. To this, 1ml of 1M DTT stock was added fresh just before use and mixed.
14. Staining solution: Coomassie brilliant blue R_{250} (250mg) was dissolved in 45ml of water and 45ml of methanol, and 10 ml of glacial acetic acid was added to it.
15. Destaining solution: This was prepared by freshly mixing together 45ml water, 45ml methanol and 10ml glacial acetic acid.

1-D Gel Electrophoresis

The glass plates were set without any leakage. The resolving gel was allowed to polymerize followed by the stacking gel with the Teflon comb in the vertical position. Samples were prepared by heating to 100°C for 3 minutes in the sample loading buffer. After polymerization, gel plates were mounted in the apparatus.

Sample Preparation and Incubation

BSA (0.1g) was dissolved in 1ml of PBS. Aliquots (0.1ml) of this protein mix solution were pipetted out into clean dry test tubes. The proteins were then treated with H_2O_2 (at a final concentration of 200µM) in the presence and absence of *C. pulcherrima* flower extracts (0.1 mg) and the total volume of each tube was made up to 1ml with PBS. The tubes were then incubated at 37°C with gentle shaking for one hour.

Electrophoresis

Fifty microliters of the reaction mixture was mixed with an equal volume of gel loading buffer containing DTT in a microfuge tube. The tubes were tightly corked and kept in a boiling water bath for exactly one minute and were then allowed to cool to room temperature followed by pulse spinning to bring the solution down. The protein samples were then loaded into the wells of the gel at a concentration of 100µg protein / well (50µl) and electrophoresed at 8V / cm (0.65mA / cm^2) till the tracking dye reached a distance of 1cm from the bottom of the gel (3 – 4 hours). The leads were disconnected and the plates were dismantled and gently pried apart with a flat spatula. After marking the direction of run by clipping off the gel at the right hand corner, SDS was removed by rinsing the gel quickly in tris buffer. The gel was then gently immersed in the staining solution kept in a staining box and incubated overnight at room temperature using a gel rocker. After draining off the staining solution, the gel was destained using freshly prepared destaining solution after several rinses with the destaining solution. The destaining solution was changed several times at a minimum interval of 30 to 45 minutes, till the bands were clearly visible in a transparent background of the gel. The gel was then documented in a digital gel documentation system (Alpha Digidoc, USA) and the integrated density values of the bands were recorded.

From the results of the above experiments, it was clear that the *C. pulcherrima* flowers exhibited good radical scavenging effects and also rendered significant protection against oxidative stress, both in cell-free systems and in intact cells.

PHASE II

The results of phase I (presented in the next chapter) showed that the extracts of 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 damage under *in vitro* conditions.

In phase II, the antioxidant potential of the flowers of *C. pulcherrima* was evaluated in an *in vitro* system subjected to oxidative stress. Liver is an important organ that plays a pivotal role in the regulation of various physiological processes such as metabolism, secretion and storage. It plays a major role in detoxifying toxic substances and synthesizing useful principles (Kalaiselvi *et al.,* 2011). The major advantage of using liver slices compared to cell culture is that it mimics the *in vivo* situation of the liver due to presence of its physiological extracellular matrix (Van de Bovenkamp *et al.,* 2007). Precision-cut liver slices is a widely used *in vitro* model to elucidate the pharmacological metabolism and to investigate the toxicology and efficacy of novel substances under standardized conditions (de Graaf *et al.,* 2007). Thus, in the present study, the precision-cut goat liver slices were used as an *in vitro* model to study the effect of the flower extracts in intact cells against oxidative stress by analysing the antioxidant status, as explained below.

3.3. DETERMINATION OF THE EFFECTS OF *C. pulcherrima* FLOWER EXTRACTS ON THE ANTIOXIDANT STATUS OF GOAT LIVER SLICES

3.3.1. Preparation of Goat Liver Slices

Fresh goat liver was obtained from the local slaughterhouse immediately after sacrifice of the animal and transported on ice to the laboratory. The liver was quickly plunged in ice-cold PBS and maintained at 4ºC till use. Thin slices of the liver of about 1mm thickness were cut using a sterile scalpel and the slices were taken in PBS at a proportion of 0.25g in 1ml, in broad, flat bottomed flasks for each treatment group.

3.3.2. Induction of Oxidative Stress

Hydrogen peroxide (H_2O_2) was used as the oxidising agent to induce oxidative stress at a final concentration of 200µM. The liver slices were treated with H_2O_2 both in the presence and the absence of the flower extracts (20µl) and incubated at room temperature for one hour with mild shaking. After incubation, the mixture was homogenized using a motorised Teflon homogenizer, followed by centrifugation to remove the debris. The supernatant was used for the analysis of various enzymic and non-enzymic antioxidants.

The treatment groups set up for each assay were,

1. Untreated control
2. Slices treated with H_2O_2 (standard oxidant)
3. Slices treated with methanolic extract of yellow flowers of *C. pulcherrima* (CPY)
4. Slices treated with methanolic extract of CPY + H_2O_2
5. Slices treated with methanolic extract of pink flowers of *C. pulcherrima* (CPP)
6. Slices treated with methanolic extract of CPP + H_2O_2
7. Slices treated with methanolic extract of orange flowers of *C. pulcherrima* (CPO)
8. Slices treated with methanolic extract of CPO + H_2O_2

3.3.3. Determination of Enzymic Antioxidants

The enzymic antioxidants, namely superoxide dismutase, catalase, peroxidase, glutathione S-transferase and glutathione reductase were assayed to evaluate the enzymic antioxidant status of the liver slices. The liver slice homogenate was used as the enzyme source. The methodology to analyse the enzymic antioxidant activities is given below.

i) Assay of Superoxide dismutase (SOD)

SOD was assayed according to the method outlined by Misra and Fridovich (1972).

Principle

Superoxide dismutase uses the photochemical reduction of riboflavin as O_2 generating system and catalyses the inhibition of NBT reduction, the extent of which can be assayed spectrophotometrically at 600nm.

Reagents

1. 50mM potassium phosphate buffer (pH 7.4)
2. 45µM of methionine
3. 5.3µM riboflavin
4. 84µM nitro blue tetrazolium (NBT)
5. 20mM potassium cyanide

Procedure

The incubation medium contained 300µl of each reagent. To the test tubes 300 µl of sample was added. The final volume was made up to 3ml with water. The tubes were then placed in an aluminium foil lined box maintained at 25°C and equipped with 15W fluorescent lamp. Reduced NBT was measured spectrophotometrically at 600nm. The maximum reduction was evaluated in the absence of the enzyme (liver slice homogenate). One Unit of enzyme activity is defined as the amount of enzyme causing a 50% reduction in NBT oxidation in one minute.

ii) Assay of Catalase

The enzyme-catalysed decomposition of H_2O_2 was measured by the method of Luck (1974).

Principle

The UV absorption of hydrogen peroxide can be easily measured between 230 and 250 nm. On decomposition of hydrogen peroxide by catalase, the absorption decreases with time. The enzyme activity can be arrived at from this decrease.

Reagents

1. Phosphate buffer (0.067M, pH 7.0)
2. Hydrogen peroxide in phosphate buffer (2mM)

Procedure

H_2O_2-phosphate buffer (2.9ml) was pipetted out into a quartz cuvette and the baseline was set at 240nm. Liver slice homogenate (0.1ml) was added quickly and mixed thoroughly. The time required for a decrease in absorbance by 0.05 units was recorded. The H_2O_2-phosphate buffer served as control. One enzyme unit was calculated as the amount of enzyme required to decrease the absorbance at 240nm by 0.05 units. The concentration of H_2O_2 was calculated using the extinction coefficient 0.036 per µM/cm.

iii) Assay of Peroxidase

Peroxidase catalyses the oxidation of some organic substrates in the presence of hydrogen peroxide. It was estimated by the method of Reddy *et al.* (1995).

Principle

In the presence of the hydrogen donor pyrogallol, peroxidase converts hydrogen peroxide to water and oxygen. The oxidation of pyrogallol to a coloured product called purpurogalli can be quantified spectrophotometrically at 430 nm. The formation of the product is proportional to the activity of the enzyme and can be used as a measure of the same.

$$\text{Pyrogallol} + H_2O_2 \longrightarrow \text{oxidised pyrogallol} + H_2O + O_2$$

Reagents

1. Phosphate buffer (0.1 M, pH 6.5).
2. Pyrogallol (0.05M in 0.1M phosphate buffer, pH 6.5).
3. H_2O_2 (1% in 0.1M phosphate buffer, pH 6.5).

Procedure

The assay mixture containing 3.0ml of pyrogallol solution and 0.1ml of liver slice homogenate were taken in a cuvette. The baseline was adjusted to read zero at 430nm. To this, 0.5ml of 1% H_2O_2 in phosphate buffer was added and the change in absorbance was recorded for every 30 seconds, up to 3 minutes. One unit of peroxidase activity is defined as the change in absorbance per minute at 430nm.

iv) Assay of Glutathione S-transferase (GST)

GSTs play an important role in the detoxification and metabolism of many xenobiotic and endobiotic compounds (Mourad and Noor, 2011). It catalyses the conjugation of GSH with a variety of endogenic and exogenic electrophilic compounds, including several carcinogens and antineoplastics. It can also function as peroxidases and isomerases (Gulubova *et al.,* 2011). The method of Habig *et al*. (1974) was employed for the assessment of glutathione S-transferase activity.

Principle

The enzyme was assayed by its ability to conjugate with GSH and CDNB. The extent of conjugation causes a proportionate change in the absorption at 340nm, which can be recorded spectrophotometrically.

Reagents

1. Chloro-2,4-dinitrobenzene (CDNB) (1mM in ethanol)
2. Reduced glutathione (1mM)
3. Phosphate buffer (0.1M, pH 6.5)

Procedure

The substrate mixture (total volume of 2.9ml) containing 0.1ml of reduced glutathione (GSH), 0.1ml of chloro-2,4-dinitrobenzene (CDNB) and phosphate buffer was taken in a cuvette. The reaction was initiated by adding 0.1ml of liver slice homogenate and the readings were recorded against distilled water blank for a minimum of three minutes. The whole assay mixture without the enzyme served as the control. The enzyme activity was determined by monitoring the changes in absorbance at 340nm in a spectrophotometer. One unit of GST activity is defined as the nmoles of CDNB conjugated/minute.

v) Assay of Glutathione reductase

Glutathione reductase (GR) reduces oxidized glutathione (GSSG) to reduced glutathione (GSH). Glutathione reductase activity was determined by the method proposed by David and Richard (1983).

Principle

The enzyme glutathione reductase catalyses the conversion of oxidized glutathione to its reduced form using NADPH as a substrate. The amount of NADPH utilized is a direct measure of enzyme activity.

Reagents

1. EDTA (15mM)
2. Sodium azide (10mM)
3. Oxidized glutathione (6.3mM)
4. NADPH (9.6mM)

Procedure

The assay mixture containing sodium azide, oxidized glutathione, EDTA, liver homogenate and water of volume 0.1ml each was incubated for 3 minutes at room temperature followed by the addition of 0.1ml NADPH. The absorbance at 340nm was recorded at an interval of 15 seconds for 3 minutes. One unit of GR is expressed as the μmoles of NADPH oxidized per minute.

3.3.4. Determination of Non-Enzymic Antioxidants

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. The non-enzymic antioxidants analysed in the liver slice homogenate were ascorbic acid, tocopherol, vitamin A, reduced glutathione, total phenol and protein thiol. The liver slice homogenate was used as the non-enzymic antioxidant source.

i) Estimation of Ascorbic acid

Vitamin C, a scavenger of oxyradicals, was estimated by the method of Roe and Keuther (1943).

Principle

Ascorbate is converted to dehydroascorbate by treatment with activated charcoal, which reacts with 2,4-dinitrophenyl hydrazine to form osazones, which dissolve in sulphuric acid to give an orange coloured solution whose absorbance can be measured spectrophotometrically at 540nm.

Reagents

1. Trichloroacetic acid (4%)
2. Sulphuric acid (9N)
3. 2,4-dinitrophenyl hydrazine reagent (2% in 9N sulphuric acid)
4. Thiourea solution (10%)
5. Sulphuric acid (85%)
6. Standard ascorbate solution: 10mg ascorbate in 100ml of 4% TCA.

Procedure

Enzyme extract (liver slice homogenate, 0.4 ml) and standard ascorbic acid (0.2 to 1ml) were aliquoted into test tubes and made up to 2ml with 0.5ml of 4% TCA. DNPH reagent (0.5 ml) followed by 2 drops of 10% thiourea solution were added and the tubes were incubated at 37°C for 3 hours. The osazone crystals formed were dissolved in 85% sulphuric acid (2.5ml) on ice. To the blank alone, DNPH reagent and thiourea were added after the addition of H_2SO_4. The absorbance was read at 540nm after incubation for 30 minutes at room temperature. The concentration of ascorbic acid was calculated and expressed as mg ascorbate/g liver.

ii) Estimation of Tocopherol

The levels of tocopherol were estimated based on Emmerie-Engel reaction using the method of Rosenberg (1992).

Principle

Tocopherol can be estimated using Emmerie - Engel reaction, which is based on the reduction of ferric to ferrous ions by tocopherol, which then forms a red colour with 2,2'-dipyridyl. Tocopherol and carotenes are first extracted with xylene and the absorbance read at 460nm to measure carotenes. A correction is made for this after adding ferric chloride and is read at 520nm.

Reagents

1. Absolute alcohol
2. Xylene
3. 2,2'-dipyridyl (1.2g/L in n-propanol)
4. Ferric chloride (1.2g/L in ethanol)
5. Standard solution of D,L-α tocopherol: 10mg/L in absolute alcohol (91mg of α-tocopherol is equivalent to 100mg of tocopherol acetate).
6. Sulphuric acid (0.1N)

Procedure

Into three stoppered centrifuge tubes (test, standard and blank), 1.5ml of liver slice homogenate, standard and water respectively were pipetted out. To all the tubes, 1.5ml each of ethanol and xylene were added, stoppered, mixed well and centrifuged. The xylene layer (0.1ml) was taken in another set of stoppered centrifuge tubes, to which 1 ml of 2,2'-dipyridyl reagent was added, stoppered and mixed well. This reaction mixture was taken in the spectrophotometric cuvettes and the extinctions of the test and the standard were read against the blank at 460nm. To all the tubes, 0.33ml of ferric chloride solution was added, mixed well and after exactly 15 minutes, the absorbance was measured at 520nm.

The levels of tocopherol in the sample were calculated using the formula

$$\text{Tocopherol } (\mu\text{g}) = \frac{\text{Reading at 520nm} - \text{Reading at 450nm}}{\text{Reading of standard at 520nm}} \text{ x } 0.29 \text{ x } 15$$

The results are expressed as µg tocopherol/g of liver.

iii) Estimation of Vitamin A

Vitamin A plays an important role in strengthening immunity against infection. Vitamin A was estimated by the method proposed by Bayfield and Cole (1980).

Principle

The colour produced by vitamin A, its acetate or palmitate with TCA is proportionate in intensity to its concentration. This property is used for the spectrophotometric estimation.

Reagents

1. Saponification mixture (2N KOH in 90% alcohol)
2. Petroleum ether (40 – 60°C)
3. Anhydrous sodium sulphate
4. Chloroform
5. Vitamin A palmitate
6. TCA reagent (60% TCA in chloroform) - freshly prepared.

Procedure

Extraction of Vitamin A from the liver

The incubated liver slices were homogenized. To 1ml of homogenate, 1ml of saponification mixture (2N KOH in 90% alcohol) was added and gently refluxed for 20 minutes at 60°C in the dark. Vitamin A was extracted twice with 10ml portions of petroleum ether (40 to 60°C). The extracts were pooled and washed thoroughly with water. The layers were separated using a separating funnel. Sodium sulphate was added to remove the moisture. 1ml of ether extract was then taken and evaporated to dryness at 60°C. The residue was dissolved in 1ml of chloroform.

Assay

Different concentrations of the standard (vitamin A palmitate) ranging from 0 to 7.5µg were pipetted out in to a series of clean, dry test tubes and were made up to 1ml with chloroform. 2ml of TCA reagent was added rapidly and mixed. The absorbance was read immediately at 620nm. The procedure was repeated for the sample tubes and the vitamin A level was expressed as µg/g tissue.

iv) Estimation of Reduced Glutathione (GSH)

GSH is one of the major intracellular defences against free radical induced damage. The method proposed by Moron *et al.* (1979) was used for the estimation of reduced glutathione.

Principle

GSH is measured by its reaction with DTNB (5,5'-dithiobis-2-nitrobenzoic acid) (Ellman's reaction) to give a yellow coloured product that absorbs at 412 nm.

Reagents

1. TCA (5% and 25%)
2. Phosphate buffer (0.2M, pH 8.0)
3. DTNB (0.6mM in 0.2M phosphate buffer)
4. Standard GSH (10 nmoles/ml in 5% TCA)

Procedure

Liver homogenate (0.5ml) was acidified immediately after homogenization with 125µl of 25% TCA to achieve a final concentration of 5%. The tubes were placed on ice for 5 minutes and the mixture was diluted further with 0.6ml of 5% TCA. The precipitates were centrifuged down at 1000rpm for 10 minutes. Then, the tubes were again transferred to ice and 0.1ml of the supernatant was taken for the assay.

Assay

The volume of the aliquot was made up to 1ml with 0.2M sodium phosphate buffer (pH 8). Freshly prepared DTNB (2ml) was added to the tubes and the intensity of the yellow colour formed was read at 412nm in a spectrophotometer after 10 minutes. A standard curve of GSH was prepared using concentrations ranging from 2 to 10 nanomoles. The concentration of GSH in the samples was calculated from this and the results were expressed as moles GSH / g liver.

v) Estimation of Total Thiols

Total thiols play a significant role in the defence against reactive oxygen species. They were estimated by the method of Sedlack and Lindsay (1968).

Principle

The sulphydryl groups in tissues were determined using Ellman's reagent. In this method, DTNB was reduced by SH group to form 1 mole of 2-nitro-5-mercapto benzoic acid per mole of SH.

Reagents

1. 0.2M Tris - EDTA (pH 8.0)
2. 0.01M DTNB
3. Methanol

4. Standard glutathione - 50mg of reduced glutathione was dissolved in 100ml of 0.2M Tris - EDTA (pH 8.0)

Procedure

The homogenate (0.2 ml) was mixed with 1.5ml of buffer and 0.2M EDTA and 1.0ml of DTNB. The mixture was made up to 10ml with methanol. A reagent blank without the sample and sample blank without DTNB were prepared in the same manner. The tubes were stoppered and allowed to stand for 15 minutes at room temperature. Then, the mixture was centrifuged at 3000rpm for 15 minutes. The absorbance of the yellow colour produced was measured at 420nm. Calibration curves were obtained with reduced glutathione as standard.

v) Estimation of Protein Thiols

Protein thiols are a target of oxidative stress. They play an important role in the scavenging of free radicals. The levels of protein thiols were calculated as the difference between total thiols and reduced glutathione.

PHASE III

The results of phase II (presented in the next chapter) signified that the treatment with the methanolic extract of the three different flowers of *Caesalpinia pulcherrima* significantly improved the antioxidant status of the goat liver slices induced with oxidative stress *in vitro*. Precision-cut goat liver slices were chosen as an *in vitro* model and was maintained and treated in an environment that simulates the conditions *in vivo*, thus it is believed that the similar protective effects could also occur in the intact system.

Under normal physiological conditions, reactive oxygen or nitrogen species (ROS/RNS) are produced endogenously and act as messengers in normal cell functions. Oxidative stress induces excessive production of ROS/RNS, thereby causing mutations and loss of the critical regulatory mechanisms that, in turn, results in defective cell death and aberrant proliferation, which has been implicated in the development of cancer (Bogeski *et al.*, 2011).

Further studies were carried out mainly to examine the influence of *C. pulcherrima* flower extracts on oxidative stress-induced apoptotic events. In recent years, the use of animals in research, teaching and testing has become an important ethical and political issue. Alternative scientific tests are being developed, which are more efficient and reliable than animal tests. Hence, the use of alternative experimental systems has been standardized for studying plant extracts and products in our laboratory, in order to minimize the use of live animals in research.

Many research studies have been carried out to identify plants with significant antioxidant and anticancer potential by analysing their cytotoxic, antiproliferative, apoptotic and radical scavenging activities using *in vitro* systems (Ghosh *et al.,* 2010; Liu *et al.,* 2010a; Mishra *et al.,* 2010; Tang *et al.,* 2010).

In the present study, *Saccharomyces cerevisiae* cells, human peripheral blood lymphocytes and cancer cell line were used as various alternative systems to study the antioxidant potential and apoptosis related events. 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 standard oxidant [H_2O_2 (for yeast) or etoposide (peripheral blood lymphocytes and KB cells)] at a final concentration of 200µM, while the concentration of plant extract used was 100µg. The treatment period for the exposure of the oxidant and the plant extract was one hour at 37ºC for H_2O_2 and 24 hours for etoposide.

Parameters Analyzed

The cell viability was quantified by MTT and SRB assays and the cytotoxicity was quantified by lactate dehydrogenase (LDH) assay in all the groups. The characteristic features of apoptosis were studied by analysing various parameters like morphological changes (Giemsa staining), apoptotic index (propidium iodide staining) and nuclear events (EtBr, AO/EtBr and DAPI staining).

Promoting tumour cell apoptosis is one of most important methods towards tumour treatment (Feng *et al.,* 2011). In order to determine the possible mechanisms of cell cycle alterations and induction of apoptosis elicited by the extracts on oral cancer cells, immunocytochemical analysis of expression of TP53, Bcl-2 and Bax proteins that are involved in apoptosis and cell cycle regulation and cell cycle analysis using flow cytometry were carried out.

The parameters were carried out in the following treatment groups:

- Untreated (negative) control cells
- Cells treated with standard oxidant (H_2O_2 / etoposide)
- Cells treated with methanolic extract of yellow flowers of *C. pulcherrima* (CPY)
- Cells treated with methanolic extract of CPY + H_2O_2 / etoposide

- Cells treated with methanolic extract of pink flowers of *C. pulcherrima* (CPP)
- Cells treated with methanolic extract of CPP + H_2O_2 / etoposide
- Cells treated with methanolic extract of orange flowers of *C. pulcherrima* (CPO)
- Cells treated with methanolic extract of CPO + H_2O_2 / etoposide

3.4. CULTURING OF YEAST CELLS

Saccharomyces cerevisiae is a widely studied eukaryotic organism. The major advantages of using *S. cerevisiae* as a model system are that it can be easily grown in laboratory and is unicellular that can be grown as a haploid to isolate the mutants (Lushchak, 2006). It is the first eukaryotic organism whose genome has been completely sequenced and is the most preferred model system to analyze the molecular mechanisms of oxidative stress protection (Costa *et al.,* 2007).

Yeast extract-peptone-dextrose (YPD) medium of pH 6.5 containing 10g of yeast extract, 20g of peptone and 20g of dextrose for 1000ml, was prepared and sterilized by autoclaving after aliquoting the medium in conical flasks. The aliquots were cooled and stored at room temperature till use. The medium was checked regularly for contamination.

Yeast cells were inoculated into the medium on the penultimate day of each assay and the flask was incubated in a temperature–controlled orbital shaker at 30ºC overnight. The cells were pelleted by centrifugation at 1000g for 15 minutes. The pellet was then washed with saline and resuspended in a specific volume of assay medium for each experiment. Aliquots containing 10^6 cells (counted using Neubauer ruling) were treated with or without H_2O_2 in the presence and the absence of the plant extract and incubated for one hour at 37ºC. After incubation, the treatment mixture containing the cells was centrifuged, and the pellet was resuspended in a specific volume of PBS. The cell suspension was used for cell viability assay whereas a spread was made from the suspension and used for various staining techniques.

3.5. CULTURING OF HUMAN PERIPHERAL BLOOD LYMPHOCYTES

The peripheral blood, a source of human cells, is abundant with mitotic cells and the cell culture technique is simple. Thus, culturing of human peripheral blood lymphocytes has been used as the most convenient method to study human chromosomes analysis in both clinical studies and research (Benn and Delach, 2008). Lymphocytes were isolated from the peripheral blood of the healthy individuals and cultured in RPMI 1640 medium by the method proposed by Boyum (1968). The cells were cultured in sealed sterile universal flasks and incubated at 37ºC in an incubator with moderate shaking (Heraeus, Germany).

Reagents

1. Complete medium [RPMI 1640 supplemented with 10% FBS, 1% phytohaemagglutinin-P (PHAP) and antibiotics (10,000 IU Penicillin and 10,000µg/mL Streptomycin at a final concentration of 100 IU/mL of Penicillin and 100µg/mL of Streptomycin)].
2. Phosphate buffered saline (PBS)
3. Alcohol (70%)
4. Lymphocyte separation medium (Lymphosep)

Procedure

Isolation of lymphocytes

Fresh blood was drawn from healthy individuals by venipuncture under aseptic conditions, using a heparinized syringe. The blood was then diluted 1:1 with RPMI 1640 medium or PBS or balanced salt solution. About 4 ml of lymphosep was dispensed into a siliconized glass centrifuge tube or 15 ml centrifuge tube. About 2 ml of the diluted blood was carefully layered on the top of the lymphosep medium and centrifuged for 30 minutes at 400xg at 18 - 20˚C. After centrifugation, the lymphocytes which has formed a gray coloured layer at the interface of the blood plasma and the separation medium was carefully aspirated without disturbing the separation medium. The lymphocytes were then transferred into a centrifuge tube containing atleast three times the volume of PBS or balanced salt solution and suspended evenly. The lymphocyte suspension was then centrifuged at 100xg for 10 - 15 minutes. The supernatant was aspirated and discarded. Using a cut microtip, the cells were then resuspended in fresh PBS and centrifuged at 100xg (RCF) for 10 minutes. This process was repeated thrice. Finally the cells were resuspended in fresh RPMI medium supplemented with 10% FBS.

Culture of lymphocytes

From the isolated cell suspension, 10µl was taken to determine cell count and viability by trypan blue exclusion in a haemocytometer. Then the cells (10^5 live cells / ml) were seeded in sealed sterile universal bottles containing complete medium. The tissue culture bottles were tightly closed and the suspension culture was incubated at 37ºC for 24 hours with occasional shaking. At the end of 24 hours incubation, the cells were treated with the oxidant, in the presence and the absence of the flower extract. The exposure of etoposide was given for 24 hours at 37ºC. The treated cells were harvested by centrifugation at 2000 rpm for 5 minutes, washed thrice with PBS and finally suspended in PBS. The cell suspension was then used for the viability assays and staining analysis.

3.6. CULTURING OF KB CELL LINE

The cell line was procured from National Centre for Cell Science, Pune, India. The cells were maintained in a CO_2 incubator with 5% CO_2 and 95% humidity, supplemented with DMEM and 10% FBS. Penicillin and streptomycin (PAA) were also added to the medium to 1X final concentration from a 100X stock. Once the cells had attained confluent growth, the cells were trypsinized using Trypsin-EDTA (PAA) and 10^5 cells required for various assays were seeded into sterile 6-well and 96-well plates. A sterile coverslip was placed in each well of the 6-well plate before the cells were seeded. The plates were then incubated in a CO_2 incubator with 5% CO_2 and 95% humidity atmosphere.

The cells were treated with the oxidant, in the presence and the absence of the flower extract. The exposure of etoposide was given for 24 hours at 37ºC. The time point was arrived at by conducting a time related response analysis of each cell type. After treatment, the medium in the 96-well plates was replaced with fresh medium and were used for cell viability assays. For staining analysis, the coverslips from the 6-well plates were removed and placed on a glass slide, sealed with Vaseline and were used for various staining techniques.

3.7. EVALUATING THE EFFECT OF *C. pulcherrima* FLOWER EXTRACT ON OXIDATIVE STRESS-INDUCED APOPTOSIS

Apoptosis, the programmed cell death, is characterized by distinct morphological and biochemical features. Most chemotherapeutic drugs exert their anti-tumour effects by inducing apoptosis. It is important to examine the process of apoptosis in disease conditions, which enables the researchers to understand the pathogenesis of a disease and explore the ways for the treatment (Wong, 2011).

In order to determine the effects of the flower extracts in transformed and non-transformed cells, the influence of *C. pulcherrima* flower extracts on oxidative stress induced apoptotic events were analysed in the yeast cells, peripheral lymphocytes and cancer cells. The apoptotic events that occurred in the treated cells were analysed by cytotoxic assays, morphological and nuclear staining techniques. The extent of DNA damage caused by oxidative stress was determined by diphenylamine assay in yeast cells and by single cell gel electrophoresis (comet) in peripheral blood lymphocytes and cancer cells.

3.7.1. Cell Viability and Cytotoxicity Assays

i) MTT Dye Reduction Assay

The MTT [3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide] reduction assay was used to evaluate the cell viability as described by Igarashi and Miyazawa (2001).

Principle

Metabolically active cells convert MTT into purple coloured formazan crystals, which are insoluble in aqueous solutions. Acidified isopropanol is used to dissolve the crystals and the resulting purple solution is measured spectrophotometrically.

Reagents

1. PBS
2. MTT (3mg/ml in PBS)
3. Acid-propanol (isopropanol containing 0.4% of 0.04N HCl)
4. HCl (0.04N)

Procedure

For *S. cerevisiae* cells, 100 µl of the treated cells was taken and 50 µl of MTT was added to it and incubated at 37ºC for 3 hours with mild shaking. After centrifugation, the cells were washed thrice with 20 µl of PBS followed by centrifugation at 10,000 rpm for 3 minutes. The supernatant was aspirated and 200 µl of 2-propanol containing 0.04N HCl was added to the cells and kept overnight in the dark. The absorbance was read at 650 nm in a microtitre plate reader (Anthos 2020, Austria).

In the case of lymphocyte culture, the cells were pelleted out by centrifugation of the cell suspension and for cancer cells, the medium was completely removed. The treated cells were incubated with 50µl of MTT at 37ºC for 3 hours with mild shaking. At the end of the incubation period, 200µl of PBS was added to all the samples and the liquid was carefully aspirated. Acid-propanol (200µl) was added and left overnight in dark. The absorbance was read at 650nm in a microtitre plate reader (Anthos 2020, Austria). The percent viabilities in the treated groups were calculated by fixing the optical density of the untreated control as 100 per cent viability.

ii) Sulphorhodamine B (SRB) Assay

The SRB assay explained by Skehan *et al.* (1990) was employed to determine the cell viability in the presence and the absence of flower extracts in the oxidant-treated cells.

Principle

SRB is used to determine cell density based on the measurement of cellular protein content. Under mild acidic conditions, sulphorhodamine B (SRB), a pink coloured aminoxanthane dye with two sulphonic groups, binds to the basic amino acid residues in the proteins of trichloroacetic acid-fixed cells. Under basic conditions, the bound dye was extracted from cells and solubilized for measurement. The amount of dye obtained from the stained cells is directly proportional to the cell number and cellular protein content.

Reagents

1. TCA (40%)
2. SRB (0.4% in 1% TCA)
3. Acetic acid (1%)
4. Tris (10mM, pH 10.5)
5. PBS

Procedure

For *S. cerevisiae* cells and lymphocyte culture, the treated cells were collected by centrifugation whereas for KB cell line, after the treatment, the medium was completely removed and washed with 200µl PBS to remove the traces of medium and serum. The treated cells were layered with 350µl of ice cold 40% TCA and were gently suspended in TCA followed by incubation at 4ºC for 1 hour. After incubation, the pellets were collected and washed 5 times with cold water. The excess water was drained off. SRB stain (350µl) was added to each tube and incubated for 30 minutes at room temperature. The cells were washed 4 times with 1 ml portions of 1% acetic acid. Then 350µl of 10mM tris (pH 10.5) was added to each tube to solubilize the dye. The pellets were shaken gently for 20 minutes on a gyratory shaker. The debris was spun down and the absorbance of the tris layer in each group was measured in a 96-well plate in a microtitre plate reader (Anthos 2020, Austria) at 492 nm. Cell survival was measured as the percentage absorbance compared to the control (untreated cells).

iii) Lactate Dehydrogenase Release

Principle

Lactate dehydrogenase (LDH), which is a soluble cytosolic enzyme present in most eukaryotic cells, releases into culture medium upon cell death due to damage of plasma membrane. The increase of the LDH activity in culture supernatant is proportional to the number of lysed cells. The LDH activity in the culture supernatant is measured by a substrate reaction and quantitated with an ELISA plate reader.

The Cytoscan™ LDH Assay Kit is a colorimetric method of assaying cellular cytotoxicity. The assay quantitatively measures LDH, which is released upon cell lysis. The released LDH is measured with a coupled enzymatic reaction that results in the conversion of a tetrazolium salt (INT) into a red coloured formazan. The LDH activity is determined as NADH oxidation or INT reduction over a defined time period. The resulting formazan absorbs maximally at 492nm and can be measured quantitatively at 490nm using a microplate reader or spectrophotometer.

Reagents

1. PBS
2. LDH assay kit (Cytoscan, India)

Procedure

The cells were treated with the methanolic extract of the *C. pulcherrima* flowers in the presence and the absence of the oxidant. For *S. cerevisiae* cells, the oxidative stress was induced by H_2O_2 treatment for 1 hour at 37°C whereas etoposide treatment for 24 hours was given for peripheral blood lymphocytes and KB cells. Similarly, a spontaneous control (medium alone) and maximum LDH release control (cells in medium lysed using lysis buffer) were also taken. After incubation, the cells were centrifuged and 50µl of the supernatant was taken into a new ELISA reader plate; 50µl of the reconstituted substrate mix (prepared by dissolving one vial of the provided substrate mix in 11.4ml water and adding 0.6ml of assay buffer provided with the kit) was added to each well and incubated at room temperature for 30 minutes. The reaction was stopped by adding 50µl of stop solution, the absorbance was recorded at 490nm and the per cent cytotoxicity was calculated as follows:

$$\text{Per cent Cytotoxicity} = \frac{\text{Experimental }(\text{OD}_{490}) - \text{Spontaneous }(\text{OD}_{490})}{\text{Maximum LDH release }(\text{OD}_{490})} \times 100$$

3.7.2 Morphological Changes of Apoptosis

The morphological changes of the cells were analysed by Giemsa staining using phase contrast microscope (Nikon, Japan) as explained by Chih *et al*. (2001).

Principle

Giemsa stain is used to differentiate nuclear and/or cytoplasmic morphology of cells. During apoptosis, the cells undergo a series of well-documented morphological changes such as loss of focal adhesions, cell membrane blebbing and decreased total cell volume, which can be observed after staining with Giemsa stain.

Reagents

1. Phosphate buffered saline (PBS), pH 7.4
2. Liquid Giemsa stain (1:2 dilution in PBS)

Procedure

The treated cells were stained with diluted Giemsa stain (10µl) and were observed under a phase contrast microscope (Nikon, Japan) at 400X magnification. The numbers of cells showing apoptotic morphological changes were counted in each experimental group per 100 cells in three different fields. The apoptotic ratio was calculated using the formula,

$$\text{Apoptotic ratio} = \frac{\text{Number of apoptotic cells}}{\text{Number of normal cells}}$$

3.7.3. Nuclear Changes of Apoptosis

The nuclear events occurring during apoptosis include changes at the morphological level such as chromatin condensation, nuclear shrinkage, DNA fragmentation and apoptotic body formation. The nuclear changes that occur during apoptosis are condensation of nuclear content into clumps of heterochromatin at the nuclear periphery, nuclear fragmentation and packaging of the nuclear fragments into membrane enclosed apoptotic bodies. The nuclear changes during apoptosis were studied in the presence and the absence of flower extract and/or oxidant by propidium iodide (PI), ethidium bromide (EtBr), DAPI and acridine orange/ethidium bromide (AO/EtBr) staining.

i) Propidium Iodide Staining

The method proposed by Sarker *et al.* (2000) was employed.

Principle

Apart from regular nuclear changes during apoptosis, like DNA fragmentation, the loss of nuclear DNA content can be analysed using a fluorochrome such as propidium iodide that intercalates into nucleic acid and stains it.

Reagents

1. PBS
2. Acetone:methanol (1:1)
3. Propidium iodide (5µg/ml in PBS)

Procedure

The treated cells were permeabilised with 50µl of acetone:methanol (1:1) mixture and incubated at -20ºC for 10 minutes. The slides were taken out and dried at room temperature. PI (10µl) stain was added to each slide, spread with a coverslip and incubated at 37ºC for 30 minutes in the dark. The apoptotic cells were detected using G-2A filter of the fluorescence microscope (Nikon, Japan) at 400X magnification and for each treatment group, 100 cells were scored. The apoptotic ratio was calculated by the formula mentioned earlier.

ii) Ethidium Bromide Staining

The nuclear changes that occur during apoptosis were analysed by ethidium bromide staining based on the method proposed by Mercille and Massie (1994).

Principle

Ethidium bromide is a molecule that intercalates into nucleic acid and can be used to visualize the nuclear changes in apoptotic cells.

Reagents

1. PBS
2. Ethidium bromide (EtBr) (50µg/ml in PBS)

Procedure

Ten microlitres of EtBr solution was added to the treated cells in the slide and spread by placing a coverslip over it. The stained cells were incubated at room temperature for 5 minutes. The apoptotic cells were scored by counting the cells with condensed chromatin and fragmented nuclei under fluorescent microscope (Nikon, Japan) using G-2A filter at 400X magnification. The numbers of cells showing apoptotic morphological changes were counted in each experimental group per 100 cells in three different fields. The apoptotic ratio was calculated by the formula mentioned earlier.

iii) DAPI Staining

The oxidant-treated cells with or without the flower extracts and untreated control cells were scored for DAPI staining using an inverted fluorescent microscope as explained by Rashmi *et al*. (2003).

Principle

DAPI (4'-6-diamidino-2-phenylindole) a blue-fluorescent dye, stains dsDNA by binding with the AT clusters in the minor groove. When DAPI binds to dsDNA, a ~20-fold increase in the fluorescence is observed due to the displacement of water molecules from both DAPI and the minor groove. Thus, DAPI is used to distinguish apoptotic nuclei (intensely stained, fragmented nuclei and condensed chromatin) from normal nuclei. The presence of nuclear apoptotic bodies and chromatin migration can also be observed after DAPI staining.

Reagents

1. PBS
2. Paraformaldehyde (3%)
3. Triton X-100 (0.2% in PBS)
4. DAPI (1µg/ml in PBS)

Procedure

After treatment, the cells were fixed with 3% paraformaldehyde (50µl) for 10 minutes at room temperature. The fixed cells were permeabilised with 0.2% triton X-100 (50µl) for 10 minutes at room temperature and incubated for 3 minutes with 10µl of DAPI by placing a coverslip over the cells to enable uniform spreading of the stain. The cells were scored by counting the cells with condensed chromatin and fragmented nuclei using an inverted fluorescent microscope (Motic, Hong Kong) using DAPI filter at 400X magnification. The apoptotic ratio was calculated as given earlier.

iv) Acridine orange/ethidium bromide staining

Acridine orange/ethidium bromide (AO/EtBr) staining is used to evaluate the nuclear morphology of apoptotic cells based on the method described by Parks *et al*. (1979).

Principle

AO/EtBr staining is used to discriminate the live from dead cells on the basis of membrane integrity. Acridine orange is a vital dye that will stain both live and dead cells, whereas ethidium bromide will stain only those cells that have lost their membrane integrity.

Reagents

1. PBS
2. Stock staining (100X): AO (15mg) + EtBr (15mg) dissolved in 1ml of 95% ethanol. For working solution, 1ml of 100X stock solution was diluted 100 times with PBS.

Procedure

Ten microlitres of AO/EtBr was added to the treated cells in cover slips, and spread by placing a cover slip over it. The stained slides were incubated for 5 minutes at room temperature. The apoptotic cells with condensed chromatin and fragmented nuclei were identified by the red fluorescence whereas the normal cells were identified by the green fluorescence. The apoptotic cells were counted using an upright fluorescent microscope (Nikon, Japan) at 400X magnification.

The results showed that the cell viability of the untransformed cells (peripheral blood lymphocytes) was not reduced on treatment with flower extract alone. This has furthered the study to determine the effect of flower extract in the presence of a standard chemotherapeutic agent that induces apoptosis. The experimental groups and parameters analysed were similar to those of untransformed cells, as explained earlier.

3.8. EVALUATING THE EFFECT OF *C. pulcherrima* FLOWER EXTRACT ON OXIDATIVE DNA DAMAGE

The effect of the flower extracts on the extent of DNA damage caused by oxidative stress was evaluated using diphenylamine assay (DPA) in case of *S. cerevisiae* cells and by single cell gel electrophoresis (comet assay) for peripheral blood lymphocytes and KB cells.

i) Quantification of DNA fragmentation with diphenylamine assay

The per cent DNA fragmentation was assessed by the method proposed by Boraschi and Maurizi (1998).

Principle

The extensively fragmented dsDNA can be separated from the chromosomal DNA by centrifugal sedimentation and can be quantified colorimetrically upon reaction with diphenylamine (DPA), which binds to deoxyribose.

Reagents

1. TTE solution: TE buffer (10mM Tris and 10 mM EDTA) pH-7.4 with 0.2% Triton X-100.
2. 5% and 25% TCA

3. DPA reagent: 5g of fresh or recrystallized diphenylamine, 500 ml of glacial acetic acid and 13.75ml conc.H_2SO_4 were mixed together and stored in a brown bottle.

Procedure

Equal volume of cells were taken in tubes labelled B and treated with 200 µM H_2O_2 and/or the extracts of *C. pulcherrima* flowers for 1 hour at room temperature. After treatment, the tubes were centrifuged at 200g at 4°C for 10 minutes. The supernatant was transferred to a new tube labelled S. To the pellets in tubes labelled B, 1.0ml of TTE solution was added and vortexed to release the fragmented chromatin from the nuclei.

The tubes labelled B were then centrifuged at 20,000g for 10minutes at 4°C to separate the fragmented DNA from intact chromatin and the supernatants were carefully transferred to new tubes labelled T. To the small pellets in tubes labelled B, 1.0 ml of TTE solution was added. Then 1.0ml of 25% TCA was added to all the tubes labelled T, B and S and vortexed vigorously. The tubes were incubated overnight at 4°C to allow the precipitation. After the incubation, the precipitated DNA was recovered by pelleting for 10 minutes at 20,000g at 4°C. The supernatant was removed and the DNA in each pellet was hydrolysed by adding 160µl of 5% TCA and heating for 15 minutes at 90°C. The blank was prepared using 160µl of 5%TCA alone.

Freshly prepared DPA solution (320µl) was added to all tubes. The tubes were incubated for 4 hours at 37°C or overnight at room temperature for colour development. Aliquots (200µl) of the coloured solution from each tube was transferred to a 96-well microtiter plate and the absorbance was read at 600 nm using microtiter plate reader (Anthos 2020, Austria).

The per cent fraction of fragmented DNA was calculated using the formula:

$$\text{Per cent fragmentation } = \frac{\mathrm{T + S}}{\mathrm{T + S + B}}\ \mathrm{X}\ 100$$

Where T, S and B are the absorbance of the fragmented DNA at 600nm in the T, S and B fractions respectively.

The fragmented DNA released by the cells undergoing apoptosis and lysis is recovered in the fraction S. Since many substances present in the fraction could heavily interfere with O.D. measurement, the following formula was also applied:

$$\text{Per cent fragmentation } = \frac{\mathrm{T}}{\mathrm{T + B}}\ \mathrm{X}\ 100$$

ii) Single cell gel electrophoresis (comet assay)

Single cell gel electrophoresis under alkaline conditions was carried out as described by Singh *et al.* (1988). Prior to the assay, the cells were resuspended in Hank's Balanced Salt Solution (HBSS).

Principle

The alkaline (pH>13) single cell gel (SCG) electrophoresis assay, commonly known as comet assay, combines the simplicity of biochemical techniques for detecting DNA single strand breaks, alkali labile sites and crosslinking with the single cell approach typical of cytogenetic assays. This major advantage of this assay is that it is a highly sensitive assay to detect DNA damage.

Reagents

1. Low melting point agarose (LMPA – 0.5%)
2. Normal melting point agarose (NMPA – 1%)
3. Lysis solution (1M tris, pH 8.0, EDTA (0.5M), NaCl (2.5M), DMSO (10%), Triton X -100 (1%)) – The DMSO and triton X-100 were added fresh before use.
4. Neutralizing buffer (1M tris, pH 7.5)
5. Alkaline electrophoresis buffer (10N NaOH, 0.2M EDTA, pH >13)
6. EtBr (5µg/ml)
7. H_2O_2 (30%)
8. PBS (pH 7.4)

Procedure

After 24 hour treatment with etoposide, the lymphocyes were collected by centrifugation, washed with PBS and resuspended in 100µl PBS. For KB cells, after treatment, the medium was completely removed and the traces of medium and serum were removed by washing with PBS and finally the cells were resuspended in 100µl PBS. An aliquot (100µl) of the cell suspension was mixed with equal volumes of 0.5% molten LMPA and maintained at 37ºC. This suspension (100µl) was carefully layered onto glass slides coated with 1% NMPA and spread evenly using a coverslip (22 x 40mm). The slides were then immediately placed on slide trays held on ice in order to solidify the agarose. The coverslip was removed carefully and the cell spread was overlaid with a layer of LMPA without cells. After solidification, the 'mini gels' were kept immersed in Coplin jars filled with cold lysing solution and incubated overnight at 4ºC. The lysed cells were denatured in the alkaline electrophoresis buffer for 20 minutes and electrophoresed in the same buffer at 25V, 300mA for 20 minutes. After the electrophoresis, the gels were neutralized in Tris buffer for 10 minutes (pH 7.5) and stained with ethidium bromide solution. The slides were washed thrice with PBS or water to remove excess ethidium bromide and air-dried. The slides were then scored for the presence of comet ‘tails’ under oil immersion in a fluorescent microscope (Nikon, Japan). A total of 100 cells per slide were scored and the frequency of DNA damage was represented as the number of comets per 100 cells.

3.9. IMMUNOCYTOCHEMISTRY ASSAY OF TP53, BAX AND BCL-2 PROTEINS

The expression of proteins involved in apoptosis namely TP53, Bcl-2 and Bax were analysed using immunocytochemistry. TP53 is a transcription factor important for both regulating the cell cycle progression and the induction of apoptosis in a variety of cells (Azizi *et al.*, 2009). The Bcl-2 (B-cell lymphoma/leukemia-2) family that constitutes both pro-apoptotic proteins (Bad, Bak, and Bax) and antiapoptotic proteins (Bcl-2, Bcl-xL, Bcl-B, Bcl-W, Bfl-1, Mcl-1) plays an important role in the regulation of apoptosis (Placzek *et al.*, 2010).

Principle

Immunocytochemistry is an *in situ* identification method of cell constituents by means of a specific antigen-antibody interaction, where the antibody has been tagged with a visible label such as a fluorophore or an enzyme.

Reagents

1. Gelatin (0.1%)
2. Paraformaldehyde (4% in PBS)
3. PBT (PBS + 0.02% Tween20)
4. DAPI (4 %/ml)
5. Primary antibodies (Bcl-2, Bax, TP53)
6. FITC labeled secondary antibody
7. 10% goat serum

Procedure

Gelatin (0.1%) was added to sterile coverslips placed inside 6-well plates and incubated for 30 minutes. The gelatin was then discarded and cells were seeded onto the plates. The treatments were given according to the procedure mentioned earlier. The treated cells were then fixed with 4% paraformaldehyde (in PBS) for 30 minutes. Then the cells were washed thrice with PBT for 10 minutes each. The cells were treated with 0.5% Triton-X100 (in PBS) for 15 minutes and were washed three times with PBT for 10 minutes each. Goat serum (200µl, 10% in PBT) was added and incubated for 30 minutes followed by incubation with 200µl of primary Ab (1:100 -1:10,000, in PBS + 1% BSA) at 4°C overnight. After overnight incubation, the cells were washed six times with PBT for 10 minutes each followed by incubation with 200µl of secondary Ab (1:100~1:500, in PBS + 1% BSA) at 37°C for 1 hour. After washing with PBT, the cells were incubated with 200 µl of DAPI for 10-15 minutes. The excess stain was removed by washing with PBT. Antifading solution (5µl) was added onto clean glass slide and the coverslip with cells was mounted facing towards the slide. The expression of proteins in the cells was observed under 400X magnification in a fluorescent microscope.

3.10. CELL CYCLE ANALYSIS BY FLOW CYTOMETRY

In order to determine at which phase of cell cycle, the plant extract induces cell cycle arrest of cancer cells, cell cycle analysis using flow cytometry was performed as previously described by Krishan (1975).

Cells treated with methanolic extract of *C. pulcherrima* flowers were harvested and washed with PBS. The cells were then fixed in 70% ethanol, and kept at -20°C until analysis. Cells were then stained with propidium iodide (50µg/ml) and incubated for 30 minutes at room temperature in dark. The DNA content of the stained cells (1×10^6 cells/ml) were analysed by flow cytometry. The population of sub-G0, G0/G1, S, and G2/M were quantitated using CellQuest Software with the FACS Calibur flow cytometry (Becton Dickinson, USA) and expressed as percentage of the cells in each phase.

PHASE IV

Molecules derived from natural sources (so-called natural products) especially from plants play an important role in the discovery of leads for the development of conventional drugs for the treatment of most human diseases. There are two distinct types of biomedical research that seek to develop this potential in plants. One type of research explores the value of medicinal plants as traditionally used to determine which plants are most potent, optimize dosages and dose forms, and identify safety risks. Another type of research uses bioassays to identify single novel biologically active molecules from plants that might be useful lead compounds for the development of new pharmaceutical drugs.

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

3.11. PRELIMINARY PHYTOCHEMICAL SCREENING

The methanolic extract of the flowers of *C. pulcherrima* were screened for the presence of phytochemicals according to the method of Khandelwal (2002).

3.11.1. Detection of Alkaloids

a) **Mayer's test:** A fraction of the extract was treated with Mayer's reagent (1.36g of mercuric chlorate and 5g of potassium iodide in 100ml distilled water) and noted for a cream coloured precipitate.

b) **Dragendroff's test:** A fraction of the extract was treated with Dragendroff's reagent and observed for the formation of reddish orange precipitate.

c) **Wagner's test:** A fraction of the extract was treated with Wagner's reagent (1.27g of iodine and 2g of potassium iodide in 100ml of distilled water) and observed for the formation of reddish brown precipitate.

3.11.2. Detection of Phenolics

a) **Ferric chloride test:** A fraction of the extract was treated with 5% $FeCl_3$ solution and observed for the formation of deep blue colour.

b) **Lead acetate test:** A fraction of the extract was treated with 10% lead acetate solution and observed for the formation of white precipitate.

3.11.3. Detection of Flavonoids

a) **Aqueous NaOH test:** To a fraction of the extract, 1N aqueous NaOH was added and observed for the formation of yellow-orange colour.

b) **Concentrated H_2SO_4 test:** To a small fraction of the extract, concentrated H_2SO_4 was added and observed for the formation of orange colour.

c) **Schinodo's test:** To a small fraction of the extract, a piece of magnesium turning was added, followed by concentrated HCl, heated slightly and the formation of dark pink colour was observed.

3.11.4. Detection of Saponins

a) **Foam test:** A fraction of the extract was vigorously shaken with water and observed for persistent foam.

b) **Haemolytic test**: A fraction of the extract was added with a drop of blood placed on a glass slide and observed for the haemolytic zone.

3.11.5. Test for Steroids

Liebermann-Buchard Test: To a fraction of extract, 2 ml of chloroform, followed by 10 drops of acetic anhydride and 2 drops of concentrated sulphuric acid were added. The appearance of rose red colour, which quickly changes from blue to green, indicated the presence of steroid**.**

3.11.6. Test for Tannins

Braemer's test: To a fraction of extract, a few drops of 10% ferric chloride was added. A dark green, blue or brown colour was observed, indicating the presence of tannins.

3.11.7. Test for Terpenoids

Salkowski Test: A fraction of extract was dissolved in chloroform and shaken well with an equal volume of concentrated sulphuric acid. The appearance of red colour, in the chloroform layer and green fluorescence in the acid layer indicated the presence of steroid.

3.12. EXTRACTION OF THE PHYTOCHEMICAL FRACTIONS

The extraction procedures followed for alkaloid, phenol, flavonoid, saponin and terpenoid was proposed by Harborne (1973). The methodology of extraction followed for steroids and tannins were given by Vitale *et al*. (1995) and Obdoni and Ochuko (2001) respectively.

3.12.1. Total Alkaloid Fraction

Fresh flowers of *C. pulcherrima* (5g) were extracted with 20ml of ethanol:28% NH_4OH, (95:5) at room temperature overnight. The extract was filtered and concentrated under reduced pressure to a fuzzy residue, which was extracted twice with 1N HCl (10ml each) and filtered. Alkaloids were liberated at pH 9.8 by the addition of 0.7M Na_2CO_3. The solution was extracted with methylene chloride (3×5ml). The organic extract was dried over anhydrous sodium sulphate to yield the total alkaloid fraction.

3.12.2. Total Phenol Fraction

Fresh flowers (1g) were crushed using a mortar and pestle and extracted with 20ml of 80% ethanol at 80°C for 15 minutes. The flowers were extracted repeatedly till a clarified extract was got.

3.12.3. Flavonoid Fraction

The phenolic extract was further extracted with petroleum ether (3×5ml), when the flavonoids were present in the aqueous fraction.

3.12.4. Saponin Fraction

Fresh flowers (20g) were crushed, transferred to a conical flask, and 200ml of 20% aqueous ethanol was added. The sample was heated over a hot water bath for 4 hours with continuous stirring at 55°C. The mixture was filtered and re-extracted with another 200ml of 20% ethanol. The combined extracts were reduced to 40ml over a water bath at about 90°C. The concentrate was transferred into a 250ml separating funnel, 20ml of diethyl ether was added and shaken vigorously. The aqueous layer was recovered and the ether layer was discarded. The extraction was repeated twice with the addition of n-butanol. The combined n-butanol extract was washed twice with 10ml of 5% NaCl. The remaining solution was heated in a water bath, evaporated and dried in an oven.

3.12.5. Steroid Fraction

Fresh flowers of about 2g was weighed and added to 10ml of methanol. It was kept in a water bath for 15 minutes. The mixture was filtered, condensed and used.

3.12.6. Tannin Fraction

The plant material was suspended in methanol and allowed to stand overnight. It was refluxed for 4 hours, then filtered and the residue was washed with methanol. The filtrate was allowed to cool down, observed for any modification and an aliquot of this was used to assay tannins.

3.12.7. Terpenoid Fraction

The plant material was suspended in petroleum ether and filtered. The filtrate was condensed and an aliquot of the filtrate was used for the assay.

All the seven phytochemical fractions of the three flowers were tested for their radical quenching potential against a battery of free radicals namely DPPH, ABTS, superoxide, nitric oxide, hydroxyl and hydrogen peroxide by the methods explained in phase I of this chapter. Also, these phytochemical fractions were also subjected to TLC analysis.

3.13. SPECTRAL ANALYSIS

3.13.1. UV Absorption Spectral Analysis

A preliminary absorption spectral analysis was done by a survey scan of the methanolic extract of the three flowers of *C. pulcherrima* in a nanospectrophotometer (Optizen, Korea). The instrument was set to scan mode and the absorption spectrum was obtained in the range of 190nm to 420nm.

3.13.2. TLC of the Phytochemical Fractions

Aliquots (3µl) of the seven extracted fractions of the three flowers were spotted onto the Thin Layer Chromatography silica gel G60 F_{254} plates (EMerck) and subjected to separation using different solvent mixtures specific for the components, as given below.

i) Alkaloids

Mobile phase: Ethyl acetate:methanol:water (10:1.35:1)

Spray reagent: Dragendorff's reagent followed by 10% ethanolic sulphuric acid reagent

ii) Phenolics

Mobile phase: Toluene: acetone: formic acid (4.5:4.5:1)

Spray reagent: 20% sodium carbonate solution followed by Folin–Ciocalteu reagent

iii) Flavonoids

Mobile phase: Ethyl acetate: formic acid: glacial acetic acid: water (10:1.1:1.1:2.6)

Spray reagent: 1% ethanolic aluminium chloride reagent

iv) Saponins

Mobile phase: Chloroform: glacial acetic acid: methanol: water (6.4:3.2:1.2:0.8)

Spray reagent: Anisaldehyde sulphuric acid reagent

v) Steroids

Mobile phase: Toluene: methanol (9:1)

Spray reagent: Anisaldehyde sulphuric acid reagent

vi) Tannins

Mobile phase: Toluene: ethyl acetate: formic acid: methanol (3:3:0.8:0.2)

Spray reagent: 5% ferric chloride reagent

vii) Terpenoids

Mobile phase: n-hexane: ethyl acetate (7.2:2.9)

Spray reagent: Anisaldehyde sulphuric acid reagent

The R_f values of the spots were calculated as the ratio of the distance traveled by the solute to that by the solvent front.

3.13.3. HPTLC Analysis

i) Samples

The test solution and the standard solution (10 µl each) were loaded as 6 mm band length in the 3 x 10 Silica gel 60 F_{254}TLC plate using Hamilton syringe of CAMAG LINOMAT 5 instrument.

ii) Spot Development

The sample loaded plate was kept in the HPTLC twin trough developing chamber (after saturation with solvent vapour) with the respective mobile phase (given below) and the plate was developed up to 90 mm.

iii) Photo-documentation

The developed plate was dried by hot air to evaporate the solvents from the plate. The plate was kept in the photo-documentation chamber (CAMAG REPROSTAR 3) and the images were captured at white light, UV 254 nm and UV 366 nm.

iv) Derivatization

The developed plate was sprayed with the respective spray reagent and dried at 100°C in a hot air oven. The plate was photo-documented at daylight, UV 254 nm and UV 366 nm using photo-documentation (CAMAG REPROSTAR 3) chamber.

v) Scanning

Before derivatization, the plate was fixed the in the scanner stage and scanning was done at 254 nm. The peak table, peak display and peak densitograms were noted.

a) HPTLC Profile of Alkaloids

The mobile phase used was ethylacetate: methanol : water (10:1.35:1). The developed plates were sprayed with Dragendroff's reagent, followed by ethanol sulphuric acid. Then the plates were heated at 120°C for 5 minutes in a hot air oven. Nicotine was used as the reference standard and the presence of alkaloids was confirmed by the appearance of bright orange coloured zones in the daylight mode.

b) HPTLC Profile of Phenolics

The mobile phase used was toluene:chloroform:acetone (4:2.5:3.5). After development, the plate was sprayed with 25% aqueous Folin-Ciocalteau reagent and heated at 120°C for 5 minutes in a hot air oven. Quercetin was used as the reference standard for the analysis of phenolics. The presence of phenolics was confirmed by the appearance of blue or blue-grey coloured zones at daylight.

c) HPTLC Profile of Flavonoids

The mobile phase used was ethylacetate:butanone:formic acid:water in the ratio of 5:3:1:1. The plate was sprayed with 1% ethanol aluminium chloride reagent and heated at 120°C for 5 minutes in a hot air oven. Rutin was used as the reference standard for flavonoid analysis. The presence of flavonoids was confirmed by the appearance of yellow and yellow-green fluorescence at UV 366nm.

d) HPTLC Profile of Saponins

The mobile phase used was chloroform:acetic acid:methanol:water in the ratio of 6.4:3.2:1.2:0.8. The plate was sprayed with anisaldehyde sulphuric acid reagent and dried at 110°C for 3 minutes in a hot air oven. Saponin was used as the reference standard. The presence of saponins was confirmed by the appearance of blue or yellowish brown coloured zones in visible light.

e) HPTLC Profile of Steroids

Ethyl acetate:methanol:glacial acetic acid:water (10:2.2:1.1:2.6) was used as the mobile phase. The plate was sprayed with anisaldehyde sulphuric acid reagent and dried at 110ºC for 3 minutes in a hot air oven. Solasodine was used as the reference standard. The presence of steroids was confirmed by the appearance of blue-violet coloured zones in visible day light.

f) HPTLC Profile of Tannins

Isobutanol:acetic acid:water (3:1:1) was used as the mobile phase. The plate was sprayed with 5% ferric chloride reagent and dried at 100ºC for 3 minutes in a hot air oven. Tannic acid was used as the reference standard. The presence of tannins was confirmed by the appearance of bluish brown coloured zones in visible light.

g) HPTLC Profile of Terpenoids

n-Hexane-ethyl acetate (7.2:2.9) was used as the mobile phase. The plate was sprayed with anisaldehyde sulphuric acid reagent and dried at 100ºC for 3 minutes in a hot air oven. Artemisinin was used as the reference standard. The presence of terpenoids was confirmed by the appearance of blue, violet coloured zones in visible light.

3.13.4. HPLC Analysis

The residue of the methanolic extract of *C. pulcherrima* flowers was dissolved in an appropriate volume of HPLC grade methanol and 20 µl of the sample was injected into the reverse phase C18 column of the HPLC (Sigma-Aldrich equipped with UV detector). The sample analysis was performed at room temperature, in the wavelength range of 210-440nm at 1000 psi and the mobile phase used was methanol and water in the ratio of 50:50 with a run time of 35 minutes at a flow rate of 1ml/minute.

3.12.5. FT–IR Spectral Analysis

Infrared light from a suitable source passes through a scanning Michelson inferometer and Fourier Transformation gives a plot of intensity versus frequency. When a powdered plant sample is placed in the beam, it absorbs particular frequencies, so that their intensities are reduced in the inferogram and the ensuing Fourier transform is the infrared absorption spectrum of the sample.

3.13.6. GC–MS Spectral Analysis

The powdered plant material was analyzed using a Shimadzu Gas chromatography apparatus (Model QP 5000 GC-MS) using a DB-S capillary column (30m) equipped with QP MS detector (EI, 70 ev) with helium as a carrier gas at a flow rate of 1ml/minute. The compounds were identified using the WILEY database available in the software provided.

3.14. STATISTICAL ANALYSIS

The parameters analysed in all the phases of the study were subjected to statistical analysis using SigmaStat (Version 3.1) statistical software. Statistical significance was determined by one-way analysis of variance for parameters in phase I. For phase II and III, one-way ANOVA, followed by post-hoc Fischer analysis was used and the values with $P<0.05$ were considered to be significantly different. The results obtained for all the various parameters analysed in all the four phases and the salient observations made during the study are presented in the next chapter.