4. RESULTS

The products of normal cellular metabolism namely reactive oxygen species (ROS) and reactive nitrogen species (RNS) are physiologically beneficial at lower concentrations. When their concentration increases, they become toxic to the cell and cause oxidative damage to all major cellular constituents (membrane lipids, proteins, enzymes and DNA). Excessive or sustained production of reactive species has been implicated in the pathogenesis of chronic inflammatory conditions, atherosclerosis, malignant neoplasms, diabetes mellitus and various neurodegenerative diseases (Valavanidis *et al*., 2013).

Cells have evolved various defence systems against excessive free radicals such as preventive mechanisms, repair mechanisms, physical defences and antioxidant defences. Antioxidants protect the cells from oxidative damage, such as lipid peroxidation, protein oxidation and oxidative DNA damage (mutations), either by scavenging or by neutralizing the free radicals and their reactive intermediates (Sena and Chandel, 2012).

The necessity for the substitution of synthetic antioxidants, which might possess toxic and mutagenic effects, with natural antioxidants has increased recently (Cilerdzic *et al*., 2013). In the past two decades, the scientific evidences about the beneficial health implications of the antioxidants from plants as therapeutic agents for several diseases caused due to oxidative stress have increased significantly (Veskoukis *et al*., 2012). Plants contain active components called phytochemicals, which are known to act as antioxidants. A considerable attention has been focused on exploring phytochemicals with medicinal properties, which can inhibit, reverse or retard diseases caused by oxidative stress, especially cancer (Nikolova, 2011).

Thus, in the recent years, the discovery of new plant species with antioxidant and anticancer properties has become more inevitable and many researchers have shown greater interest in identifying the natural antioxidants from different types of plants (Ashokkumar and Ramasamy, 2013). In the present study, the antioxidant and anticancer properties of the three different flowers (yellow, pink and orange) of the candidate plant *Caesalpinia pulcherrima* (Plate 4.1) were determined and the results obtained are presented in this chapter.

Plate 4.1

Three different flowers of *Caesalpinia pulcherrima*, Swartz.

Yellow

Pink

Orange

Phase I

In phase I, the radical scavenging activity of the three different flowers (yellow, pink and orange) of *C. pulcherrima* were determined *in vitro*. The biomolecule-protective effects of the flower extracts against lipid peroxidation, oxidative DNA damage and protein oxidation were also evaluated.

4.1. RADICAL SCAVENGING EFFECTS OF *C. pulcherrima* FLOWER EXTRACTS

The flowers were individually extracted into solvents of increasing polarity (petroleum ether, benzene, chloroform, ethyl acetate and methanol) under cold conditions. Aqueous extracts of the flowers were also prepared. The radical scavenging activity of these flower extracts were determined *in vitro* against a battery of radicals namely DPPH, ABTS, H_2O_2 and hydroxyl radicals.

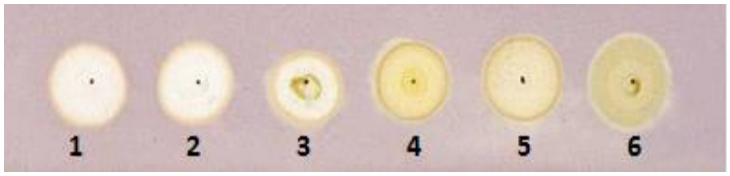
4.1.1. DPPH Radical Scavenging Activity of *C. pulcherrima* Flower Extracts

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

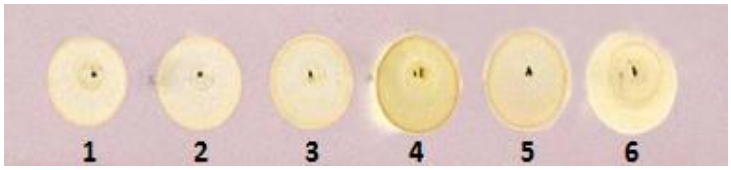
a) DPPH dot-blot assay

Plate 4.2

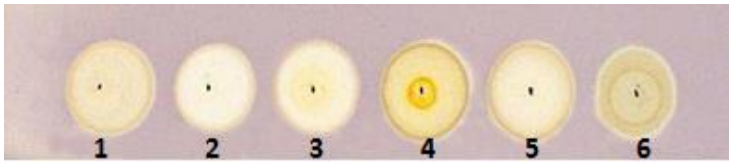
DPPH Dot Blot Assay



***C. pulcherrima* yellow flower**



***C. pulcherrima* pink flower**



***C. pulcherrima* orange flower**

1 - Petroleum ether
2 - Benzene
3 - Chloroform
4 - Ethyl acetate
5 - Methanol
6 - Water

The result obtained for the dot blot screening is shown in Plate 4.2, wherein all the six different extracts of the three flowers exhibited good DPPH radical scavenging activity. In all the three flowers, the scavenging activity was higher in extracts prepared in solvents with higher polarity (ethyl acetate, methanol and water).

b) DPPH spectrophotometric assay

In the spectrophotometric analysis, the extent of DPPH scavenging by all the extracts of the three flowers was significant, where the stable radical DPPH was effectively reduced to the yellow-coloured compound diphenylpicryl hydrazine. An increase in the DPPH scavenging activity of the flower extracts was found in solvents with increasing polarity (ethyl acetate, methanol and water). The results are presented in Figure 4.1.

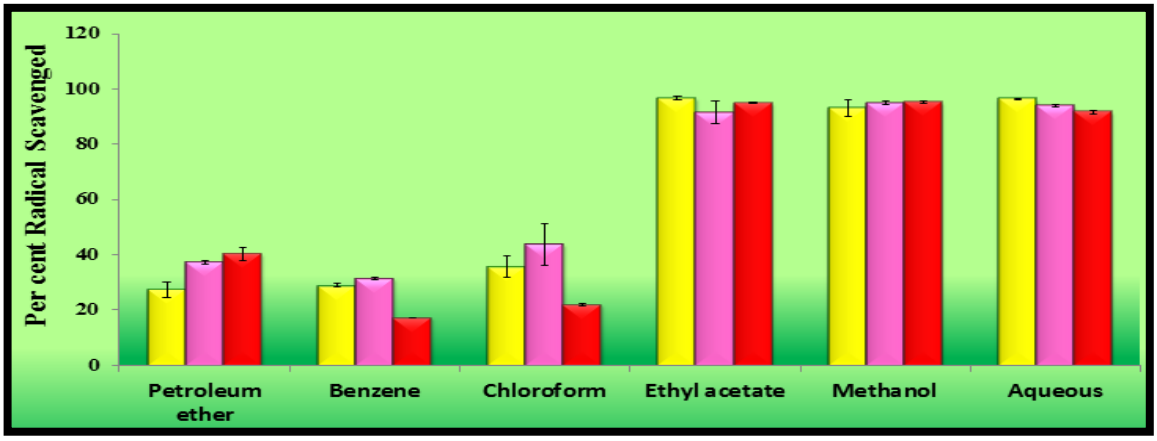
4.1.2. ABTS Radical Scavenging Activity of *C. pulcherrima* Flower Extracts

The antioxidant activity of the flower extracts were analysed using another type of stable free radical, namely ABTS, and the results obtained are shown in Figure 4.2. The different solvent extracts of all the three flowers effectively scavenged the ABTS free radicals. The extent of ABTS radical scavenging was maximum in the methanolic extract of all the three flowers followed by the ethyl acetate and aqueous extracts. The benzene extract of all the three flowers showed the lowest effect.

4.1.3. Hydrogen Peroxide Scavenging Activity of *C. pulcherrima* Flower Extracts

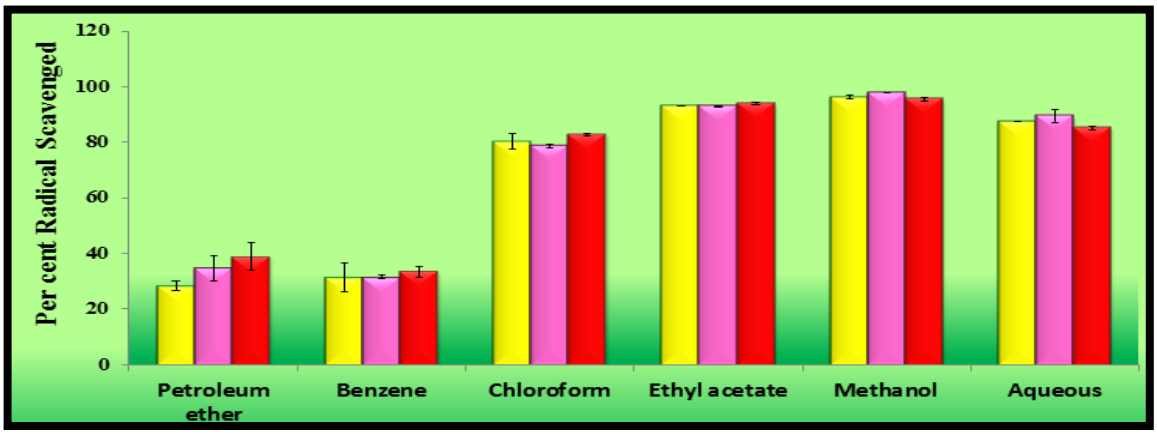
The ability of the *C. pulcherrima* flower extracts to scavenge H_2O_2 in an *in vitro* system was followed in the present study and the results are shown in Figure 4.3. All the different solvent extracts of the three flowers of *C. pulcherrima* elicited strong H_2O_2-scavenging activity. The extent of H_2O_2-scavenging varied with the different solvent extracts, among which the methanolic extract of all three flowers exhibited the maximum effect, followed by the petroleum ether and the aqueous extracts. The least scavenging activity was observed in the chloroform extracts.

FIGURE: 4.1
DPPH Scavenging Effects of *C. pulcherrima* Flowers



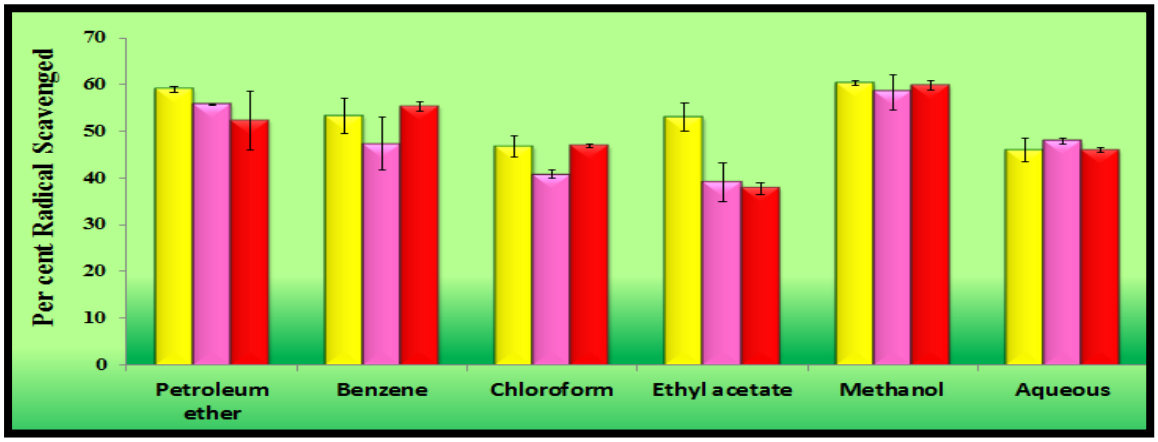
The values are Mean ± S.D. of triplicates

FIGURE: 4.2
ABTS Scavenging Effects of *C. pulcherrima* Flowers



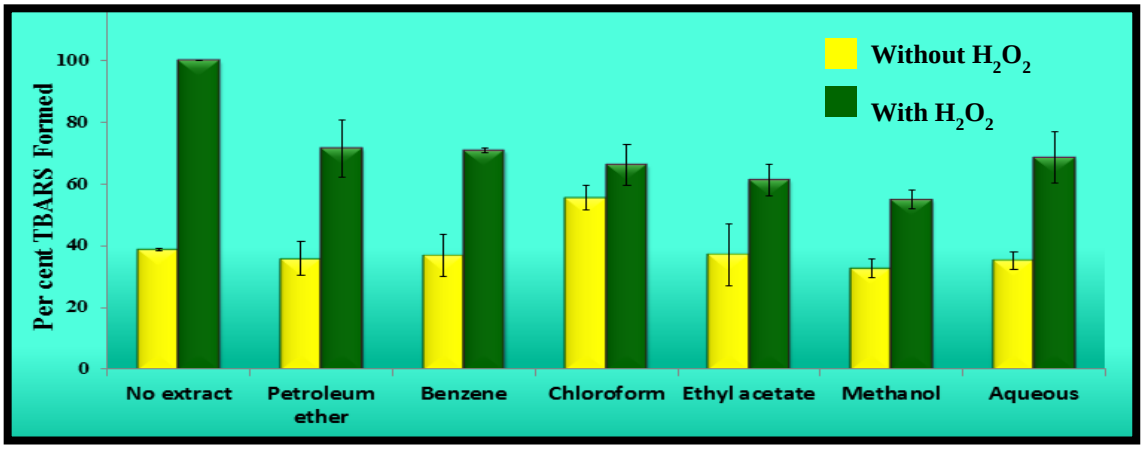
The values are Mean ± S.D. of triplicates

FIGURE: 4.3
H_2O_2 Scavenging Effects of *C. pulcherrima* Flowers



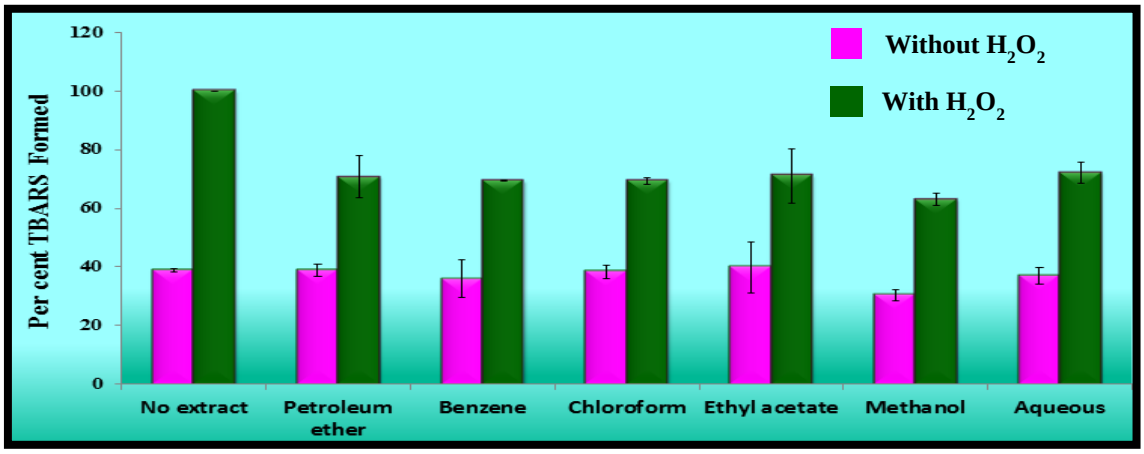
The values are Mean ± S.D. of triplicates

FIGURE 4.4
Hydroxyl Radical Scavenging Effects of the Yellow Flowers of *C. pulcherrima*



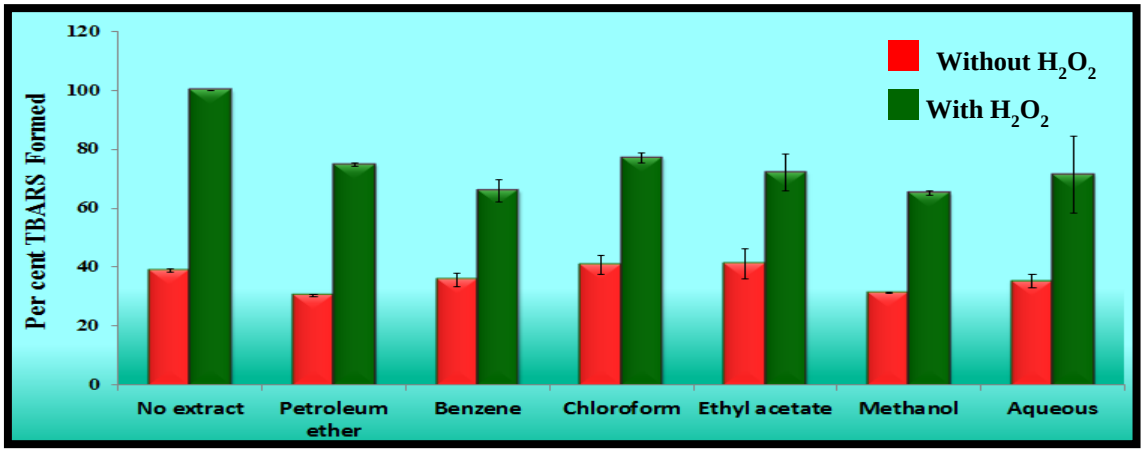
The values are Mean ± S.D. of triplicates

FIGURE 4.5
Hydroxyl Radical Scavenging Effects of the Pink Flowers of *C. pulcherrima*



The values are Mean ± S.D. of triplicates

FIGURE 4.6
Hydroxyl Radical Scavenging Effects of the Orange Flowers of *C. pulcherrima*



The values are Mean ± S.D. of triplicates

The values of H_2O_2-treated groups were fixed as 100 per cent and the relative values in percentage were calculated for the other groups

4.1.4. Hydroxyl Radical Scavenging Activity of *C. pulcherrima* Flower Extracts

Hydroxyl radical is the most reactive oxygen radical known, with a highly positive reduction potential of +2310 mV. It has a very short half-life (~10^{-9}s) and reacts very rapidly with all types of molecules found in living cells (Berg *et al*., 2011). In this assay, the extent of TBARS formed from the oxidative reaction of the substrate 2'-deoxyribose was taken as a measure of the hydroxyl radical formation. Thus, hydroxyl radical scavenging activity of the flowers was determined from the extent of inhibition of TBARS production.

H_2O_2 exposure significantly increased the TBARS formation, which was effectively counteracted by the presence of the flower extracts. The methanolic extract of all the three flowers exhibited the maximum extent of radical scavenging activity (Figures 4.4 – 4.6), followed by the aqueous extracts. All the other solvent extracts also showed good H_2O_2 scavenging activity.

The results of the above assays revealed that the methanolic extract of all the three flowers exhibited the maximum scavenging activity for all the radicals tested, compared to all the other extracts. Therefore, the methanolic extract alone was chosen for further analysis. The minimum concentration of the methanolic extract that exhibited the maximum radical scavenging response was determined, by conducting a dose-response study using concentrations ranging from 0.01 mg to 1.0 mg.

TABLE 4.1

DPPH RADICAL SCAVENGING EFFECTS OF *C. pulcherrima* FLOWER EXTRACTS FOR DOSE OPTIMIZATION

Flower extract (mg)	Percent DPPH Radical Scavenging		
	Yellow	Pink	Orange
0.01	42.68 ± 2.61	32.82 ± 9.16	43.86 ± 8.36
0.05	67.13 ± 3.54	77.45 ± 3.16	72.72 ± 1.83
0.10	78.78 ± 3.45	80.21 ± 2.96	79.32 ± 3.91
0.25	79.99 ± 2.96	82.87 ± 1.67	84.23 ± 1.78
0.50	93.48 ± 0.88	94.70 ± 0.07	93.26 ± 0.88
0.75	94.69 ± 0.70	94.80 ± 1.17	93.16 ± 0.51
1.0	93.25 ± 1.51	94.93 ± 1.16	94.47 ± 1.02

The values are Mean ± S.D. of triplicates

TABLE 4.2

ABTS RADICAL SCAVENGING EFFECTS OF *C. pulcherrima* FLOWER EXTRACTS FOR DOSE OPTIMIZATION

Flower extract (mg)	Percent ABTS Radical Scavenging		
	Yellow	Pink	Orange
0.01	20.96 ± 0.15	21.76 ± 0.57	18.20 ± 0.36
0.05	23.86 ± 0.43	22.87 ± 1.18	22.36 ± 0.01
0.10	41.82 ± 1.59	46.20 ± 2.26	44.12 ± 4.27
0.25	53.28 ± 1.58	54.73 ± 0.93	50.26 ± 0.05
0.50	73.61 ± 0.94	81.27 ± 1.94	76.09 ± 0.74
0.75	92.35 ± 0.52	97.76 ± 0.20	89.99 ± 1.36
1.0	94.66 ± 0.24	96.70 ± 0.08	93.56 ± 0.44

The values are Mean ± S.D. of triplicates

TABLE 4.3

H_2O_2 RADICAL SCAVENGING EFFECTS OF *C. pulcherrima* FLOWER EXTRACTS FOR DOSE OPTIMIZATION

Flower extract (mg)	Percent H_2O_2 Radical Scavenging		
	Yellow	Pink	Orange
0.01	45.15 ± 2.63	45.49 ± 0.69	47.30 ± 2.82
0.05	46.81 ± 4.90	47.85 ± 1.12	47.44 ± 0.47
0.10	52.34 ± 0.39	48.04 ± 3.13	54.34 ± 1.29
0.25	50.34 ± 2.17	53.98 ± 3.39	51.98 ± 2.88
0.50	48.44 ± 4.31	52.41 ± 0.89	52.31 ± 2.65
0.75	51.49 ± 0.88	52.30 ± 2.10	53.82 ± 1.48
1.0	50.46 ± 1.64	52.65 ± 0.82	53.22 ± 0.33

The values are Mean ± S.D. of triplicates

The dose was optimized using free radical scavenging assays (DPPH, ABTS, hydrogen peroxide and hydroxyl), in which different concentrations (0.01 mg to 1.0 mg) of the methanolic extracts were compared. The results obtained are depicted in Tables 4.1 – 4.4.

TABLE 4.4

HYDROXYL RADICAL SCAVENGING EFFECTS OF THE *C. pulcherrima* FLOWER EXTRACTS FOR DOSE OPTIMIZATION

Flower extract (mg)	Percent TBARS formed					
	Yellow		Pink		Orange	
	Without H_2O_2	With H_2O_2	Without H_2O_2	With H_2O_2	Without H_2O_2	With H_2O_2
No extract	16.76 ± 0.13	100	16.76 ± 0.13	100	16.76 ± 0.13	100
0.01	20.48 ± 1.23	51.97 ± 2.78	19.92 ± 0.44	53.78 ± 2.97	20.19 ± 4.72	57.15 ± 1.80
0.05	19.99 ± 1.92	48.81 ± 2.48	20.41 ± 0.26	52.38 ± 0.60	19.21 ± 3.33	43.62 ± 3.46
0.10	17.25 ± 0.56	45.58 ± 0.69	19.21 ± 3.33	50.91 ± 2.68	17.74 ± 1.25	38.15 ± 1.51
0.25	19.28 ± 1.85	48.74 ± 0.99	19.28 ± 1.85	47.83 ± 3.86	18.23 ± 1.95	38.08 ± 3.00
0.50	17.88 ± 1.72	46.63 ± 0.79	20.48 ± 1.23	48.18 ± 0.19	19.84 ± 1.05	41.86 ± 0.41
0.75	17.81 ± 0.23	46.07 ± 0.01	19.77 ± 2.54	48.32 ± 3.17	20.33 ± 1.74	42.85 ± 1.80
1.0	17.74 ± 1.15	47.19 ± 1.58	20.47 ± 1.23	48.52 ± 3.47	20.48 ± 1.23	41.30 ± 1.20

The values are Mean ± S.D. of triplicates

The values of H_2O_2-treated groups were fixed as 100 per cent and the relative values in percentage were calculated for the other groups

The results showed that the extent of scavenging increased upto the dose of 100µg for all the three flowers, after which a plateau in their activity was observed. Therefore, further studies were carried out with the optimal dose of 100 µg of the cold methanolic extract of all the three flowers.

4.2. EFFECT OF *C. pulcherrima* FLOWERS ON OXIDATIVE DAMAGE TO BIOMOLECULES

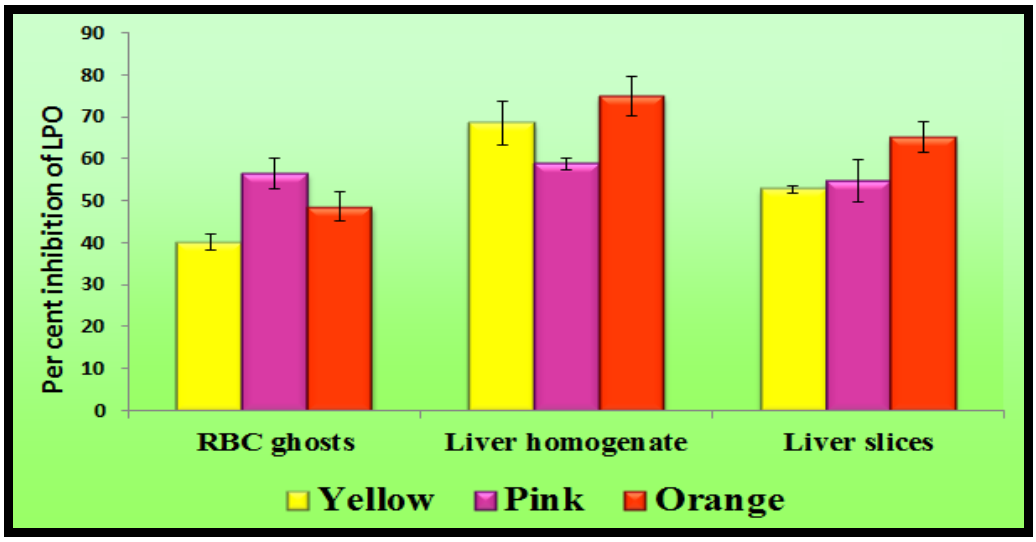
In the human body, all the biological molecules are susceptible to attack by free radicals. ROS can react with cellular components and cause damage to biomolecules such as lipids, proteins and DNA that leads to lipid peroxidation, protein modification and DNA oxidation. Such detrimental reactions cause alterations in the intrinsic membrane properties like fluidity, ion transport, loss of enzyme activity, protein cross-linking, inhibition of protein synthesis and DNA damage, all of which ultimately result in cell death (Sharma *et al*., 2012). Thus, it becomes essential to study the effects of the flower extracts on oxidant-induced damage to lipids, DNA and proteins.

4.2.1. Effects of the Flower Extracts of *C. pulcherrima* against *In vitro* Lipid Peroxidation

The biomolecule-protective effects of *C. pulcherrima* flower extracts against lipids were investigated first. Three different membrane models namely, goat RBC ghosts (plasma membrane lipids), goat liver homogenate (plasma membrane and intracellular lipids) and liver slices (intact cells) were used to assess the extent of lipid peroxidation and the protection rendered by the flower extracts against induced oxidative stress. The per cent inhibition of *in vitro* lipid peroxidation by the flower extracts in all the three membrane systems is presented in Figure 4.7.

The results obtained showed that all the three flowers substantially decreased the extent of lipid peroxidation in all the three membrane preparations. Pink flower extracts rendered better protection to plasma membrane lipids (RBC ghosts) and almost equal protection to intracellular lipids (liver homogenate) and intact cells (liver slices), whereas the orange flower extract rendered the maximum protection against lipid peroxidation in liver homogenate and slices. In the yellow flower extract treated groups, the maximum response was observed in goat liver homogenate, followed by liver slices and RBC ghosts.

FIGURE 4.7
Inhibition of Lipid Peroxidation by the Flower Extracts of *C. pulcherrima* in Different Membrane Preparations



The values are Mean ± S.D. of triplicates

4.2.2. Effects of the Flower Extracts of *C. pulcherrima* against Oxidative DNA Damage

In cells, DNA molecules are the primary targets for oxidative stress. Continuous oxidative damage of the DNA has been implicated in the pathophysiology of various diseases such as cancer, atherosclerosis and aging (Neofytou *et al*., 2012). The effect of the flowers of the candidate plant on oxidant induced DNA damage was assessed *in vitro* in commercially available DNA preparations. DNA from different hierarchies of evolutionary development were selected for the analysis, which included the commercially available preparations of viral DNA (λ DNA), bacterial plasmid (pUC18) and DNA of animal origin (herring sperm DNA). Hydrogen peroxide was used to induce oxidative stress in all the types of DNA both in the presence and the absence of the flower extract.

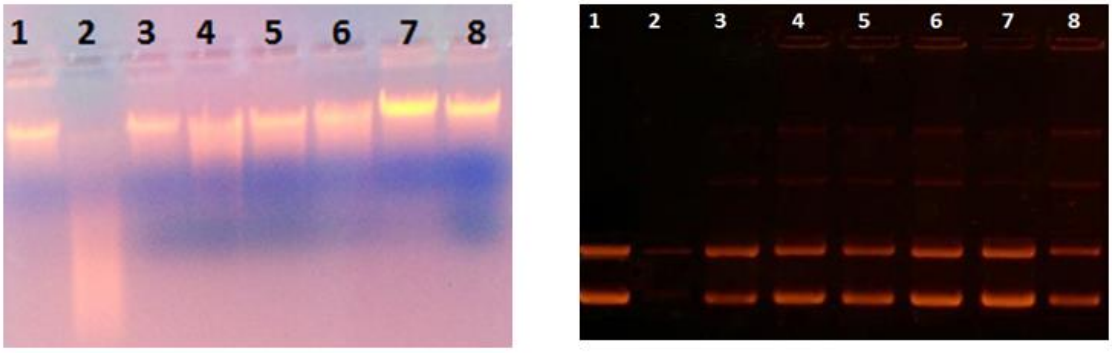
a) Protective Effects of the Flower Extracts of *C. pulcherrima* to λ DNA and pUC18 DNA

The extent of DNA damage in λ and pUC18 DNA was analysed using agarose gel electrophoresis in which the migration of DNA was observed. The results are presented in Plate 4.3. In both λ and pUC18 DNA, the absence of specific bands in lane 2, wherein the DNA was treated with oxidant alone indicated the significant damage induced by H_2O_2. The treatment with the flower extracts alone did not cause any

damage to λ and pUC18 DNA (Lanes 3, 5 and 7). The exposure to the oxidant in the presence of the flower extracts significantly inhibited the oxidant-induced damage of both λ and pUC18 DNA, which is evident from the intact DNA bands (Lanes 4, 6 and 8).

Plate 4.3

Migration patterns of λ DNA and pUC18 DNA Treated with H_2O_2 with and without *C. pulcherrima* Flower Extracts



(a) Lambda DNA **(b) pUC18 DNA**

Lane 1 : Untreated control
Lane 2: DNA + H_2O_2
Lane 3: DNA + CPY
Lane 4: DNA + CPY + H_2O_2
Lane 5: DNA + CPP
Lane 6: DNA + CPP + H_2O_2
Lane 7: DNA + CPO
Lane 8: DNA + CPO + H_2O_2

CPY – *C. pulcherrima* yellow flower
CPP – *C. pulcherrima* pink flower
CPO – *C. pulcherrima* orange flower

TABLE 4.5

IDV OF THE BANDS IN THE AGAROSE GEL OF DNA DAMAGE IN λ DNA and pUC18 DNA

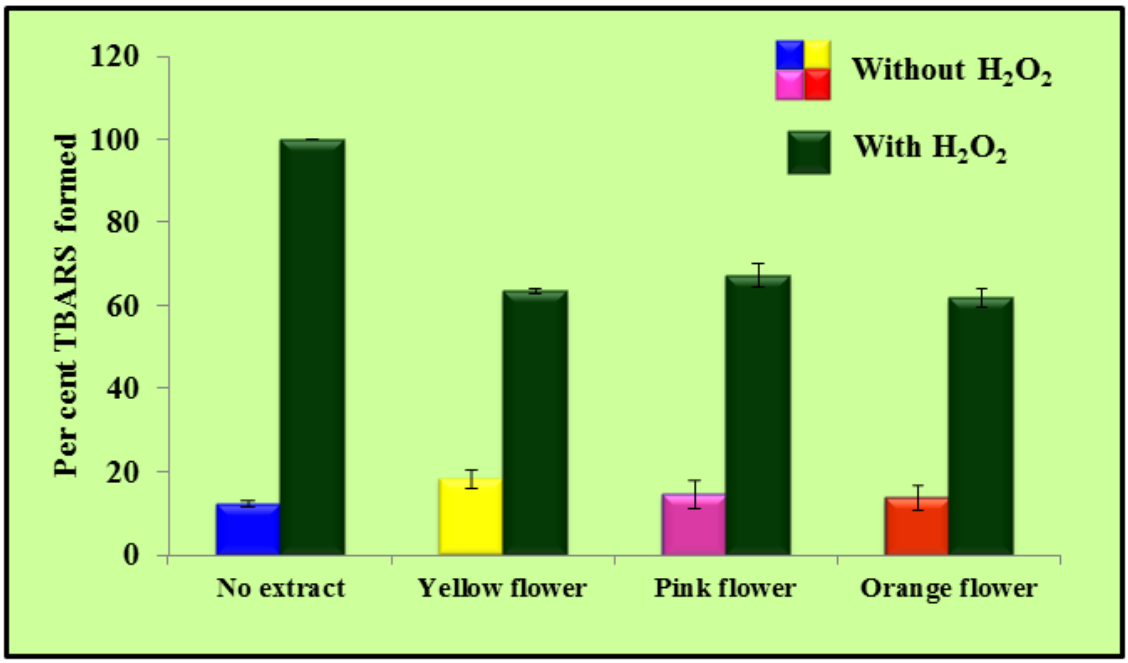
Sample	IDV of the bands of λ DNA		IDV of the bands of pUC18 DNA	
	Without H_2O_2	With H_2O_2	Without H_2O_2	With H_2O_2
No Extract	609039	423522	37840	4305
Yellow Flower Extract	609348	530250	31694	21504
Pink Flower Extract	564425	467460	38976	29400
Orange Flower Extract	549608	491625	44100	24346

In λ DNA, the orange flower extract rendered the maximum protection followed by the pink and yellow flower extracts, whereas in the case of pUC 18 DNA, all the three flower extracts showed significant protection, among which the pink flower exhibited the maximum protection. These observations were further confirmed by the Integrated Density Values (IDV) of the bands, recorded using the digital gel documentation software (Alpha Ease FC of Alpha Digidoc 1201) and the respective values are presented in Table 4.5.

b) Protective Effect of The Flower Extracts of *C. pulcherrima* on H_2O_2 Induced Damage to Herring Sperm DNA

The extent of DNA damage in herring sperm DNA was measured by spectrophotometric analysis of TBARS formation and the results are depicted in Figure 4.8. The extent of damage to herring sperm DNA was increased markedly on exposure to H_2O_2, which was significantly decreased on co-treatment with the flower extracts. The protection rendered by the methanolic extract of the orange flower was more pronounced than that of the pink and yellow flower extracts.

FIGURE 4.8: Inhibition of Oxidant-Induced Damage to Herring Sperm DNA by *C. pulcherrima* Flower Extracts



The values are Mean ± S.D. of triplicates

The value of H_2O_2-treated group was fixed as 100 per cent and the relative values in percentage were calculated for the other groups

4.2.3. Protective Effect of *C. pulcherrima* Flower Extracts on Oxidative Damage to Proteins

i) Effect of *C. pulcherrima* Flower Extracts on Protein Carbonyl Formation

The effect of the flower extracts on protein oxidation is depicted in Table 4.6. The formation of protein carbonyl was significantly increased in the presence of the oxidant. On co-treatment with the methanolic extracts of the three flowers of *C. pulcherrima*, a significant decrease in the oxidation of proteins was observed compared to that of oxidant alone treated group. This observation signifies the protective effect of the extracts of all three flowers of *C. pulcherrima* against protein oxidation.

TABLE 4.6

EFFECT OF *C. pulcherrima* FLOWER EXTRACTS ON PROTEIN CARBONYL FORMATION

Sample	Protein carbonyl (nmol/mg protein)	
	Without H_2O_2	With H_2O_2
No Extract	17.33 ± 0.08	40.62 ± 0.14 [a]
Yellow Flower Extract	22.83 ± 0.08 [a]	29.39 ± 0.27 [a,b,c]
Pink Flower Extract	21.61 ± 0.90 [a]	26.79 ± 0.70 [a,b,c]
Orange Flower Extract	20.96 ± 1.78 [a]	26.32 ± 0.12 [a,b,c]

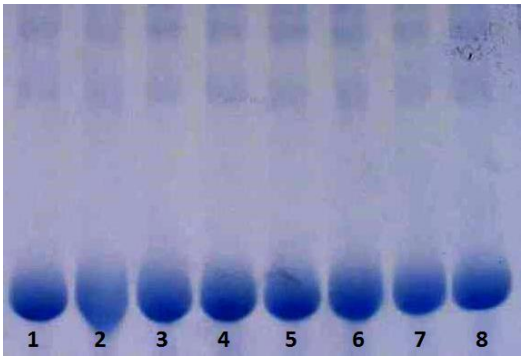
The values are Mean ± S.D. of triplicates
a – statistically significant ($p<0.05$) compared to untreated control
b – statistically significant ($p<0.05$) compared to H_2O_2 control
c – statistically significant ($p<0.05$) compared to the respective plant control

ii) Effect of *C. pulcherrima* Flower Extracts on Protein Migration on 1D Gel

The effect of the flower extracts on protein oxidation *in vitro* was evaluated by 1D gel probing of oxidized proteins. The differences in the electrophoretic mobility of the protein bovine serum albumin subjected to oxidative stress *in vitro* were determined in the presence and/or absence of H_2O_2 and the flower extract.

It is evident from the results of the SDS-PAGE depicted in Plate 4.4, that the intensity of the bands in the H_2O_2-treated group (lane 2) showed a significant decrease when compared to that of the untreated control (lane 1). This effect was counteracted by the co-treatment with the flower extracts (Lanes 4, 6 and 8).

PLATE 4.4
Effect of *C. pulcherrima* Flower Extracts on the Migration of Proteins Subjected to Oxidative Stress



Lane 1: Untreated control
Lane 2: BSA + H_2O_2
Lane 3: BSA + CPY
Lane 4: BSA + CPY + H_2O_2
Lane 5: BSA + CPP
Lane 6: BSA + CPP + H_2O_2
Lane 7: BSA + CPO
Lane 8: BSA + CPO + H_2O_2

CPY – *C. pulcherrima* yellow flower
CPP – *C. pulcherrima* pink flower
CPO – *C. pulcherrima* orange flower

TABLE 4.7
IDV OF THE BANDS IN THE POLYACRYLAMIDE GEL OF PROTEINS SUBJECTED TO OXIDATIVE STRESS

Sample	IDV of bands		
	Band 1	Band 2	Band 3
Control	92988	73920	125020
H_2O_2	78720	63336	96750
CPY	92736	72320	114121
CPY + H_2O_2	80640	75012	105570
CPP	79560	62566	105570
CPP + H_2O_2	59160	58968	96585
CPO	60333	68310	123114
CPO + H_2O_2	62100	58800	100878

The results of phase I, thus, showed that the methanolic 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 stress, both in cell-free systems and in intact cells.

Phase II

4.3 Effects of the Flower Extracts on Antioxidant Levels in Liver Slices Subjected to Oxidative Stress *in vitro*

In phase II, the protective effects of the flowers of *C. pulcherrima* against H_2O_2 induced oxidative stress was investigated using precision-cut goat liver slices. Both enzymic and non-enzymic antioxidants were assessed in the liver slices subjected to oxidative stress in the presence and the absence of the flower extracts.

i) Enzymic Antioxidant Status in Liver Slices Exposed to Oxidant and Flower Extracts *in vitro*

The activities of enzymic antioxidants superoxide dismutase (SOD), catalase (CAT) and peroxidase (GPx) in the liver slices treated with H_2O_2 and/or flower extract are represented in Tables 4.8 to 4.12. The activities of all these enzymes decreased significantly on treatment with H_2O_2, when compared to that of untreated control.

The treatment with the flower extracts alone showed no significant changes in the SOD activity (Table 4.8). The co-treatment with methanolic extract of the three flowers (yellow, pink and orange) improved the SOD activity compared to that of untreated control. The orange flower extract significantly improved the SOD activity compared to that of H_2O_2 treated group. A similar trend was observed in peroxidase activity, in which, the pink flower extract showed significant improvement in the peroxidase activity in the presence of the oxidant.

TABLE 4.8

EFFECT OF *C. pulcherrima* FLOWER EXTRACTS ON SUPEROXIDE DISMUTASE ACTIVITY IN GOAT LIVER SLICES EXPOSED *in vitro* TO H_2O_2

Sample	SOD ACTIVITY (Units$^{\$}$/g tissue)	
	Without H_2O_2	**With H_2O_2**
No Extract	15.44 ± 3.85	10.42 ± 2.87 [a]
Yellow Flower Extract	13.62 ± 2.47 [a]	11.61 ± 2.77 [a]
Pink Flower Extract	14.25 ± 3.13 [a]	11.52 ± 3.46 [a,c]
Orange Flower Extract	13.30 ± 3.23 [a]	11.14 ± 4.70 [a,b,c]

Values are expressed as Mean ± S.D of triplicates
Enzyme activity is expressed as Units/g liver tissue
$-One unit is defined as the amount of enzyme that gives 50% inhibition of NBT reduction in one minute
a – statistically significant ($p<0.05$) compared to untreated control
b – statistically significant ($p<0.05$) compared to H_2O_2 control
c – statistically significant ($p<0.05$) compared to the respective plant control

The catalase activity in the liver slices reduced significantly compared to that of the untreated group. On treatment with the orange flower extract alone, the enzyme activity was increased compared to that of untreated control and no significant changes were found in the yellow and pink flower extract treated groups. All the three flowers of *C. pulcherrima* significantly elevated the catalase activity ($P<0.05$) in the presence of the oxidant (Table 4.9).

TABLE 4.9

EFFECT OF *C. pulcherrima* FLOWER EXTRACTS ON CATALASE ACTIVITY IN GOAT LIVER SLICES EXPOSED *in vitro* TO H_2O_2

Sample	CAT ACTIVITY (Units[#]/g tissue)	
	Without H_2O_2	**With H_2O_2**
No Extract	276.06 ± 12.58	151.11 ± 16.35[a]
Yellow Flower Extract	276.06 ± 12.58	208.33 ± 7.21[a,b,c]
Pink Flower Extract	262.49 ± 20.38	200.46 ± 11.81[a,b,c]
Orange Flower Extract	291.91 ± 14.87[a]	227.34 ± 15.18[a,b,c]

Values are expressed as Mean ± S.D of triplicates
Enzyme activity is expressed as Units/g liver tissue
- One unit is defined as the amount of enzyme required to decrease the absorbance at 240nm
a – statistically significant ($p<0.05$) compared to untreated control
b – statistically significant ($p<0.05$) compared to H_2O_2 control
c – statistically significant ($p<0.05$) compared to the respective plant control

A significant reduction in GPx activity was observed in the H_2O_2 treated group compared to the untreated control. Co-treatment of liver slices with *C. pulcherrima* flower extracts elevated the GPx activity compared to that of the H_2O_2 treated group (Table 4.10).

TABLE 4.10

EFFECT OF *C. pulcherrima* FLOWER EXTRACTS ON GLUTATHIONE PEROXIDASE ACTIVITY IN GOAT LIVER SLICES EXPOSED *in vitro* TO H_2O_2

Sample	GPx ACTIVITY (Units[@]/g tissue)	
	Without H_2O_2	**With H_2O_2**
No Extract	30.12 ± 3.50	18.88 ± 0.33[a]
Yellow Flower Extract	31.70 ± 2.91	18.50 ± 1.27[a,b,c]
Pink Flower Extract	30.58 ± 2.97	22.70 ± 0.08[a,c]
Orange Flower Extract	32.14 ± 0.87	28.12 ± 0.08[b]

Values are expressed as Mean ± S.D of triplicates
Enzyme activity is expressed as Units/g liver tissue
@ - One unit is defined as the change in absorbance at 430 nm/minute
a – statistically significant ($p<0.05$) compared to untreated control
b – statistically significant ($p<0.05$) compared to H_2O_2 control
c – statistically significant ($p<0.05$) compared to the respective plant control

The activities of glutathione S-transferase (GST) and glutathione reductase (GR) in the liver slices treated with H_2O_2 and/or flower extract are represented in Table 4.11 and 4.12. H_2O_2 significantly reduced the activities of GST and GR compared to

untreated control. The liver slices treated with the flower extracts alone showed a significant increase in GST and GR activities than the untreated control. The toxic effect of H_2O_2 was counteracted upon co-treatment with the three flower extracts.

TABLE 4.11

EFFECT OF *C. pulcherrima* FLOWER EXTRACTS ON GLUTATHIONE S-TRANSFERASE ACTIVITY IN GOAT LIVER SLICES EXPOSED *in vitro* TO H_2O_2

Sample	GST ACTIVITY (Units[#]/g tissue)	
	Without H_2O_2	**With H_2O_2**
No Extract	0.042 ± 0.001	0.015 ± 0.002[a]
Yellow Flower Extract	0.030 ± 0.004[a]	0.021 ± 0.004[a,c]
Pink Flower Extract	0.026 ± 0.002[a]	0.020 ± 0.002[a]
Orange Flower Extract	0.026 ± 0.004[a]	0.020 ± 0.002[a]

Values are expressed as Mean ± S.D of triplicates
Enzyme activity is expressed as Units/g liver tissue
- One unit is defined as milli moles of NADPH oxidized/minute
a – statistically significant ($p<0.05$) compared to untreated control
b – statistically significant ($p<0.05$) compared to H_2O_2 control
c – statistically significant ($p<0.05$) compared to the respective plant control

TABLE 4.12

EFFECT OF *C. pulcherrima* FLOWER EXTRACTS ON GLUTATHIONE REDUCTASE ACTIVITY IN GOAT LIVER SLICES EXPOSED *in vitro* TO H_2O_2

Sample	GR ACTIVITY (Units[$]/g tissue)	
	Without H_2O_2	**With H_2O_2**
No Extract	2.53 ± 0.14	1.49 ± 0.28[a]
Yellow Flower Extract	2.30 ± 0.20	2.16 ± 0.14[a,b]
Pink Flower Extract	2.12 ± 0.08[a]	1.97 ± 0.06[a,b]
Orange Flower Extract	2.38 ± 0.13	2.17 ± 0.12[a,b]

Values are expressed as Mean ± S.D of triplicates
Enzyme activity is expressed as Units/g liver tissue
$ - One unit is defined as nano moles of CDNB conjugated/minute
a – statistically significant ($p<0.05$) compared to untreated control
b – statistically significant ($p<0.05$) compared to H_2O_2 control
c – statistically significant ($p<0.05$) compared to the respective plant control

ii) Non-enzymic Antioxidant Levels in Liver Slices Exposed to Oxidant and Flower Extracts *in vitro*

The effect of the methanolic extract of the three different flowers of *C. pulcherrima* on non-enzymic antioxidant levels in liver slices exposed to oxidative stress were analysed and the results are represented in Tables 4.13 to 4.18. H_2O_2 significantly decreased the levels of ascorbic acid, tocopherol and vitamin A ($P<0.05$). The treatment with *C. pulcherrima* flower extracts significantly ($P<0.05$) increased their level, which remained elevated even in the presence of the oxidant. A similar trend was observed for reduced glutathione, total thiols and protein thiols.

TABLE 4.13

EFFECT OF *C. pulcherrima* FLOWER EXTRACTS ON ASCORBIC ACID LEVELS IN GOAT LIVER SLICES EXPOSED *in vitro* TO H_2O_2

Sample	Ascorbic acid (mg/g tissue)	
	Without H_2O_2	With H_2O_2
No Extract	0.14 ± 0.02	0.10 ± 0.002
Yellow Flower Extract	0.17 ± 0.01 [a]	0.12 ± 0.001 [c]
Pink Flower Extract	0.16 ± 0.005	0.13 ± 0.01 [b,c]
Orange Flower Extract	0.16 ± 0.003	0.12 ± 0.001 [c]

Values are expressed as Mean ± S.D of triplicates
a – statistically significant ($p<0.05$) compared to untreated control
b – statistically significant ($p<0.05$) compared to H_2O_2 control
c – statistically significant ($p<0.05$) compared to the respective plant control

TABLE 4.14

EFFECT OF *C. pulcherrima* FLOWER EXTRACTS ON VITAMIN E LEVELS IN GOAT LIVER SLICES EXPOSED *in vitro* TO H_2O_2

Sample	Vitamin E (µg/g tissue)	
	Without H_2O_2	With H_2O_2
No Extract	4.17 ± 0.47	0.80 ± 0.03 [a]
Yellow Flower Extract	2.84 ± 0.24 [a]	1.88 ± 0.30 [a,b,c]
Pink Flower Extract	3.09 ± 0.59 [a]	1.92 ± 0.11 [a,b,c]
Orange Flower Extract	3.01 ± 0.23 [a]	1.94 ± 0.14 [a,b,c]

Values are expressed as Mean ± S.D of triplicates
a – statistically significant ($p<0.05$) compared to untreated control
b – statistically significant ($p<0.05$) compared to H_2O_2 control
c – statistically significant ($p<0.05$) compared to the respective plant control

TABLE 4.15

EFFECT OF *C. pulcherrima* FLOWER EXTRACTS ON VITAMIN A LEVELS IN GOAT LIVER SLICES EXPOSED *in vitro* TO H_2O_2

Sample	Vitamin A (µg/g tissue)	
	Without H_2O_2	With H_2O_2
No Extract	95.95 ± 0.77	58.70 ± 2.96[a]
Yellow Flower Extract	84.50 ± 2.54[a]	71.85 ± 2.05[a,b,c]
Pink Flower Extract	85.05 ± 4.31[a]	70.40 ± 0.70[a,b,c]
Orange Flower Extract	88.90 ± 1.55[a]	75.05 ± 1.20[a,b,c]

Values are expressed as Mean ± S.D of triplicates

TABLE 4.16

EFFECT OF *C. pulcherrima* FLOWER EXTRACTS ON REDUCED GLUTATHIONE LEVELS IN GOAT LIVER SLICES EXPOSED *in vitro* TO H_2O_2

Sample	GSH (mg/g tissue)	
	Without H_2O_2	With H_2O_2
No Extract	2.16 ± 0.20	1.30 ± 0.03[a]
Yellow Flower Extract	1.97 ± 0.18	1.63 ± 0.02[a,b,c]
Pink Flower Extract	1.95 ± 0.04[a]	1.58 ± 0.01[a,b,c]
Orange Flower Extract	2.03 ± 0.30[a]	1.72 ± 0.19[a,b,c]

Values are expressed as Mean ± S.D of triplicates

TABLE 4.17

EFFECT OF *C. pulcherrima* FLOWER EXTRACTS ON TOTAL THIOLS LEVELS IN GOAT LIVER SLICES EXPOSED *in vitro* TO H_2O_2

Sample	Total thiols (mg/g tissue)	
	Without H_2O_2	With H_2O_2
No Extract	21.82 ± 0.08	9.24 ± 0.22[a]
Yellow Flower Extract	19.45 ± 0.08[a]	13.45 ± 0.32[a,b,c]
Pink Flower Extract	19.45 ± 0.16[a]	15.44 ± 0.97[a,b,c]
Orange Flower Extract	20.16 ± 0.24[a]	15.80 ± 0.74[a,b,c]

Values are expressed as Mean ± S.D of triplicates

a – statistically significant ($p<0.05$) compared to untreated control
b – statistically significant ($p<0.05$) compared to H_2O_2 control
c – statistically significant ($p<0.05$) compared to the respective plant control

TABLE 4.18

EFFECT OF *C. pulcherrima* FLOWER EXTRACTS ON PROTEIN THIOLS LEVELS IN GOAT LIVER SLICES EXPOSED *in vitro* TO H_2O_2

Sample	Protein thiols (mg/g tissue)	
	Without H_2O_2	With H_2O_2
No Extract	19.66 ± 0.13	7.93 ± 0.19[a]
Yellow Flower Extract	17.48 ± 0.26[a]	11.91 ± 0.35[a,b,c]
Pink Flower Extract	17.49 ± 0.11[a]	13.86 ± 0.96[a,b,c]
Orange Flower Extract	18.13 ± 0.06[a]	14.07 ± 0.94[a,b,c]

Values are expressed as Mean ± S.D of triplicates
a – statistically significant ($p<0.05$) compared to untreated control
b – statistically significant ($p<0.05$) compared to H_2O_2 control
c – statistically significant ($p<0.05$) compared to the respective plant control

The results, thus, showed that the flower extracts of *C. pulcherrima* can improve the antioxidant status in the goat liver slices exposed *in vitro* to oxidative stress. Since the *in vitro* model (liver slices) that have been chosen for the present study can simulate the *in vivo* conditions more precisely, similar protective effects of the flowers of *C. pulcherrima* can be anticipated in the intact systems.

Phase III

In all living systems, a homeostatic balance between proliferation of cells and their death is maintained for development and maintenance of the biological system. Apoptosis, a physiological cell suicide program is characterized by distinct morphological and biochemical features such as cytoplasmic membrane blebbing, chromosomal condensation and aggregation around the nuclear periphery and formation of small apoptotic bodies (Cheung *et al*., 2012).

The third phase of the present study was formulated to examine the apoptosis-modulating effects of *C. pulcherrima* flower extracts on oxidative stress-induced apoptotic events in both transformed and non-transformed cells. Two different untransformed cell types (*Saccharomyces cerevisiae* cells and peripheral blood lymphocytes) and one transformed cells (KB oral carcinoma cells) were used to examine the oxidative stress-induced apoptosis, and the effect of the flower extracts on them. Oxidative stress was induced by H_2O_2 for yeast cells and by etoposide for peripheral blood lymphocytes and KB cells. The effect of flower extracts on cell viability was determined by MTT, SRB and LDH release assays. The characteristic features of apoptosis were analysed using various staining methods namely Giemsa

(morphological changes), PI, EtBr, AO/EtBr (nuclear changes) and DAPI (apoptotic index) staining. The extent of DNA damage caused by oxidative stress was determined by diphenylamine assay in yeast cells and by single cell gel electrophoresis (comet) in peripheral blood lymphocytes and cancer cells. The results obtained for the various parameters analysed are presented below.

4.4 EFFECT OF *C. pulcherrima* FLOWER EXTRACTS ON H_2O_2-INDUCED APOPTOSIS IN *S. cerevisiae* CELLS

Yeast cell culture is an easily manipulated model system that is used to determine the preliminary cytotoxicity of compounds (Limberger *et al*., 2011). In the present study, yeast cells were chosen to study the influence of flower extracts on oxidant (H_2O_2)-induced apoptotic events.

4.4.1. Effect of *C. pulcherrima* Flower Extracts on the Viability of *S. cerevisiae* Cells

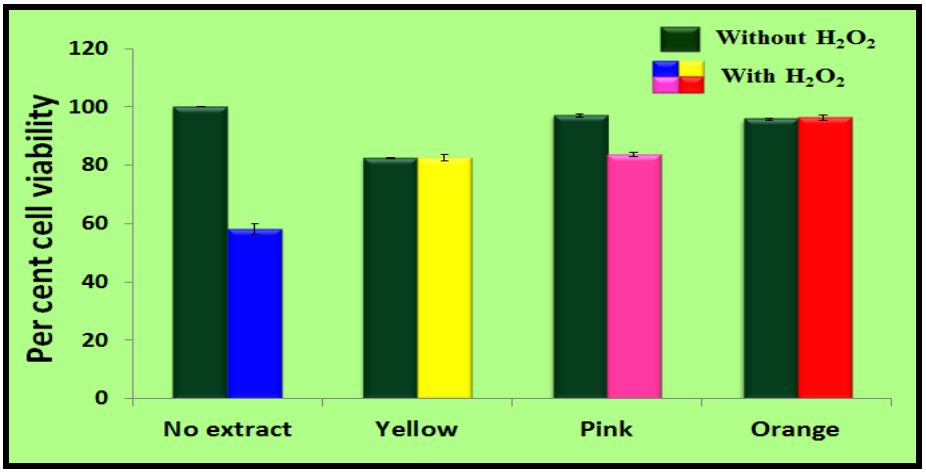
The influence of the methanolic extract of the three different flowers of *C. pulcherrima* on the *S. cerevisiae* cell survival was determined by quantifying the per cent cell viability using MTT. The extent of viability in the different treatment groups are presented in Figure 4.9. Exposure to H_2O_2 caused a marked decrease in the viability of the *S. cerevisiae* cells. All the three flower extracts increased the viability of the cells subjected to oxidative stress. The maximum cytoprotection was rendered by the methanolic extract of the orange flower followed by the pink and yellow flower. When compared to the untreated control group, a minimal decrease in the cell viability of yeast cells in groups treated with the flower extracts alone was also observed.

The Sulphorhodamine B (SRB) assay, which was developed in 1990, is one of the most widely used methods for *in vitro* cytotoxicity screening (Vichai and Kirtikara, 2006). In the present investigation, SRB assay was used as an additional parameter to determine the cell viability and proliferative efficiency of the yeast cells subjected to oxidative stress in the presence and the absence of the flower extracts.

The results obtained upon determining the cell viability using SRB assay (Figure 4.10) showed that, when compared to the untreated control, the viability of the cells decreased drastically in the H_2O_2 treated group. Treatment with the flower extracts improved the viability of the yeast cells in the presence of the oxidant, which proved the protective effects of the flowers of *C. pulcherrima* against oxidative stress-induced apoptosis in untransformed cells.

FIGURE 4.9

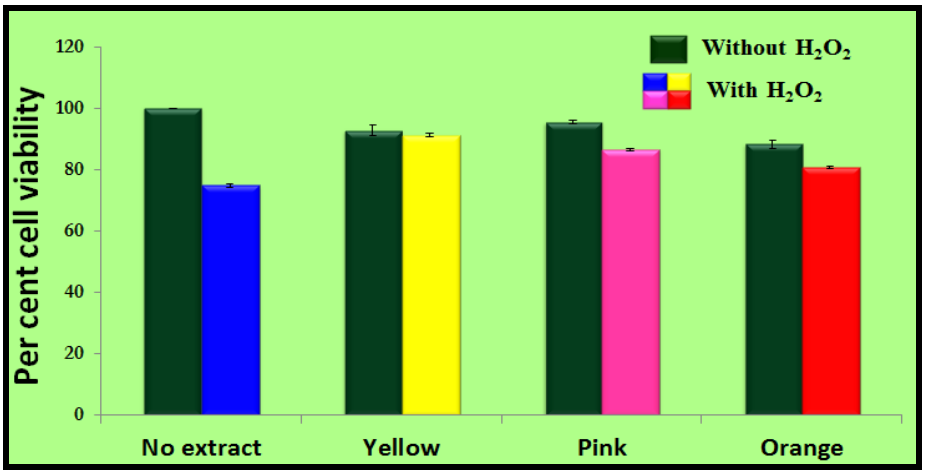
Effect of *C. pulcherrima* Flower Extracts on the Viability of *S. cerevisiae* Cells Subjected to Oxidative Stress as Determined by MTT assay



Values are expressed as Mean ± S.D of triplicates

FIGURE 4.10

Effect of *C. pulcherrima* Flower Extracts on the Viability of *S. cerevisiae* Cells Subjected to Oxidative Stress as Determined by SRB assay



Values are expressed as Mean ± S.D of triplicates

The values of the untreated (negative) control group were fixed as 100% and the per cent viabilities in the other groups were calculated relative to this

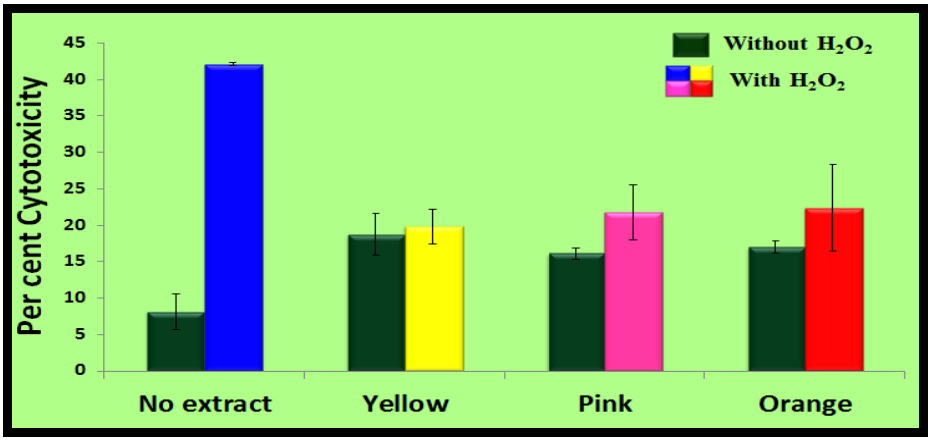
4.4.2. Effect of *C. pulcherrima* Flower Extracts on the Viability of *S. cerevisiae* Cells Subjected to Oxidative Stress (LDH Assay)

The lactate dehydrogenase (LDH) assay is one of the most popular methods used to detect cell viability. The major advantage of the LDH assay is that the LDH enzyme is stable for up to 36 hours after cell death, which therefore eliminates any false negative viability results due to cell processing (Jähn and Stoddart, 2011). The

cytotoxicity in the cells treated with or without H_2O_2, in the presence or absence of *C. pulcherrima* flower extracts was determined using LDH release assay.

FIGURE 4.11

Effect of *C. pulcherrima* Flower Extracts on Percent Cytotoxicity in *S. cerevisiae* Cells as Determined by LDH Release



Values are expressed as Mean ± S.D of triplicates

The results obtained (Figure 4.11) showed that the extent of apoptosis in yeast cells increased drastically on exposure to H_2O_2. All the three flower extracts significantly counteracted the H_2O_2-induced cytotoxicity, which is evident from the reduced level of LDH release.

4.4.3. Effect of *C. pulcherrima* Flower Extracts on the Morphological Changes in *S. cerevisiae* Cells subjected to Oxidative Stress

Giemsa staining, a rapid staining method was used to characterize the morphological changes in apoptotic cells in the presence and the absence of the flower extracts and/or H_2O_2. The characteristic morphological changes that occur in yeast cells undergoing apoptosis, such as membrane blebbing, cell shrinkage and margination of nuclear chromatin were analysed by Giemsa staining (Plate 4.5) using a phase contrast microscope. The number of cells exhibiting apoptotic morphological changes was counted in each treatment group per 100 cells in three different fields and the results are presented in Table 4.19.

The number of apoptotic cells increased in the oxidant treated group, which decreased significantly on co-treatment with the methanolic extract of all the three flowers. The three flower extracts, by themselves, increased the number of apoptotic cells, but to a minimal extent. Among the three flower extracts, orange flower extract rendered the maximum protection against H_2O_2-induced apoptosis followed by the yellow and the pink flower extracts.

TABLE 4.19

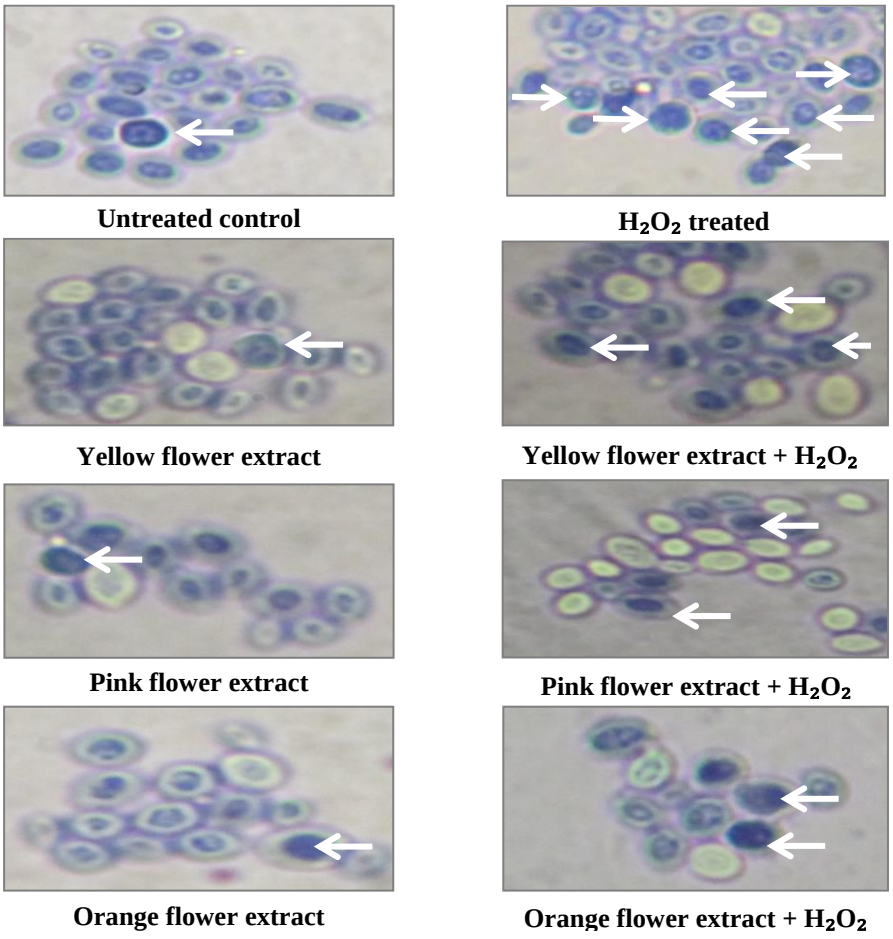
EFFECT OF *C. pulcherrima* FLOWER EXTRACTS ON THE MORPHOLOGICAL CHANGES IN *S. cerevisiae* CELLS SUBJECTED TO OXIDATIVE STRESS AS DETERMINED BY GIEMSA STAINING

TREATMENT GROUPS	Number of Apoptotic Cells / 100 Cells		Apoptotic Ratio	
	Without H_2O_2	With H_2O_2	Without H_2O_2	With H_2O_2
No Extract	14 ± 1	74 ± 4[a]	0.16	2.90
Yellow Flower Extract	22 ± 1[a]	32 ± 1[a,b,c]	0.28	0.47
Pink Flower Extract	24 ± 2[a]	35 ± 2[a,b,c]	0.36	0.54
Orange Flower Extract	21 ± 1[a]	22 ± 2[a,b]	0.27	0.37

The values are mean ± S.D of triplicates
a – Statistically significant (P<0.05) compared to untreated control
b – Statistically significant (P<0.05) compared to H_2O_2 alone treated group
c – Statistically significant (P<0.05) compared to the respective plant extract treated group

PLATE 4.5

Morphological (Giemsa Staining) Changes Induced by H_2O_2 in *S. cerevisiae* Cells



Untreated control | **H_2O_2 treated**

Yellow flower extract | **Yellow flower extract + H_2O_2**

Pink flower extract | **Pink flower extract + H_2O_2**

Orange flower extract | **Orange flower extract + H_2O_2**

4.4.4. Effect of *C. pulcherrima* Flower Extracts on the Nuclear Changes in *S. cerevisiae* Cells subjected to Oxidative Stress

The nuclear changes in yeast cells undergoing apoptosis such as 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 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.

The fragmented nuclei of the apoptotic cells were viewed using PI staining (Plate 4.6a). The other characteristic features of apoptosis, such as nuclear shrinkage, chromatin condensation, nuclear fragmentation and apoptotic body formation were detected using EtBr (Plate 4.6b) and DAPI (Plate 4.7a) staining methods. The AO/EtBr staining (Plate 4.7b) was used to distinguish normal cells from apoptotic cells through two distinct coloured fluorescences.

The number of apoptotic cells was determined along with the respective apoptotic ratio for all the treatment groups and the results are shown in Table 4.20 (PI staining), Table 4.21 (EtBr staining), Table 4.22 (DAPI staining) and Table 4.23 (AO/EtBr staining). The results showed that the nuclei of the yeast cells in the untreated control group were intact, whereas an extensive nuclear fragmentation with the formation of apoptotic bodies were observed in cells treated with the oxidant.

TABLE 4.20

EFFECT OF *C. pulcherrima* FLOWER EXTRACTS ON THE NUCLEAR CHANGES IN *S. cerevisiae* CELLS SUBJECTED TO OXIDATIVE STRESS AS DETERMINED BY PI STAINING

TREATMENT GROUPS	Number of Apoptotic Cells / 100 Cells		Apoptotic Ratio	
	Without H_2O_2	With H_2O_2	Without H_2O_2	With H_2O_2
No Extract	13 ± 1	74 ± 3 [a]	0.15	2.87
Yellow Flower Extract	20 ± 3 [a]	30 ± 1 [a,b,c]	0.25	0.43
Pink Flower Extract	25 ± 1 [a]	35 ± 3 [a,b,c]	0.33	0.54
Orange Flower Extract	22 ± 1 [a]	28 ± 1 [a,b,c]	0.28	0.39

The values are mean ± S.D of triplicates

a – Statistically significant ($P<0.05$) compared to untreated control

b – Statistically significant ($P<0.05$) compared to H_2O_2 alone treated group

c – Statistically significant ($P<0.05$) compared to the respective plant extract treated group

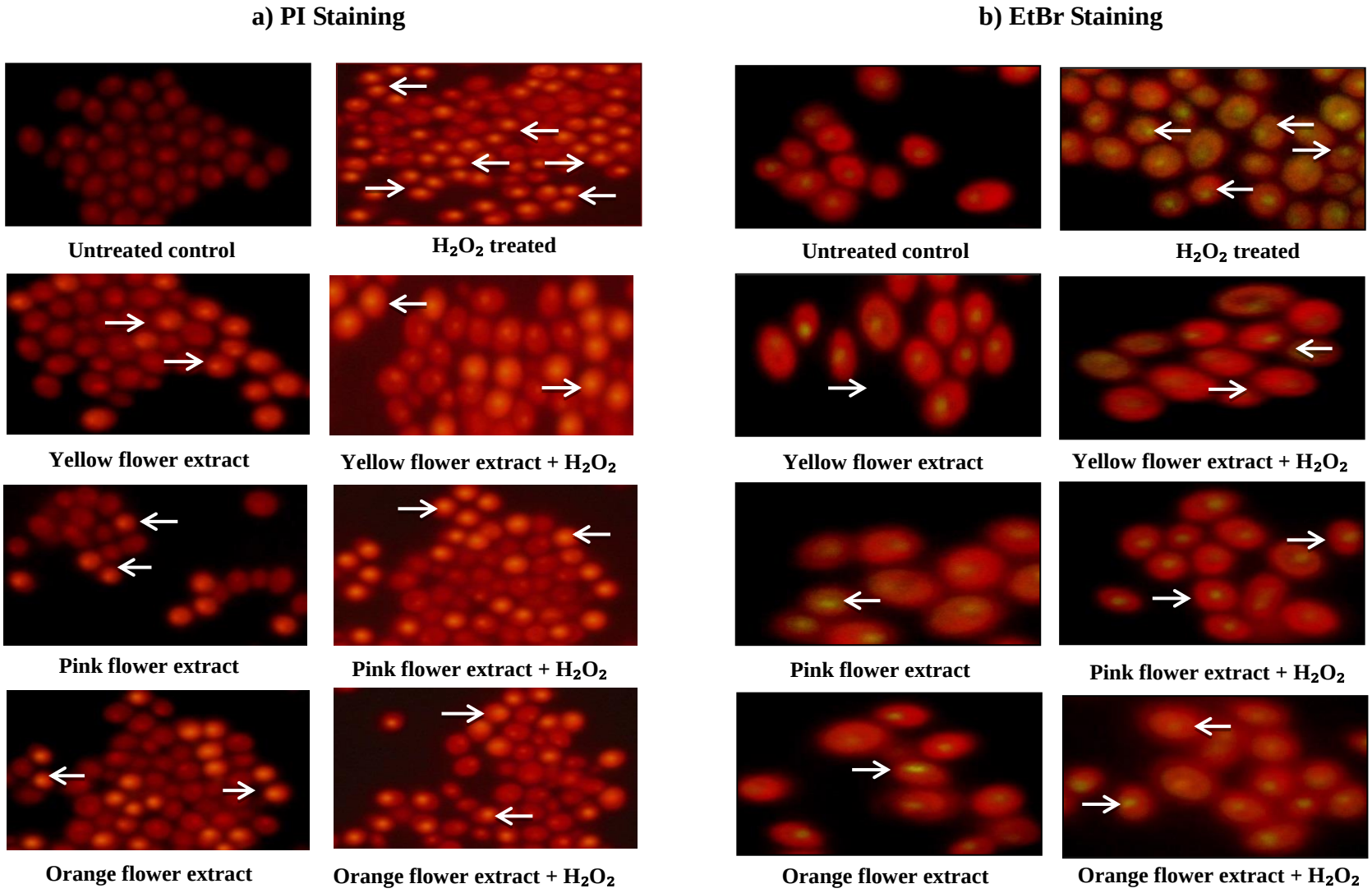


PLATE 4.6
Nuclear (PI and EtBr Staining) Changes Induced by H_2O_2 in *S. cerevisiae* Cells

TABLE 4.21

EFFECT OF *C. pulcherrima* FLOWER EXTRACTS ON THE NUCLEAR CHANGES IN *S. cerevisiae* CELLS SUBJECTED TO OXIDATIVE STRESS AS DETERMINED BY EtBr STAINING

TREATMENT GROUPS	Number of Apoptotic Cells / 100 Cells		Apoptotic Ratio	
	Without H_2O_2	With H_2O_2	Without H_2O_2	With H_2O_2
No Extract	10 ± 1	70 ± 1[a]	0.11	2.34
Yellow Flower Extract	17 ± 1[a]	27 ± 3[a,b,c]	0.20	0.37
Pink Flower Extract	16 ± 1[a]	25 ± 5[a,b,c]	0.19	0.34
Orange Flower Extract	15 ± 3[a]	22 ± 1[a,b,c]	0.18	0.30

The values are mean ± S.D of triplicates

a – Statistically significant ($P<0.05$) compared to untreated control

b – Statistically significant ($P<0.05$) compared to H_2O_2 alone treated group

c – Statistically significant ($P<0.05$) compared to the respective plant extract treated group

TABLE 4.22

EFFECT OF *C. pulcherrima* FLOWER EXTRACTS ON THE NUCLEAR CHANGES IN *S. cerevisiae* CELLS SUBJECTED TO OXIDATIVE STRESS AS DETERMINED BY DAPI STAINING

TREATMENT GROUPS	Number of Apoptotic Cells / 100 Cells		Apoptotic Ratio	
	Without H_2O_2	With H_2O_2	Without H_2O_2	With H_2O_2
No Extract	13 ± 2	76 ± 3[a]	0.15	3.20
Yellow Flower Extract	23 ± 1[a]	35 ± 2[a,b,c]	0.30	0.54
Pink Flower Extract	19 ± 2[a]	34 ± 1[a,b,c]	0.24	0.52
Orange Flower Extract	20 ± 2[a]	32 ± 2[a,b,c]	0.25	0.47

The values are mean ± S.D of triplicates

a – Statistically significant ($P<0.05$) compared to untreated control

b – Statistically significant ($P<0.05$) compared to H_2O_2 alone treated group

c – Statistically significant ($P<0.05$) compared to the respective plant extract treated group

The number of apoptotic cells in all the three flower extract treated groups were significantly decreased ($P<0.05$) in the presence of the oxidant. The methanolic extract of the orange flower showed the maximum inhibitory effect against oxidative stress-induced apoptosis, while the pink and the yellow flower extracts rendered a moderate protection. Thus the above observations show that all the three flowers of *C. pulcherrima* provide significant protection for *S. cerevisiae* against oxidant-induced apoptotic cell death.

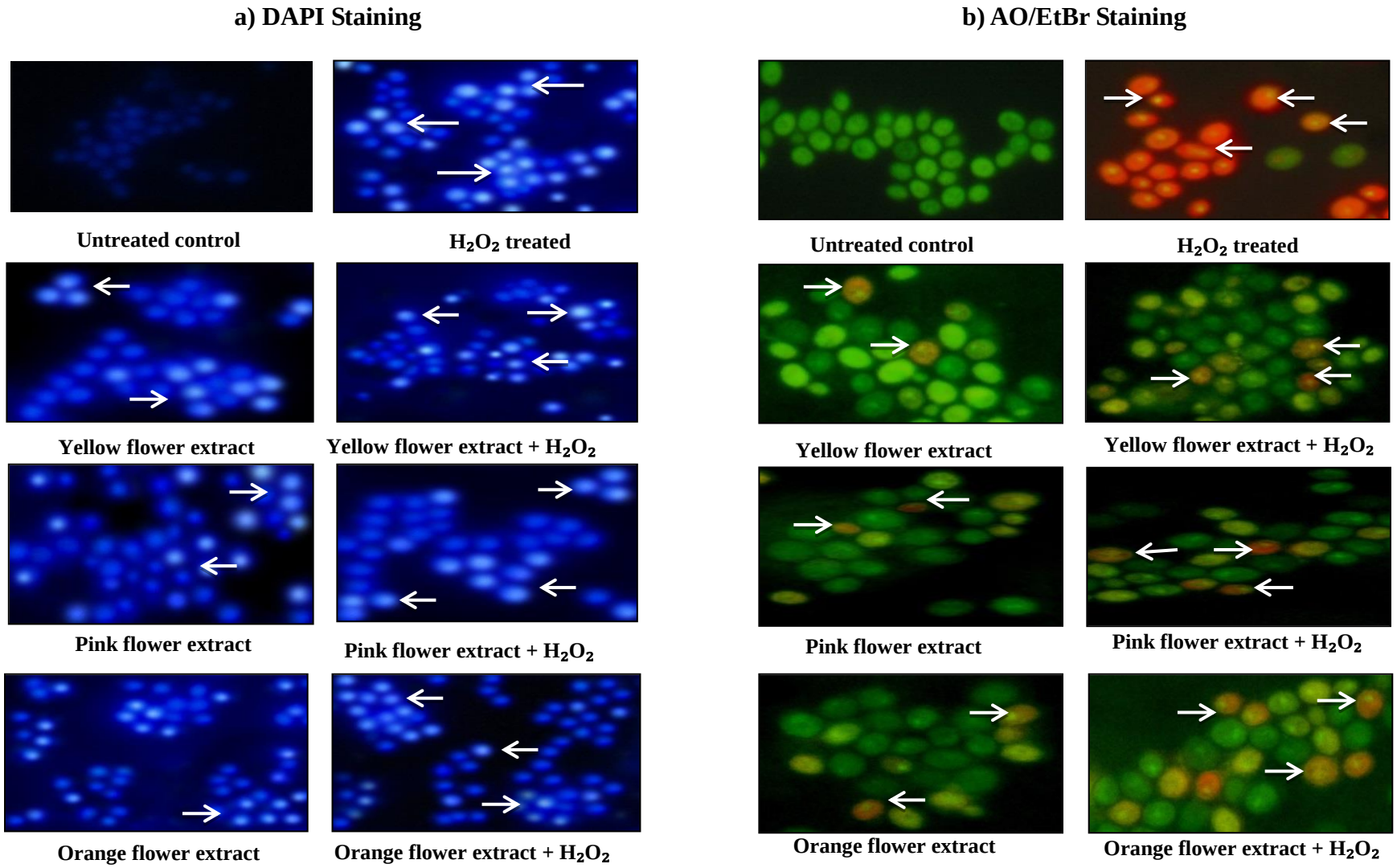


PLATE 4.7

Nuclear (DAPI and AO/EtBr Staining) Changes Induced by H_2O_2 in *S. cerevisiae* Cells

TABLE 4.23

EFFECT OF *C. pulcherrima* FLOWER EXTRACTS ON THE NUCLEAR CHANGES IN *S. cerevisiae* CELLS SUBJECTED TO OXIDATIVE STRESS AS DETERMINED BY AO/EtBr STAINING

TREATMENT GROUPS	Number of Apoptotic Cells / 100 Cells		Apoptotic Ratio	
	Without H_2O_2	With H_2O_2	Without H_2O_2	With H_2O_2
No Extract	12 ± 2	77 ± 3[a]	0.14	3.38
Yellow Flower Extract	22 ± 1[a]	35 ± 1[a,b,c]	0.28	0.54
Pink Flower Extract	18 ± 1[a]	32 ± 1[a,b,c]	0.22	0.47
Orange Flower Extract	15 ± 1	28 ± 1[a,b,c]	0.18	0.39

The values are mean ± S.D of triplicates

a – Statistically significant ($P<0.05$) compared to untreated control

b – Statistically significant ($P<0.05$) compared to H_2O_2 alone treated group

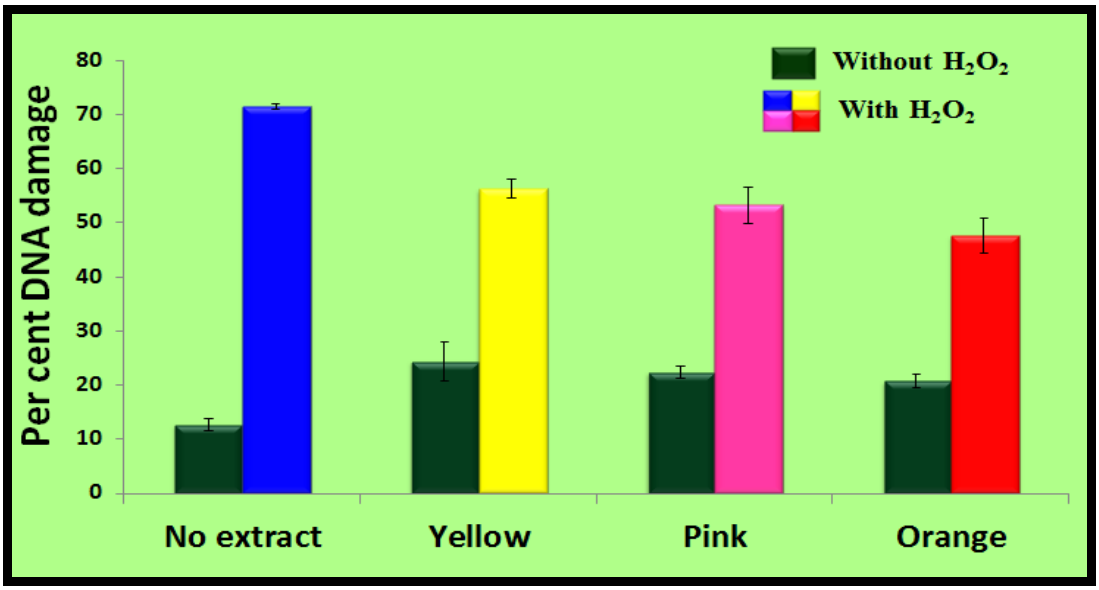
c – Statistically significant ($P<0.05$) compared to the respective plant extract treated group

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

The extent of DNA fragmentation in apoptotic *S. cerevisiae* cells was determined using a spectrophotometric method both in the absence and the presence of the flower extracts. The per cent DNA damage quantified in each treatment group is shown in Figure 4.12.

FIGURE 4.12

Effect of *C. pulcherrima* Flower Extracts on DNA Damage in *S. cerevisiae* cells Subjected to Oxidative Stress



The values are means ± S.D. of triplicates

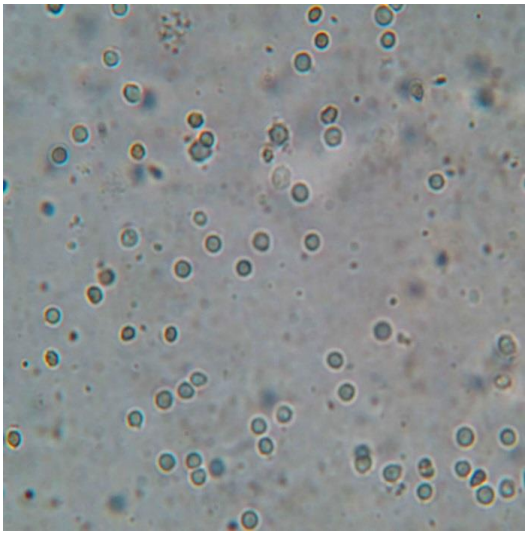
A significant extent of DNA fragmentation was found in *S. cerevisiae* cells exposed to the oxidant, which was counteracted by the methanolic extract of all the three flowers of *C. pulcherrima.* The protective effect rendered by the orange flower extract against oxidative DNA damage in *S. cerevisiae* cells was slightly higher than that of the pink and yellow flower extracts.

4.5. EFFECT OF C. *pulcherrima* FLOWER EXTRACTS ON ETOPOSIDE INDUCED STRESS IN HUMAN PERIPHERAL BLOOD LYMPHOCYTES AND ORAL CARCINOMA (KB) CELLS

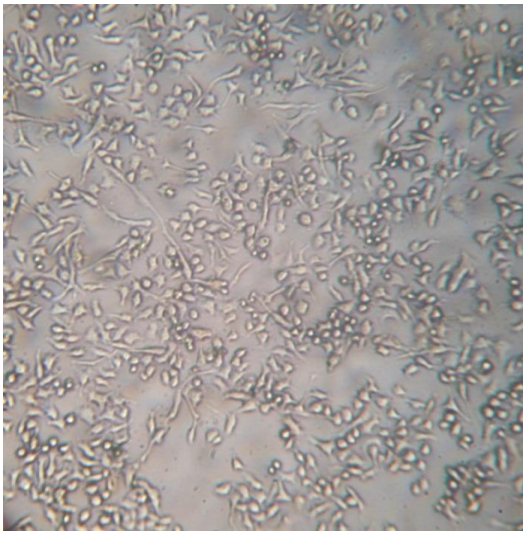
The results obtained from the apoptotic studies using *S. cerevisiae* cells showed that all the three flower extracts of *C. pulcherrima* can protect the non-transformed *S. cerevisiae* cells from oxidative stress-induced cell death. These findings have furthered the present study to determine the effects of *C. pulcherrima* on transformed cells. Apoptosis is closely related with anti-cancer therapy and many anticancer drugs exert their action by inducing apoptosis of cancer cells (Grimm *et al*., 2011).

Earlier studies in our laboratory have shown that the aqueous extract of the three flowers of *C. pulcherrima* decreased the viability of the cancerous (Hep2) cells (Yamuna, 2004). Thus, in order to find whether the flowers possess anticancer potential, their influence on oxidant-induced apoptosis in oral carcinoma (KB) cells was analysed. Peripheral blood lymphocytes from healthy subjects were used as the non-transformed control cells.

PLATE 4.8



a) Peripheral Blood Lymphocytes



b) KB cells

Peripheral blood lymphocyte culture is a simple, efficient and inexpensive *in vitro* culture system widely used for *in vivo* cytogenetic analysis (Kligerman *et al*., 1984). In the present study, peripheral blood lymphocytes from the fresh blood of healthy individuals were isolated and cultured under aseptic conditions. Etoposide was used to induce oxidative stress in both peripheral blood lymphocytes and KB cells. Etoposide is an important chemotherapeutic agent used to treat a wide spectrum of human cancers and has been in clinical use for more than two decades. It is one of the most highly prescribed anticancer drugs in the world, which exerts its action via oxidative stress (Baldwin and Osheroff, 2005). The influence of the flower extracts on etoposide-induced cell death in peripheral blood lymphocytes (Plate 4.8a) and KB cells (Plate 4.8b) were evaluated by the cytotoxicity assays and by various (membrane and nuclear) staining methods.

4.5.1. Effect of *C. pulcherrima* Flower Extracts on the Viability of Peripheral Blood Lymphocytes and KB cells

Cell viability was determined using MTT and SRB assays. In both peripheral blood lymphocytes and KB cells, etoposide exposure resulted in a steep decline in the proportion of viable cells. In the peripheral blood lymphocytes, all the three flowers of *C. pulcherrima* reduced the cytotoxicity induced by etoposide. Treatment with the flower extracts alone exhibited a mild toxicity towards peripheral blood lymphocytes (Figure 4.13). In KB cells, the viability was decreased by the flower extracts both in the presence and the absence of etoposide (Figure 4.14). These observations, thus, show that the flower extracts of *C. pulcherrima* act synergistically with etoposide in decreasing the viability of KB cells, while protecting the peripheral blood lymphocytes from the toxicity of etoposide. A similar trend was observed for the SRB assay, which confirmed the results of MTT assay. The results are shown in Figures 4.15 and 4.16.

4.5.2. Effect of *C. pulcherrima* Flower Extracts on the LDH Release in Peripheral Blood Lymphocytes and KB cells

The extent of cell death due to oxidative stress and the effect of the flower extracts of *C. pulcherrima* on oxidative stress-induced cell death were also analysed by LDH release. The enzyme LDH is a marker for the membrane damage caused due to cell death. Thus, the extent of LDH release was measured in both peripheral blood lymphocytes and KB cells subjected to oxidative stress both in the presence and absence of the flower extracts of *C. pulcherrima*.

FIGURE 4.13

Effect of *C. pulcherrima* Flower Extracts on the Viability of Peripheral Blood Lymphocytes subjected to Oxidative Stress as Determined by MTT Assay

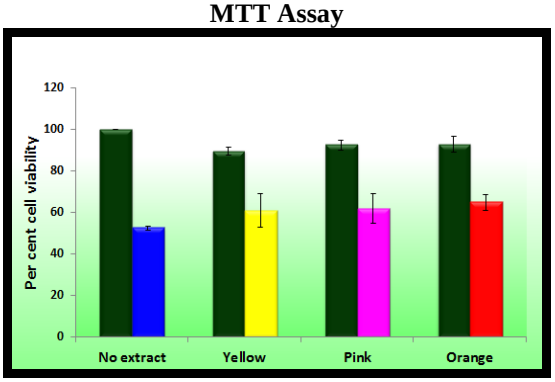


FIGURE 4.14

Effect of *C. pulcherrima* Flower Extracts on the Viability of Oral Carcinoma (KB) Cells subjected to Oxidative Stress as Determined by MTT Assay

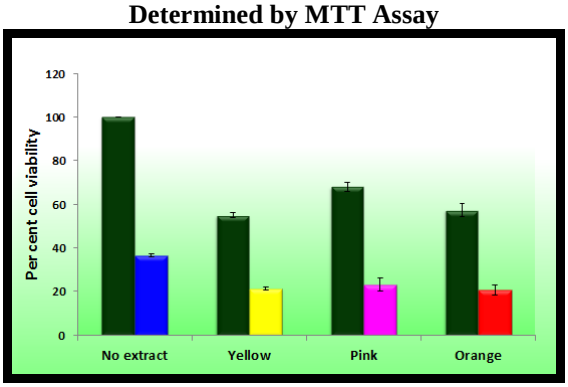


FIGURE 4.15

Effect of *C. pulcherrima* Flower Extracts on the Viability of Peripheral Blood Lymphocytes subjected to Oxidative Stress as Determined by SRB Assay

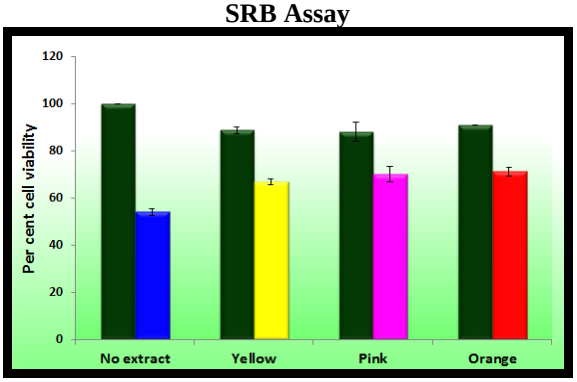


FIGURE 4.16

Effect of *C. pulcherrima* Flower Extracts on the Viability of Oral Carcinoma (KB) Cells subjected to Oxidative Stress as Determined by SRB Assay

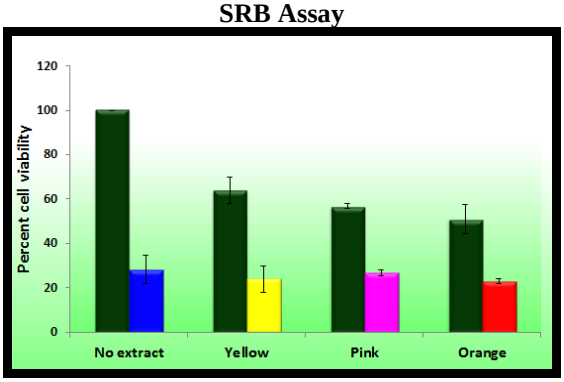


FIGURE 4.17

Effect of *C. pulcherrima* Flower Extracts on Percent Cytotoxicity in Peripheral Blood Lymphocytes as Determined by LDH Release

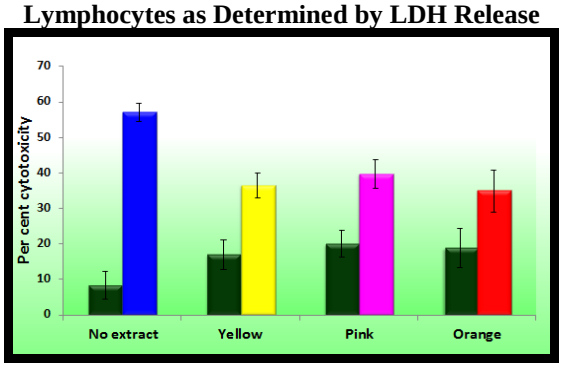
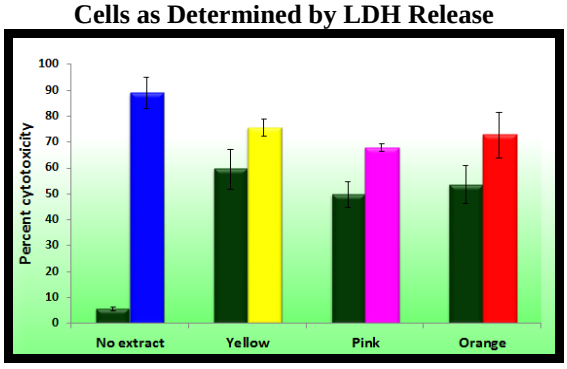


FIGURE 4.18

Effect of *C. pulcherrima* Flower Extracts on Percent Cytotoxicity in Oral Carcinoma (KB) Cells as Determined by LDH Release



The values are mean ± SD of triplicates
In MTT and SRB assays, the values of the untreated (negative) control group were fixed as 100% and the per cent viabilities in the other groups were calculated relative to this.
In LDH release, the values of etoposide treated control group were fixed as 100% and the per cent cytotoxicity in the other groups were calculated relative to this

Without etoposide **With etoposide**

In the non-transformed peripheral blood lymphocytes, etoposide treatment caused a significant increase in the cytotoxicity, which is evident from the LDH release. The etoposide-induced cytotoxicity was drastically reduced in the presence of the methanolic extract of all the three flowers (Figure 4.17). The maximum protection was observed in the orange flower extract treated group.

In the case of the cancerous (KB) cells, cytotoxicity was increased in cells treated with etoposide. The flower extracts, by themselves, also increased the cytotoxicity in KB cells both in the presence and absence of the etoposide compared to that of untreated control. This observation confirmed the anticancer potential the flower extracts of *C. pulcherrima* (Figure 4.18).

4.5.3. Effect of *C. pulcherrima* Flower Extracts on the Morphological Changes in Peripheral Blood Lymphocytes and KB cells

The morphological changes observed in the peripheral blood lymphocytes and KB cells stained with giemsa are depicted in Tables 4.24 and 4.25 respectively.

An increase in the number of apoptotic cells in etoposide treated group was found in both peripheral blood lymphocytes and KB cells. The three flower extracts, by themselves, did not cause any toxic effects to non-transformed lymphocytes, whereas they caused a steep increase in the number of apoptotic cells in KB cells.

TABLE 4.24

EFFECT OF *C. pulcherrima* FLOWER EXTRACTS ON THE MORPHOLOGICAL CHANGES IN PERIPHERAL BLOOD LYMPHOCYTES SUBJECTED TO OXIDATIVE STRESS AS DETERMINED BY GIEMSA STAINING

TREATMENT GROUPS	Number of Apoptotic Cells / 100 Cells		Apoptotic Ratio	
	Without etoposide	With etoposide	Without etoposide	With etoposide
No Extract	9 ± 2	46 ± 1[a]	0.11	0.85
Yellow Flower Extract	17 ± 1[a]	30 ± 3[a,b,c]	0.21	0.44
Pink Flower Extract	16 ± 3[a]	33 ± 2[a,b,c]	0.19	0.50
Orange Flower Extract	12 ± 2	28 ± 1[a,b,c]	0.14	0.39

The values are mean ± S.D of triplicates
a – Statistically significant (P<0.05) compared to untreated control
b – Statistically significant (P<0.05) compared to oxidant alone treated group
c – Statistically significant (P<0.05) compared to the respective plant extract treated group

TABLE 4.25

EFFECT OF *C. pulcherrima* FLOWER EXTRACTS ON THE MORPHOLOGICAL CHANGES IN KB CELLS SUBJECTED TO OXIDATIVE STRESS AS DETERMINED BY GIEMSA STAINING

TREATMENT GROUPS	Number of Apoptotic Cells / 100 Cells		Apoptotic Ratio	
	Without etoposide	With etoposide	Without etoposide	With etoposide
No Extract	13 ± 1	79 ± 4[a]	0.14	3.76
Yellow Flower Extract	36 ± 1[a]	81 ± 1[a,c]	0.57	4.26
Pink Flower Extract	37 ± 1[a]	80 ± 2[a,c]	0.58	4.00
Orange Flower Extract	41 ± 9[a]	83 ± 4[a,c]	0.69	4.88

The values are mean ± S.D of triplicates
a – Statistically significant (P<0.05) compared to untreated control
b – Statistically significant (P<0.05) compared to oxidant alone treated group
c – Statistically significant (P<0.05) compared to the respective plant extract treated group

On co-treatment with both etoposide and the flower extract, a differential effect was observed in peripheral blood lymphocytes and KB cells. In peripheral blood lymphocytes, all the three flower extracts of *C. pulcherrima* reduced the etoposide-induced apoptotic cell death (Plate 4.9a). On the other hand, in KB cells, the etoposide-induced cell death was increased by the flower extracts (Plate 4.9b). This observation indicates that the flower extracts of *C. pulcherrima* flower extracts reduced the cell viability of the cancer cells in the presence of etoposide, while protecting the non-cancerous cells from etoposide-induced cytotoxicity.

4.5.4. Effect of *C. pulcherrima* Flower Extracts on the Nuclear Changes in Peripheral Blood Lymphocytes and KB cells

The nuclear changes that occur during apoptosis in the cancerous and non-cancerous cells exposed to etoposide in the presence/absence of flower extracts were analysed using the various nuclear stains, namely EtBr, PI, DAPI and AO/EtBR. The number of apoptotic cells was counted and the apoptotic ratio was calculated for each treatment group. The results are shown in Tables 4.26 – 4.33.

In both peripheral blood lymphocytes and KB cells, a drastic increase in the number of dying cells with apoptotic nuclear morphology was found in the etoposide treated groups compared to that of untreated control. In peripheral blood lymphocytes, the co-administration of *C. pulcherrima* flower extract with etoposide, increased the number of surviving cells, compared to that of etoposide treated group.

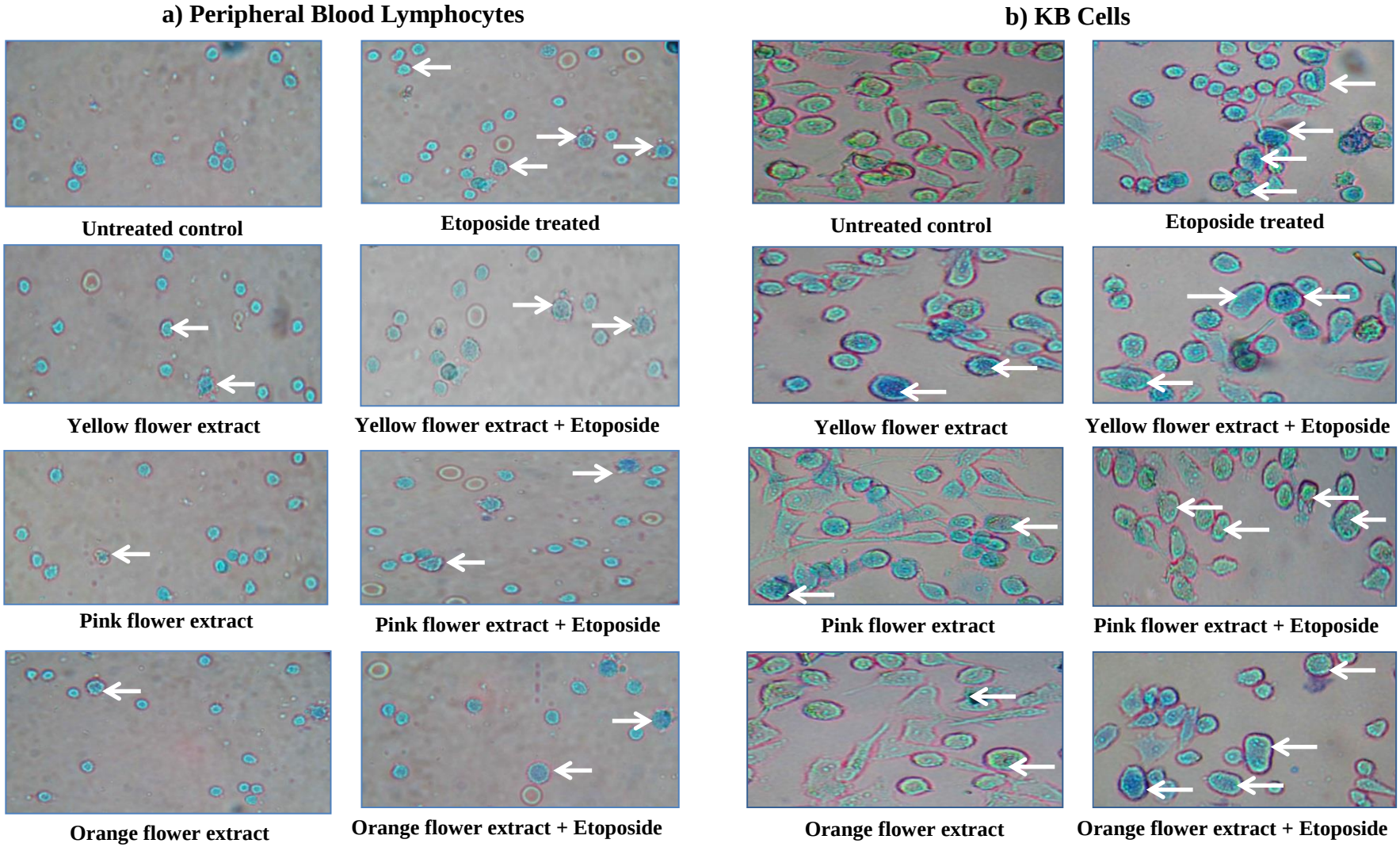


PLATE 4.9

Morphological Changes Induced by Etoposide in Peripheral Blood Lymphocytes and KB cells as Determined by Giemsa Staining

In KB cells, however, this co-treatment increased the proportion of apoptotic cells. All the three flower extracts showed similar differential response, among which the orange flower extract of *C. pulcherrima* showed the maximum response compared to the pink and the yellow flower extract.

The nuclear morphology of both peripheral blood lymphocytes and KB cells exposed to etoposide in the presence/absence of flower extracts that were viewed after various nuclear staining methods namely PI, EtBr, DAPI and AO/EtBr are presented in Plates 4.9 to 4.13 respectively.

TABLE 4.26

EFFECT OF *C. pulcherrima* FLOWER EXTRACTS ON THE NUCLEAR CHANGES IN PERIPHERAL BLOOD LYMPHOCYTES SUBJECTED TO OXIDATIVE STRESS AS DETERMINED BY PI STAINING

TREATMENT GROUPS	Number of Apoptotic Cells / 100 Cells		Apoptotic Ratio	
	Without etoposide	With etoposide	Without etoposide	With etoposide
No Extract	9 ± 3	47 ± 5[a]	0.10	0.91
Yellow Flower Extract	13 ± 1	30 ± 1[a,b,c]	0.15	0.43
Pink Flower Extract	14 ± 2	28 ± 1[a,b,c]	0.17	0.39
Orange Flower Extract	12 ± 1	27 ± 2[a,b,c]	0.14	0.38

The values are mean ± S.D of triplicates

TABLE 4.27

EFFECT OF *C. pulcherrima* FLOWER EXTRACTS ON THE NUCLEAR CHANGES IN KB CELLS SUBJECTED TO OXIDATIVE STRESS AS DETERMINED BY PI STAINING

TREATMENT GROUPS	Number of Apoptotic Cells / 100 Cells		Apoptotic Ratio	
	Without etoposide	With etoposide	Without etoposide	With etoposide
No Extract	14 ± 1	74 ± 4[a]	0.16	3.16
Yellow Flower Extract	29 ± 4[a]	78 ± 2[a,c]	0.42	3.54
Pink Flower Extract	31 ± 1[a]	77 ± 4[a,c]	0.44	3.34
Orange Flower Extract	31 ± 7[a]	80 ± 3[a,c]	0.46	4.08

The values are mean ± S.D of triplicates

a – Statistically significant (P<0.05) compared to untreated control
b – Statistically significant (P<0.05) compared to oxidant alone treated group
c – Statistically significant (P<0.05) compared to the respective plant extract treated group

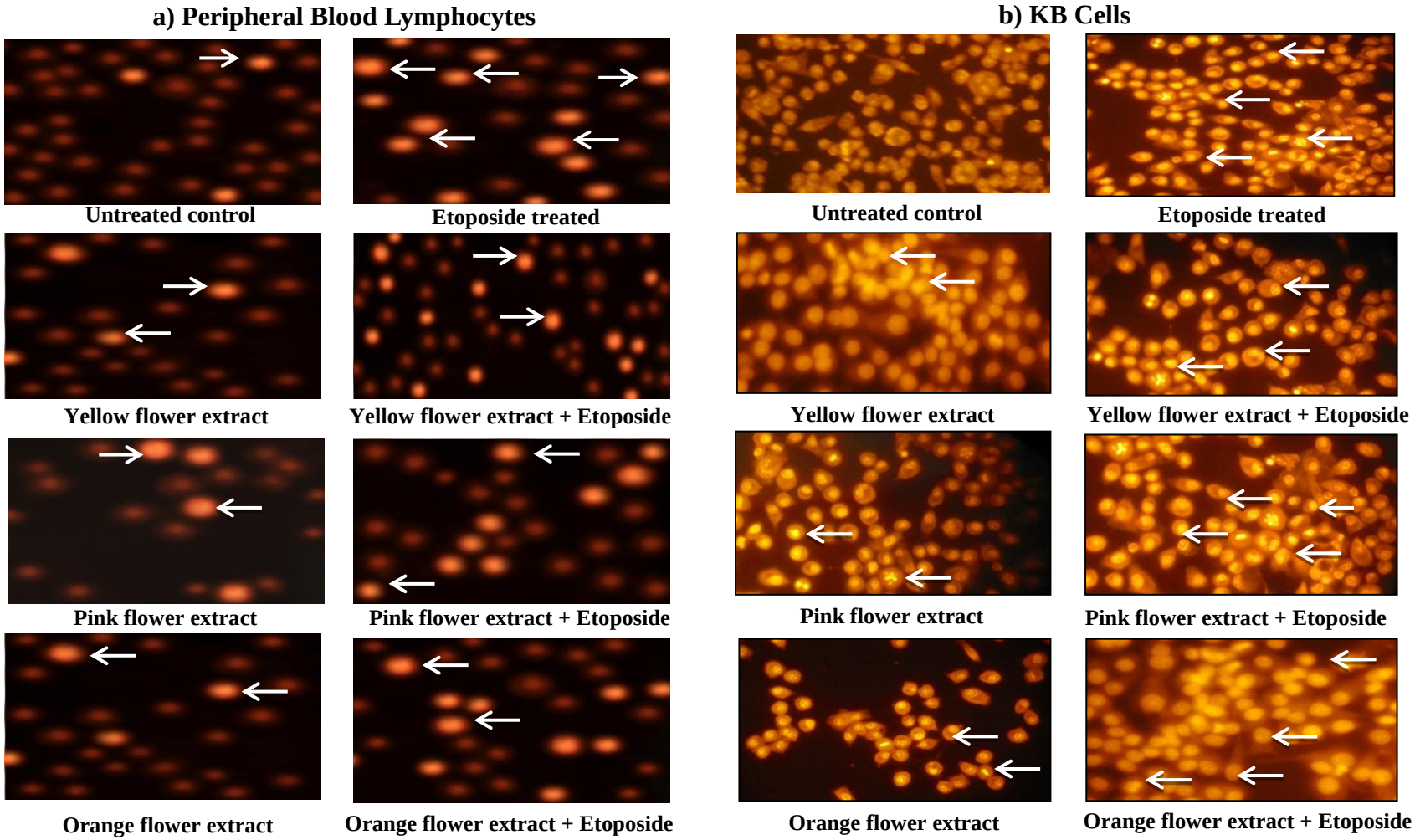


PLATE 4.10

Nuclear Changes Induced by Etoposide in Peripheral Blood Lymphocytes and KB cells as Determined by PI Staining

TABLE 4.28

EFFECT OF *C. pulcherrima* FLOWER EXTRACTS ON THE NUCLEAR CHANGES IN PERIPHERAL BLOOD LYMPHOCYTES SUBJECTED TO OXIDATIVE STRESS AS DETERMINED BY EtBr STAINING

TREATMENT GROUPS	Number of Apoptotic Cells / 100 Cells		Apoptotic Ratio	
	Without etoposide	With etoposide	Without etoposide	With etoposide
No Extract	5 ± 1	41 ± 2[a]	0.06	0.71
Yellow Flower Extract	12 ± 1	26 ± 5[a,b,c]	0.14	0.36
Pink Flower Extract	12 ± 2[a]	23 ± 4[a,b,c]	0.14	0.30
Orange Flower Extract	11 ± 1[a]	22 ± 1[a,b,c]	0.12	0.28

The values are mean ± S.D of triplicates

a – Statistically significant (P<0.05) compared to untreated control

b – Statistically significant (P<0.05) compared to oxidant alone treated group

c – Statistically significant (P<0.05) compared to the respective plant extract treated group

TABLE 4.29

EFFECT OF *C. pulcherrima* FLOWER EXTRACTS ON THE NUCLEAR CHANGES IN KB CELLS SUBJECTED TO OXIDATIVE STRESS AS DETERMINED BY EtBr STAINING

TREATMENT GROUPS	Number of Apoptotic Cells / 100 Cells		Apoptotic Ratio	
	Without etoposide	With etoposide	Without etoposide	With etoposide
No Extract	12 ± 1	76 ± 4[a]	0.14	3.28
Yellow Flower Extract	36 ± 9[a]	79 ± 4[a,c]	0.57	3.91
Pink Flower Extract	34 ± 3[a]	82 ± 1[a,c]	0.51	4.66
Orange Flower Extract	38 ± 1[a]	83 ± 2[a,c]	0.61	4.88

The values are mean ± S.D of triplicates

a – Statistically significant (P<0.05) compared to untreated control

b – Statistically significant (P<0.05) compared to oxidant alone treated group

c – Statistically significant (P<0.05) compared to the respective plant extract treated group

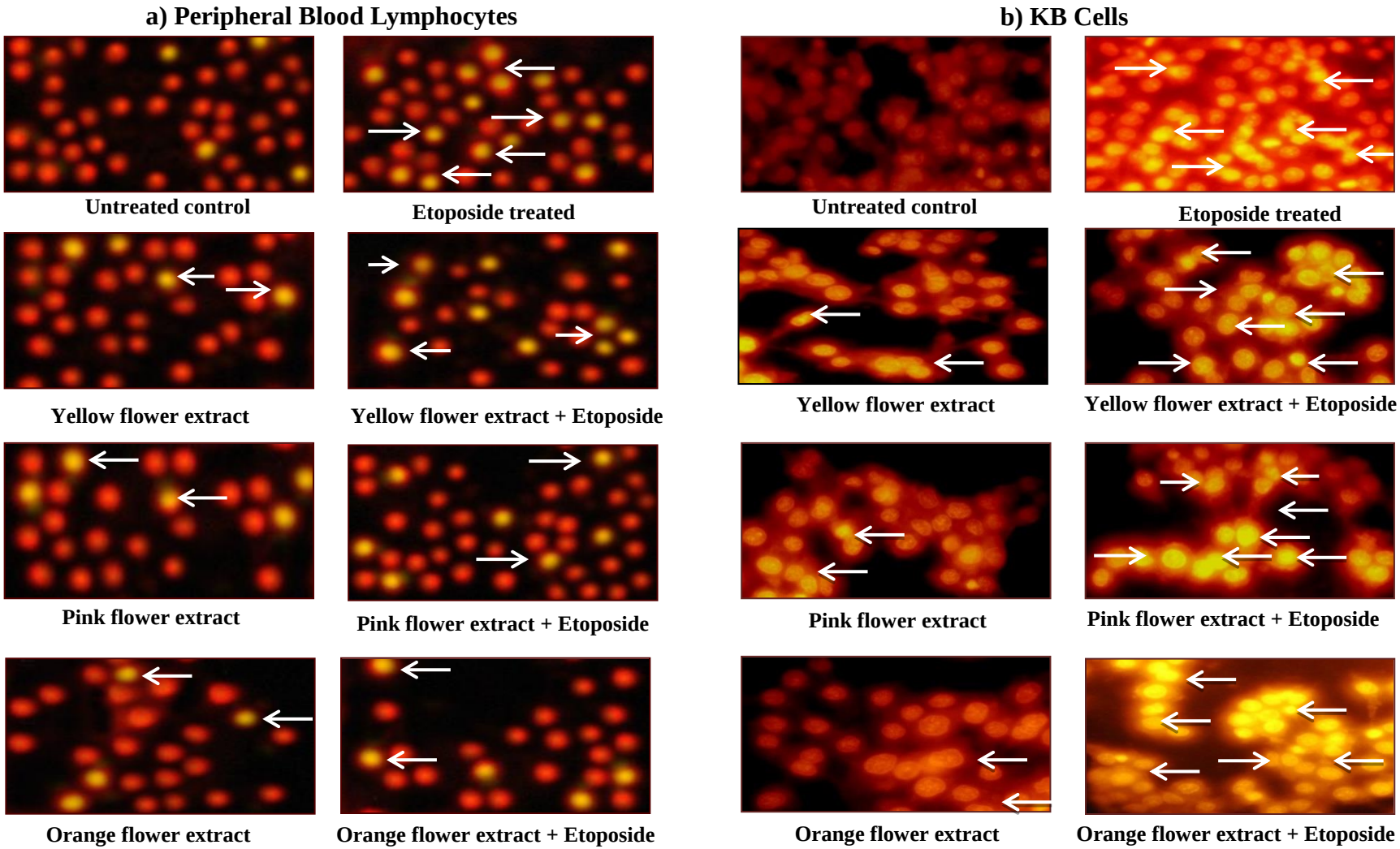


PLATE 4.11

Nuclear Changes Induced by Etoposide in Peripheral Blood Lymphocytes and KB cells as Determined by EtBr Staining

TABLE 4.30

EFFECT OF *C. pulcherrima* FLOWER EXTRACTS ON THE NUCLEAR CHANGES IN PERIPHERAL BLOOD LYMPHOCYTES SUBJECTED TO OXIDATIVE STRESS AS DETERMINED BY DAPI STAINING

TREATMENT GROUPS	Number of Apoptotic Cells / 100 Cells		Apoptotic Ratio	
	Without etoposide	With etoposide	Without etoposide	With etoposide
No Extract	10 ± 1	43 ± 1[a]	0.12	0.75
Yellow Flower Extract	15 ± 2[a]	29 ± 1[a,b,c]	0.18	0.41
Pink Flower Extract	15 ± 1[a]	32 ± 1[a,b,c]	0.18	0.47
Orange Flower Extract	13 ± 1[a]	28 ± 1[a,b,c]	0.15	0.39

The values are mean ± S.D of triplicates

a – Statistically significant (P<0.05) compared to untreated control

b – Statistically significant (P<0.05) compared to oxidant alone treated group

c – Statistically significant (P<0.05) compared to the respective plant extract treated group

TABLE 4.31

EFFECT OF *C. pulcherrima* FLOWER EXTRACTS ON THE NUCLEAR CHANGES IN KB CELLS SUBJECTED TO OXIDATIVE STRESS AS DETERMINED BY DAPI STAINING

TREATMENT GROUPS	Number of Apoptotic Cells / 100 Cells		Apoptotic Ratio	
	Without etoposide	With etoposide	Without etoposide	With etoposide
No Extract	12 ± 5	79 ± 3[a]	0.13	3.59
Yellow Flower Extract	31 ± 2[a]	80 ± 6[a,c]	0.45	3.47
Pink Flower Extract	32 ± 2[a]	79 ± 3[a,c]	0.47	3.76
Orange Flower Extract	30 ± 5[a]	86 ± 3[a,b,c]	0.45	6.14

The values are mean ± S.D of triplicates

a – Statistically significant (P<0.05) compared to untreated control

b – Statistically significant (P<0.05) compared to oxidant alone treated group

c – Statistically significant (P<0.05) compared to the respective plant extract treated group

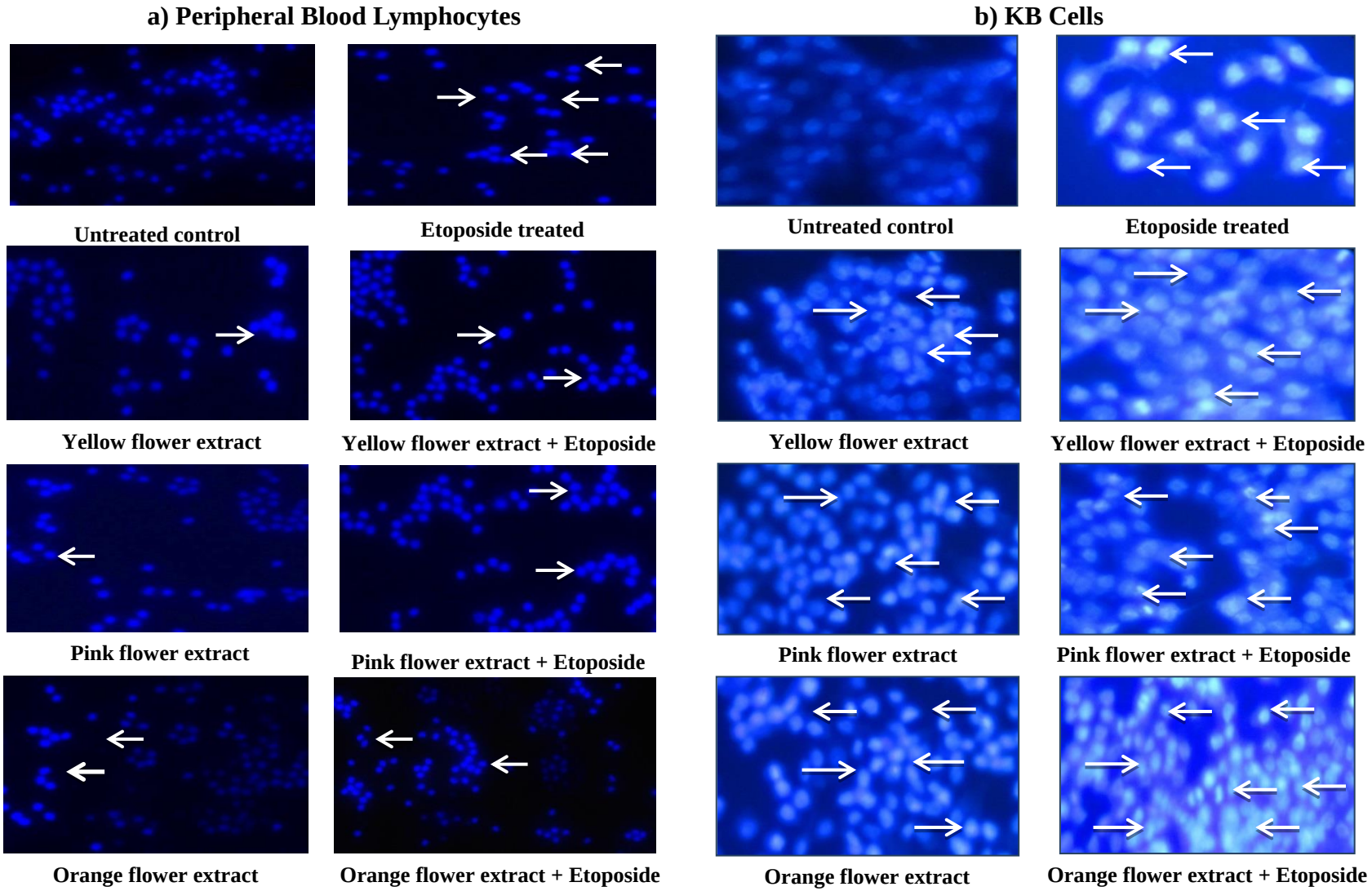


PLATE 4.12

Nuclear Changes Induced by Etoposide in Peripheral Blood Lymphocytes and KB cells as Determined by DAPI Staining

TABLE 4.32

EFFECT OF *C. pulcherrima* FLOWER EXTRACTS ON THE NUCLEAR CHANGES IN PERIPHERAL BLOOD LYMPHOCYTES SUBJECTED TO OXIDATIVE STRESS AS DETERMINED BY AO/EtBr STAINING

TREATMENT GROUPS	Number of Apoptotic Cells / 100 Cells		Apoptotic Ratio	
	Without etoposide	With etoposide	Without etoposide	With etoposide
No Extract	9 ± 1	45 ± 4	0.10	0.82
Yellow Flower Extract	13 ± 3[a]	27 ± 1[a,b,c]	0.15	0.37
Pink Flower Extract	14 ± 1[a]	30 ± 2[a,b,c]	0.16	0.42
Orange Flower Extract	11 ± 1	26 ± 2[a,b,c]	0.12	0.36

The values are mean ± S.D of triplicates

a – Statistically significant ($P<0.05$) compared to untreated control

b – Statistically significant ($P<0.05$) compared to oxidant alone treated group

c – Statistically significant ($P<0.05$) compared to the respective plant extract treated group

TABLE 4.33

EFFECT OF *C. pulcherrima* FLOWER EXTRACTS ON THE NUCLEAR CHANGES IN KB CELLS SUBJECTED TO OXIDATIVE STRESS AS DETERMINED BY AO/EtBr STAINING

TREATMENT GROUPS	Number of Apoptotic Cells / 100 Cells		Apoptotic Ratio	
	Without etoposide	With etoposide	Without etoposide	With etoposide
No Extract	14 ± 3	75 ± 5[a]	0.16	2.93
Yellow Flower Extract	34 ± 3[a]	79 ± 5[a,c]	0.50	3.29
Pink Flower Extract	30 ± 2[a]	79 ± 1[a,c]	0.41	3.09
Orange Flower Extract	32 ± 3[a]	81 ± 2[a,c]	0.43	3.11

The values are mean ± S.D of triplicates

a – Statistically significant ($P<0.05$) compared to untreated control

b – Statistically significant ($P<0.05$) compared to oxidant alone treated group

c – Statistically significant ($P<0.05$) compared to the respective plant extract treated group

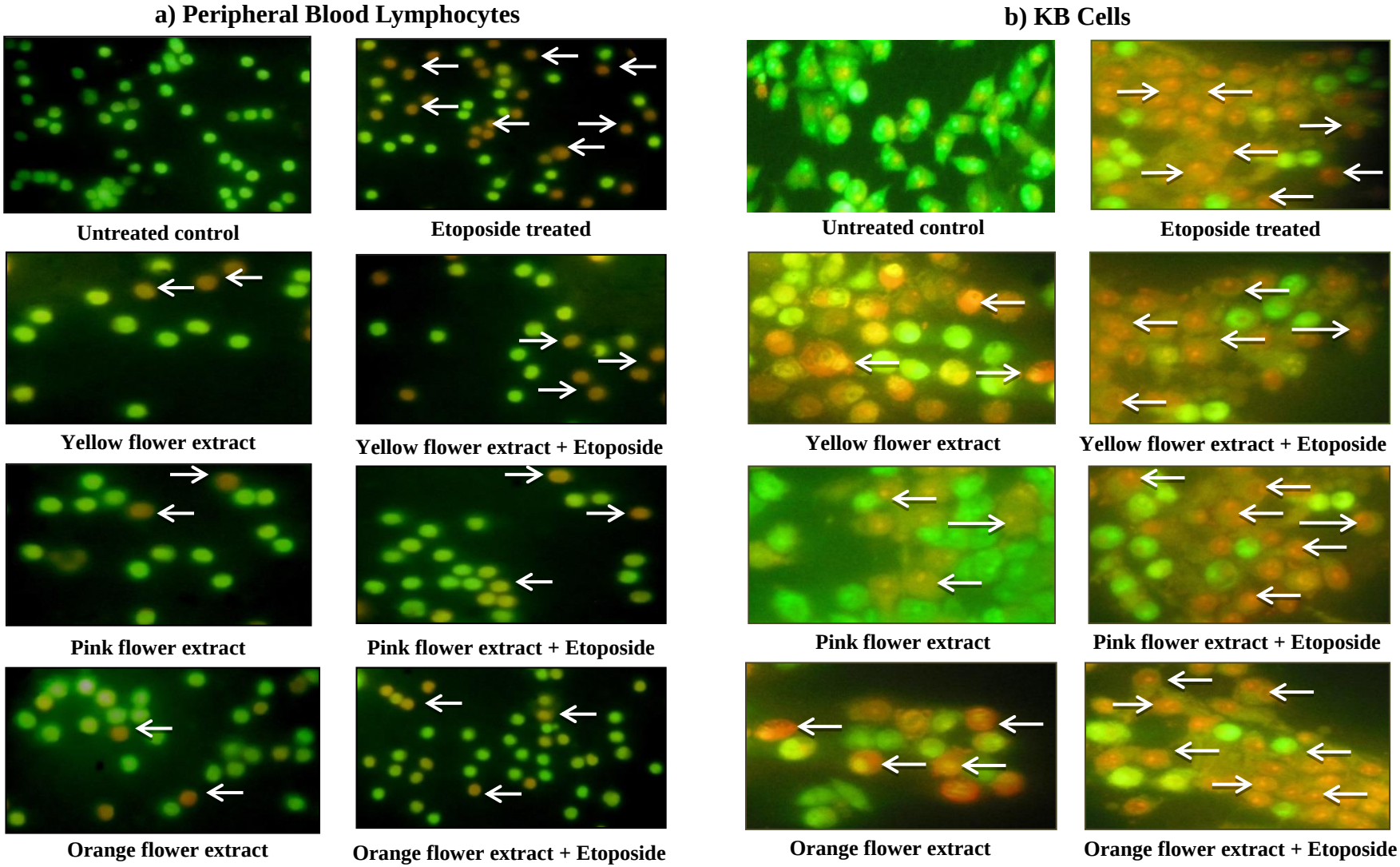


PLATE 4.13

Nuclear Changes Induced by Etoposide in Peripheral Blood Lymphocytes and KB cells as Determined by AO/EtBr Staining

4.5.5. Effect of *C. pulcherrima* Flower Extracts on Oxidative DNA Damage in Peripheral Blood Lymphocytes and KB cells

The extent of etoposide-induced DNA damage in the peripheral blood lymphocytes and KB cells was analysed by single cell gel electrophoresis (comet) both in the presence and the absence of the three flower extracts (Plate 4.14).

TABLE 4.34

EFFECT OF *C. pulcherrima* FLOWER EXTRACTS ON OXIDATIVE DNA DAMAGE IN PERIPHERAL BLOOD LYMPHOCYTES

TREATMENT GROUPS	Number of Cells with comets / 100 Cells	
	Without etoposide	With etoposide
No Extract	6 ± 1	40 ± 2[a]
Yellow Flower Extract	13 ± 1	31 ± 1[a,b,c]
Pink Flower Extract	12 ± 1	31 ± 4[a,b,c]
Orange Flower Extract	14 ± 1	27 ± 1[a,b,c]

The values are mean ± S.D of triplicates
a – Statistically significant (P<0.05) compared to untreated control
b – Statistically significant (P<0.05) compared to oxidant alone treated group
c – Statistically significant (P<0.05) compared to the respective plant extract treated group

TABLE 4.35

EFFECT OF *C. pulcherrima* FLOWER EXTRACTS ON COMET ASSAY PARAMETERS IN PERIPHERAL BLOOD LYMPHOCYTES

TREATMENT GROUPS	Tail Length (µm)	Percent Head DNA	Percent Tail DNA	Olive Tail Moment
Cells alone	3	98.53	1.46	0.23
Cells + Etoposide	81	69.37	30.62	16.63
Cells + CPY	3	95.88	4.11	0.55
Cells + CPY + Etoposide	12	91.79	8.20	3.48
Cells + CPP	4	96.47	3.52	1.55
Cells + CPP + Etoposide	9	98.04	1.95	0.95
Cells + CPO	4	98.01	1.98	0.81
Cells + CPO + Etoposide	6	94.25	5.74	0.61

CPY – *C. pulcherrima* yellow flower
CPP – *C. pulcherrima* pink flower
CPO – *C. pulcherrima* orange flower

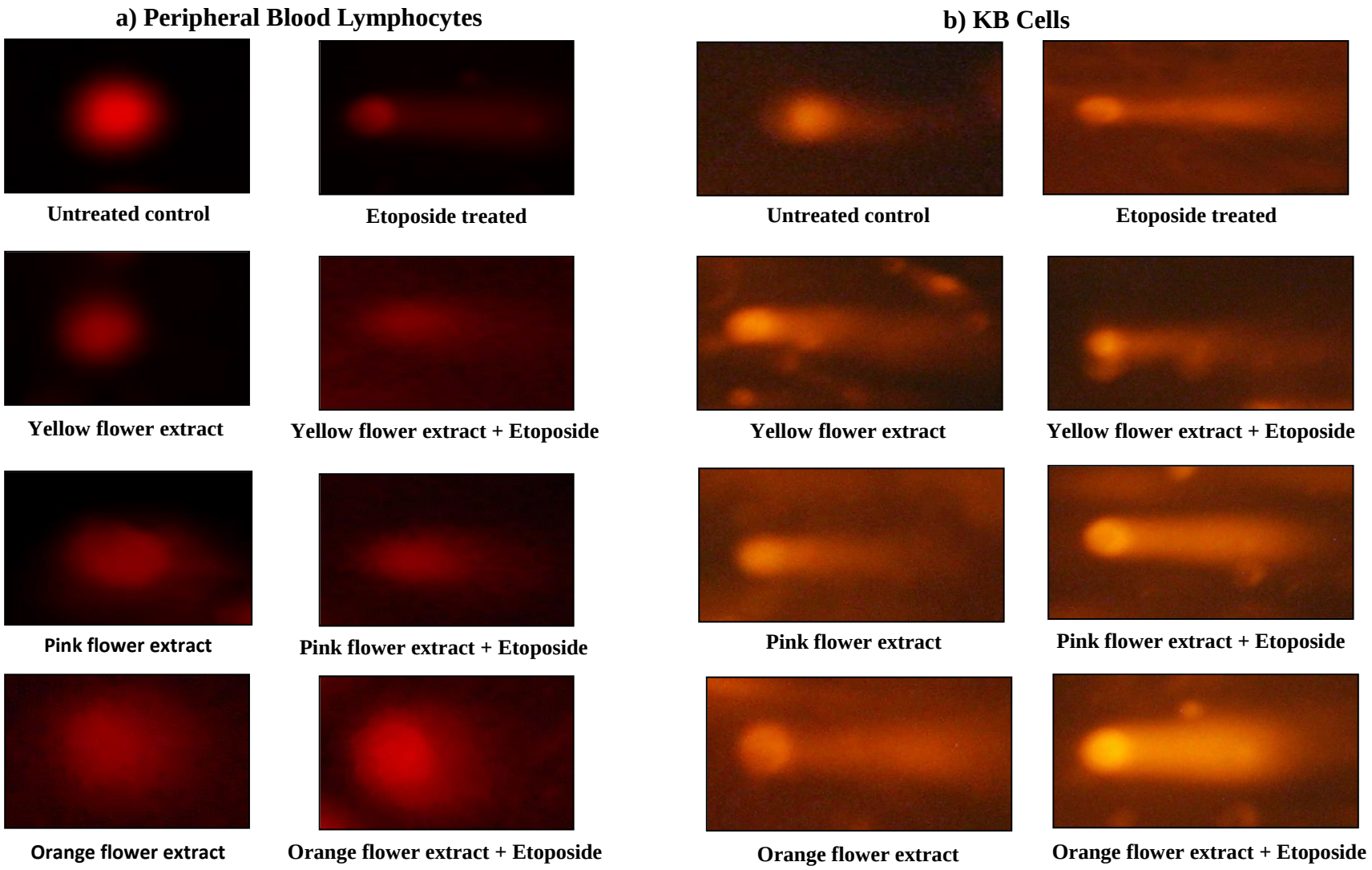


PLATE 4.14

Comet Bearing Cells in Peripheral Blood Lymphocytes and KB Cells

TABLE 4.36

EFFECT OF *C. pulcherrima* FLOWER EXTRACTS ON OXIDATIVE DNA DAMAGE IN KB CELLS

TREATMENT GROUPS	Number of Cells with comets / 100 Cells	
	Without etoposide	With etoposide
No Extract	10 ±1	67 ± 1[a]
Yellow Flower Extract	27 ± 1	76 ± 1[a,b,c]
Pink Flower Extract	29 ± 3	78 ± 1[a,b,c]
Orange Flower Extract	33 ± 1	83 ± 1[a,b,c]

The values are mean ± S.D of triplicates
a – Statistically significant (P<0.05) compared to untreated control
b – Statistically significant (P<0.05) compared to oxidant alone treated group
c – Statistically significant (P<0.05) compared to the respective plant extract treated group

TABLE 4.37

EFFECT OF *C. pulcherrima* FLOWER EXTRACTS ON COMET ASSAY PARAMETERS IN KB CELLS

TREATMENT GROUPS	Tail Length (µm)	Per cent Head DNA	Per cent Tail DNA	Olive Tail Moment
Cells alone	74	80.29	19.70	10.38
Cells + Etoposide	149	52.40	47.59	55.24
Cells + CPY	131	58.96	41.03	32.06
Cells + CPY + Etoposide	151	52.31	47.38	49.11
Cells + CPP	137	57.39	42.60	30.21
Cells + CPP + Etoposide	154	52.58	47.41	47.14
Cells + CPO	135	56.12	43.87	46.46
Cells + CPO + Etoposide	158	51.91	48.08	53.85

CPY – *C. pulcherrima* yellow flower
CPP – *C. pulcherrima* pink flower
CPO – *C. pulcherrima* orange flower

The number of comet bearing cells per 100 cells was calculated in three different fields for each treatment group using fluorescent microscope and the results are shown in Table 4.34 (peripheral blood lymphocytes) and Table 4.36 (KB cells). The extent of DNA damage was further analysed using a computerized image analysis system (CASP software).

The various comet parameters namely per cent tail DNA, per cent head DNA, tail length (µm) and olive tail moment were calculated for each treatment group and the values obtained are shown in Table 4.35 (peripheral blood lymphocytes) and Table 4.37 (KB cells).

Etoposide, a potent anti-tumor drug belongs to the class of topoisomerase poisons, which cause cytotoxicity by inducing oxidative DNA damage (Montecucco and Biamonti, 2007). Exposure to etoposide caused a steep increase in the number of comet bearing cells in both peripheral blood lymphocytes and KB cells compared to the respective untreated control. The DNA damage was severe in etoposide treated cells.

In the peripheral blood lymphocytes, all the three flower extracts significantly reduced the incidence of comets in the presence of etoposide. In KB cells, the number of comet bearing cells was increased on co-treatment with the flower extracts and etoposide. These observations, thus confirm the protective effects of the three flower extracts in non-transformed cells against oxidative DNA damage. The enhancement of etoposide-induced DNA damage in KB cells by the flower extracts reveals that the flower extracts exhibit a differential response towards cancerous and non-cancerous cells.

4.6. EFFECTS OF THE FLOWERS OF C. *pulcherrima* ON THE EXPRESSION OF APOPTOTIC PROTEINS (TP53, Bax AND Bcl-2) IN KB CELLS

The apoptosis-inducing effects of all the three flowers of *C. pulcherrima* was confirmed by analysing their effect on the expression of key proteins involved in apoptosis using immunocytochemistry. The expression of pro-apoptotic proteins (TP53 and Bax) and the anti-apoptotic protein (Bcl-2) were analysed after treatment with the flower extracts of *C. pulcherrima* and/or etoposide. The results are depicted in Plates 4.15 – 4.17.

The results obtained revealed that, when compared to the untreated control, the treatment with etoposide significantly increased the expression of pro-apoptotic proteins (TP53 and Bax), whereas, it decreased the expression of the anti-apoptotic protein Bcl-2. Similarly, treatment with the flower extracts significantly upregulated the expression of the TP53 and Bax (Plates 4.15 and 4.16) both in the presence and the absence of etoposide. On the other hand, the expression of the anti-apoptotic protein Bcl-2 was downregulated in the presence of the flower extracts (Plate 4.17).

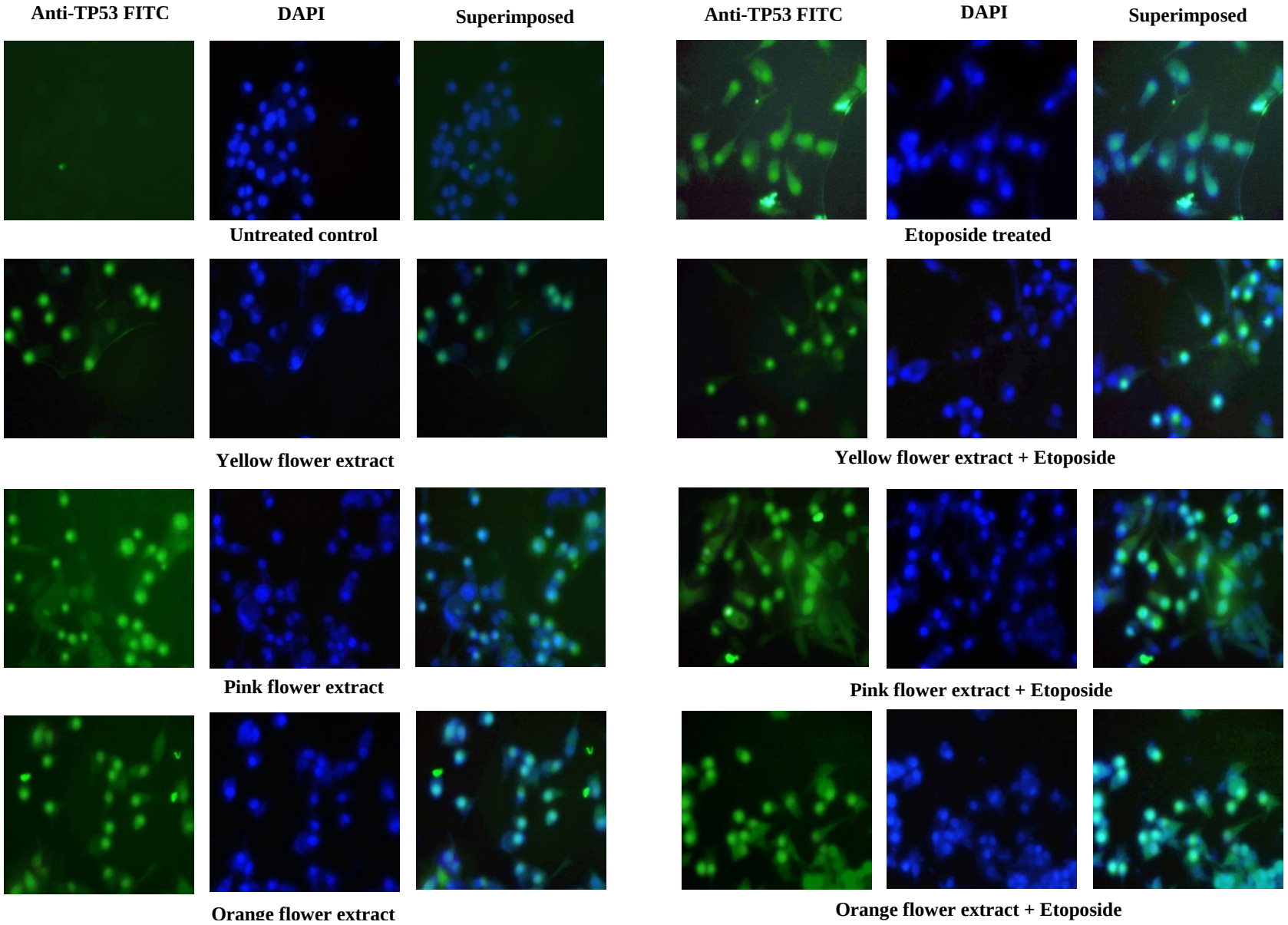


PLATE 4.15

Immunocytochemical Analysis of TP53 Protein Expression in KB Cells

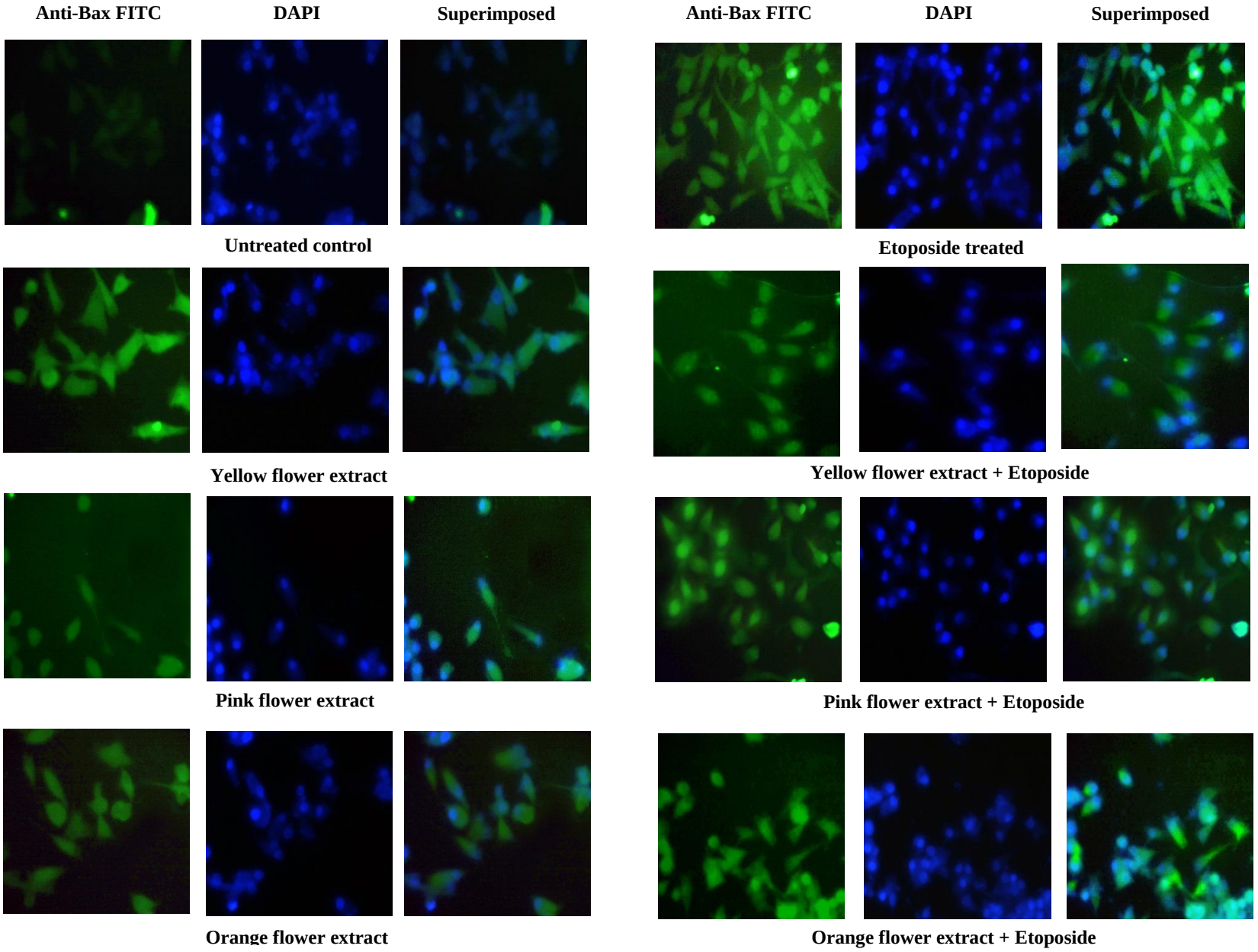


PLATE 4.16

Immunocytochemical Analysis of Bax Protein Expression in KB Cells

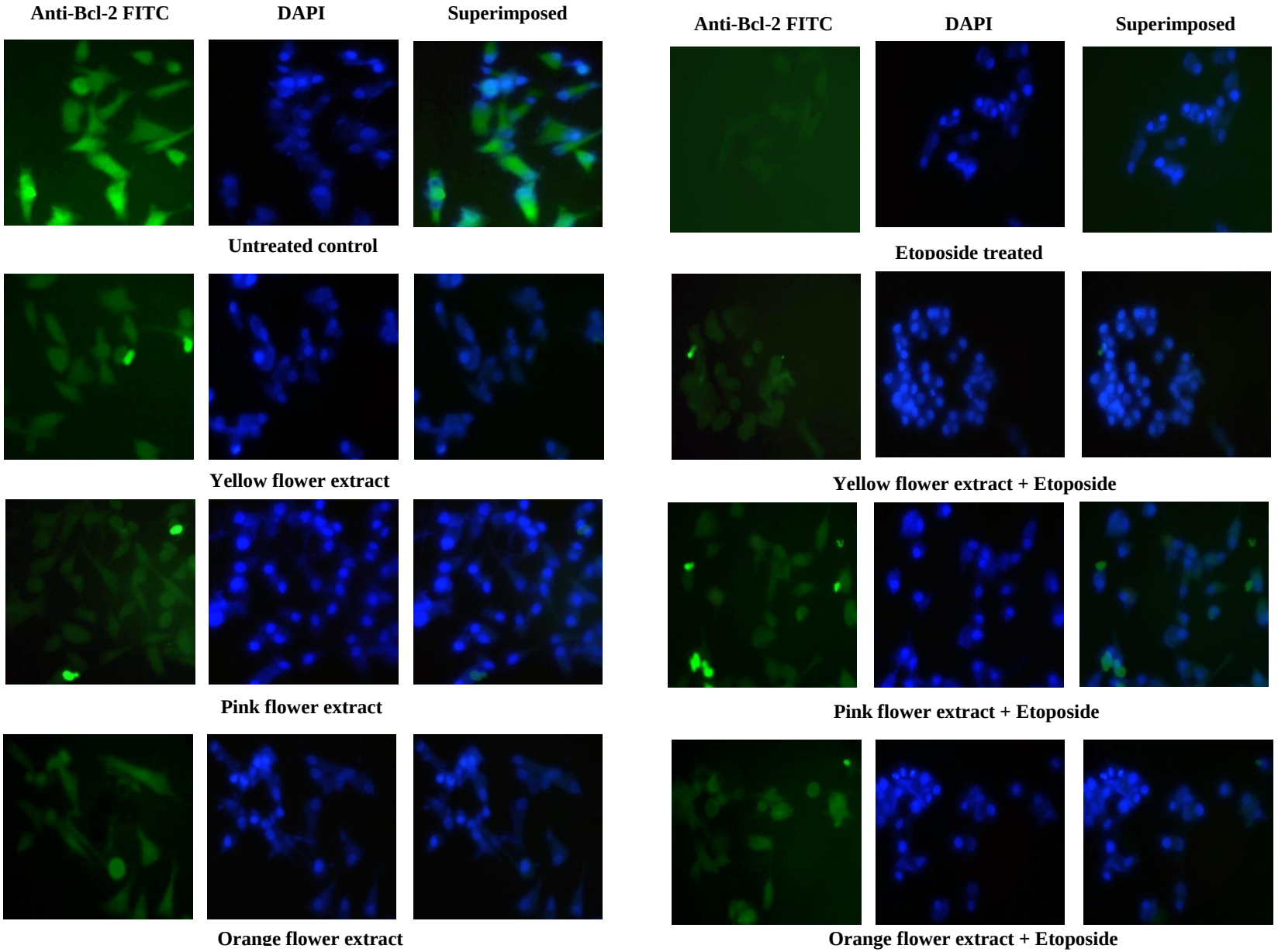


PLATE 4.17

Immunocytochemical Analysis of Bcl-2 Protein Expression in KB Cells

Thus, the differential expression of proteins associated with apoptosis validate the findings of the present study, wherein all the three flower extracts of *C. pulcherrima* inhibit cancer cell proliferation through induction of apoptosis.

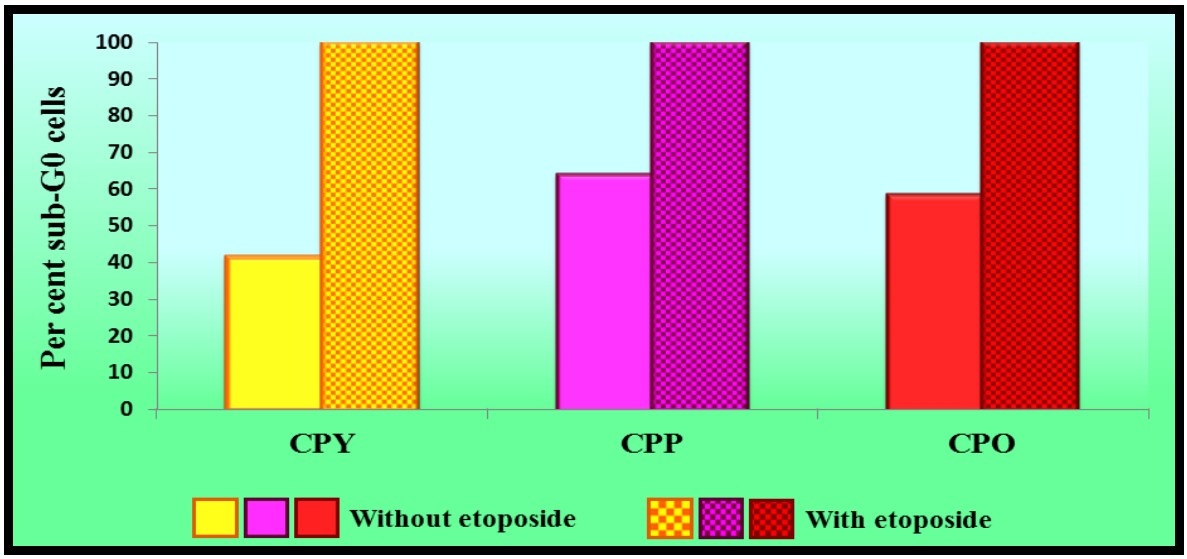
4.7. EFFECTS OF THE FLOWERS OF C. *pulcherrima* ON CELL CYCLE IN KB CELLS AS DETERMINED BY FLOW CYTOMETRY

The effect of the flower extracts of *C. pulcherrima* on cell cycle progression of oral carcinoma (KB) cells was analysed using flow cytometry both in the presence and the absence of etoposide. The relative percentage of KB cells in each phase of the cell cycle was determined. The DNA content was evaluated with propidium iodide staining and the relative fluorescence was measured. The results obtained are shown in Figures 4.19 and 4.20 a,b.

Cell cycle distribution showed that the proportion of KB cells at late apoptosis stage (sub-G0 phase) was significantly increased after treatment with etoposide. An increase in the proportion of cells in the sub-G0 phase was also observed in all three flower extract treated groups both in the presence and the absence of etoposide. Cell cycle arrest at sub-G0 phase showed that the flower extracts exerted anticancer effect by inducing apoptosis.

FIGURE 4.19

EFFECT OF *C. pulcherrima* FLOWER EXTRACTS ON CELL CYCLE DISTRIBUTION OF KB CELLS



CPY – *C. pulcherrima* yellow flower
CPP – *C. pulcherrima* pink flower
CPO – *C. pulcherrima* orange flower
The values of etoposide-treated groups were fixed as 100 per cent and the relative values in percentage of sub-G0 cells was calculated for the respective group treated without etoposide

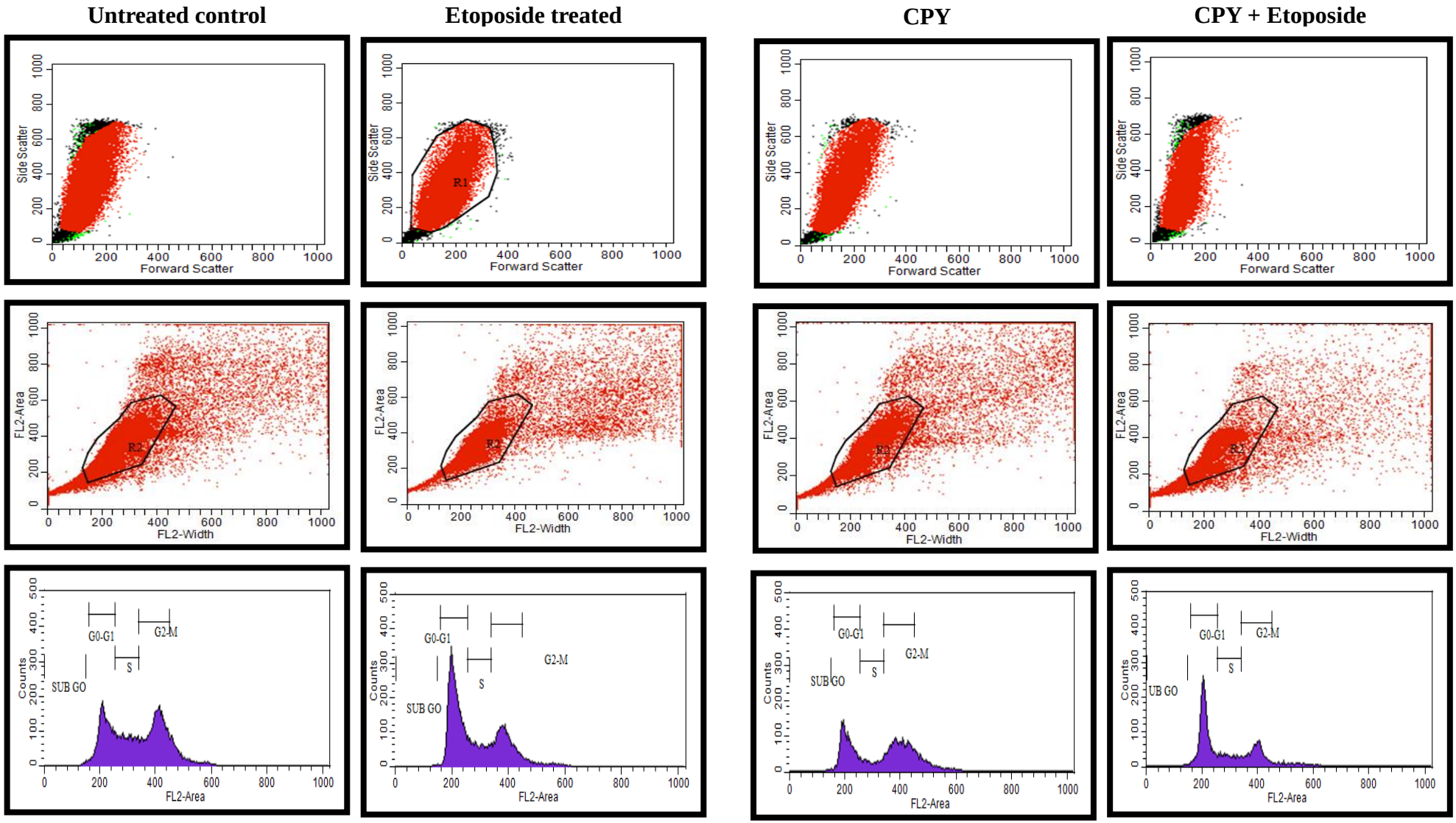


FIGURE 4.20 (a)

Effect of *C. pulcherrima* Flower Extracts on Cell Cycle Distribution in KB Cells

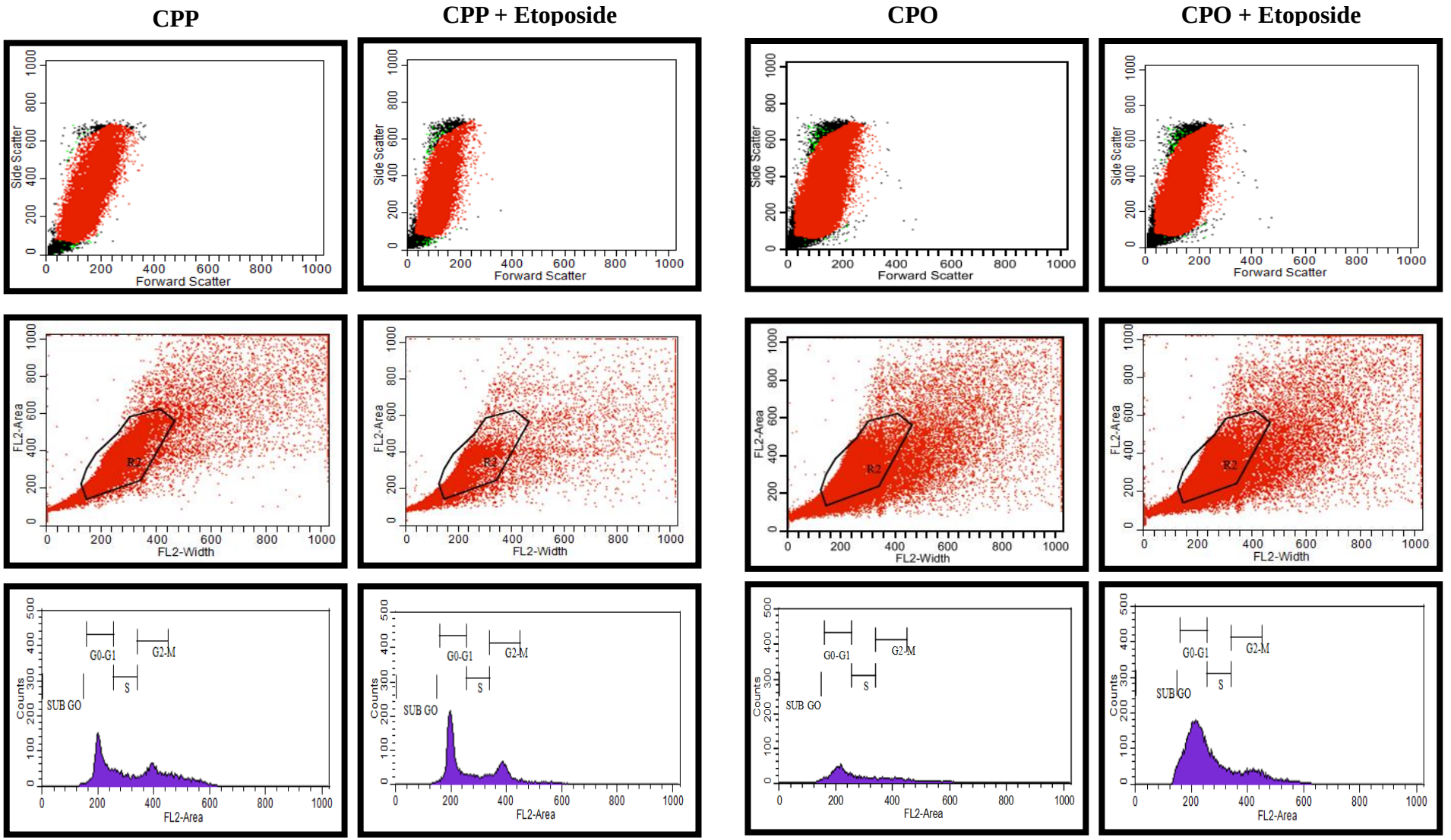


FIGURE 4.20 (b)

Effect of *C. pulcherrima* Flower Extracts on Cell Cycle Distribution in KB Cells

Phase IV

It is evident from the results of the first three phases that the methanolic extract of all the three flowers of *C. pulcherrima* possess strong antioxidant potential, render biomolecule protection against oxidative stress, modulate oxidant-induced apoptosis in non-transformed cells and reduce the viability of cancer cells under *in vivo*-simulated *in vitro* conditions. From these observations, it became essential that further research has to be carried out to identify the bioactive compounds present in the flowers that render these beneficial effects. Thus, the final phase of the study was formulated to identify the active principle(s) rendering the antioxidant responses evoked by the flower extracts against oxidative stress.

In the fourth phase, preliminary screening and qualitative phytochemical analysis, followed by various chromatographic (TLC, HPTLC and HPLC) and spectral (UV absorption, FT-IR and GC-MS) analysis were carried out. The phytochemical fractions of all the three flowers were extracted and their radical quenching activity was also evaluated.

4.8. PRELIMINARY QUALITATIVE PHYTOCHEMICAL ANALYSIS OF THE FLOWERS OF *C. pulcherrima*

Phytochemical analysis of the fresh flowers of *C. pulcherrima* was carried out to identify the presence of the major phytochemicals. The qualitative analysis of all the three flowers showed the presence of alkaloids, phenols, flavonoids, saponins, sterols and tannins (Table 4.38).

It can be inferred from the results that the active components present in all the three flowers of *C. pulcherrima* may belong to the six groups of phytochemicals, namely alkaloids, phenols, flavonoids, saponins, sterols or tannins. These six phytochemical fractions of the three flowers were isolated and their radical quenching activity was evaluated, followed by UV absorption and TLC analysis.

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

The phytochemical fractions of the three flowers namely alkaloids, phenols, flavonoids, saponins, steroids, tannins and terpenoids were isolated and their radical quenching activity was evaluated against a battery of free radicals (DPPH, ABTS, H_2O_2 and hydroxyl). The results obtained are shown in Figures 4.21 and 4.22.

TABLE 4.38

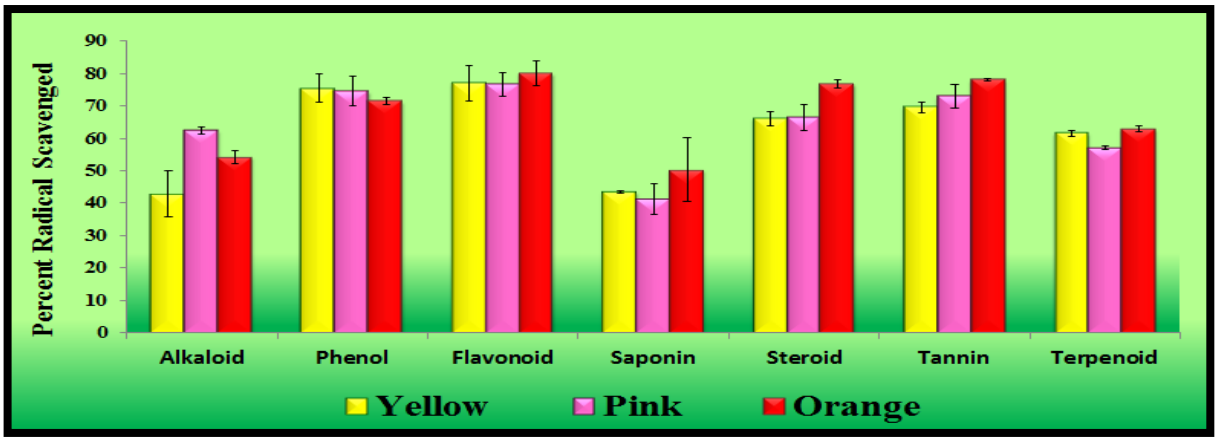
QUALITATIVE PHYTOCHEMICAL ANALYSIS OF *C. pulcherrima* FLOWERS

S. No.	COMPONENTS	CPY	CPP	CPO
1.	**ALKALOIDS**			
	Mayer's test	+	+	+
	Dragondroff's test	+	+	+
	Wagner's test	+	+	+
2.	**PHENOLS**			
	Ferric chloride test	+	+	+
	Lead acetate test	+	+	+
3.	**FLAVONOIDS**			
	Aqueous NaOH test	+	+	+
	Concentrated sulfuric acid test	+	+	+
	Schinado's test	+	+	+
4.	**SAPONINS**			
	Froth test	+	+	+
	Haemolytic test	+	+	+
5.	**STEROIDS**			
	Leibermann-Buchard test	+	+	+
6.	**TANNINS**			
	Braemer's test	+	+	+
7.	**TERPENOIDS**			
	Salkowski test	+	+	+

CPY – *C. pulcherrima* yellow flower
CPP – *C. pulcherrima* pink flower
CPO – *C. pulcherrima* orange flower

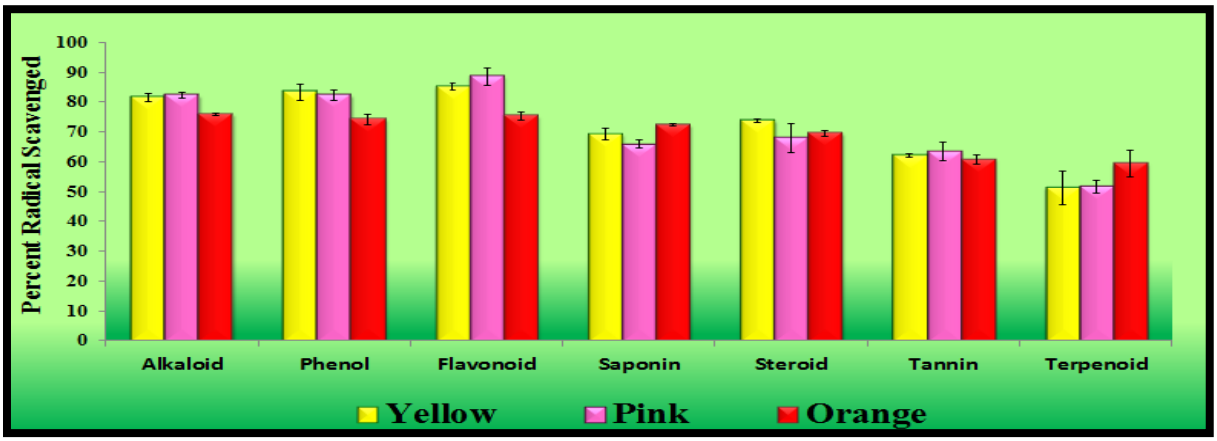
All the seven phytochemical fractions of the three flowers of *C. pulcherrima* significantly reduced the DPPH (Figure 4.21a) and ABTS (Figure 4.21b) radicals. In all the three flowers, the maximum radical scavenging was elicited by the flavonoid fraction, followed by the phenolic fraction. Alkaloid, saponin, steroid, tannin and terpenoid fractions showed moderate radical scavenging activity. A similar trend was observed for H_2O_2 (Figure 4.21c) and hydroxyl (Figures 4.22 a,b,c) scavenging activities of all the three flowers.

FIGURE 4.20 (a)
DPPH Scavenging Effects of the Phytochemical Fractions of the Flowers of *C. pulcherrima*



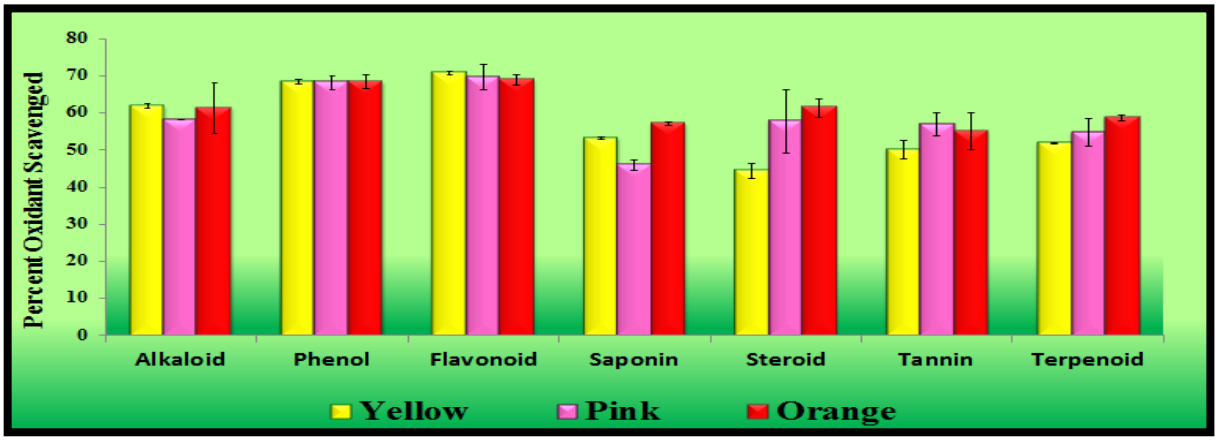
The values are mean ± SD of triplicates

FIGURE 4.20 (b)
ABTS Scavenging Effects of the Phytochemical Fractions of the Flowers of *C. pulcherrima*



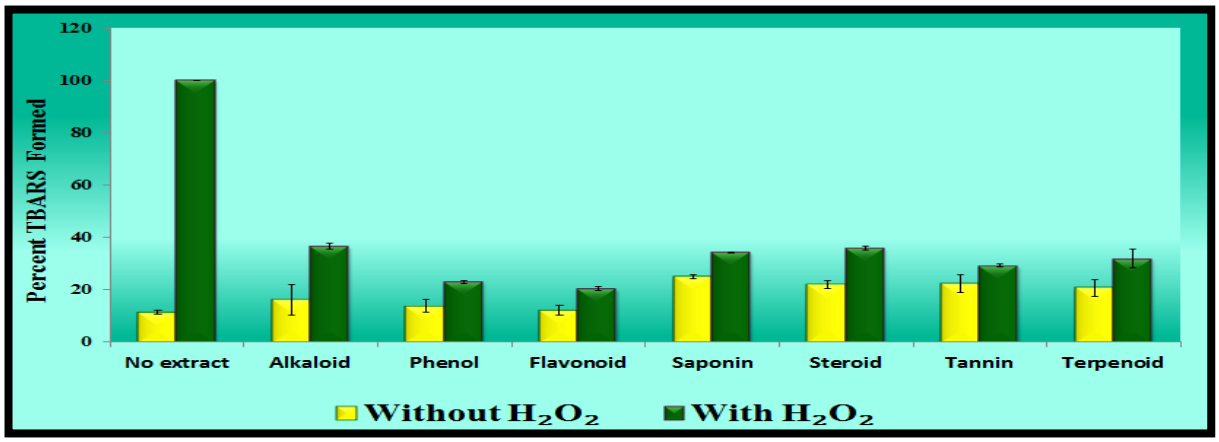
The values are mean ± SD of triplicates

FIGURE 4.20 (c)
H_2O_2 Scavenging Effects of the Phytochemical Fractions of the Flowers of *C. pulcherrima*



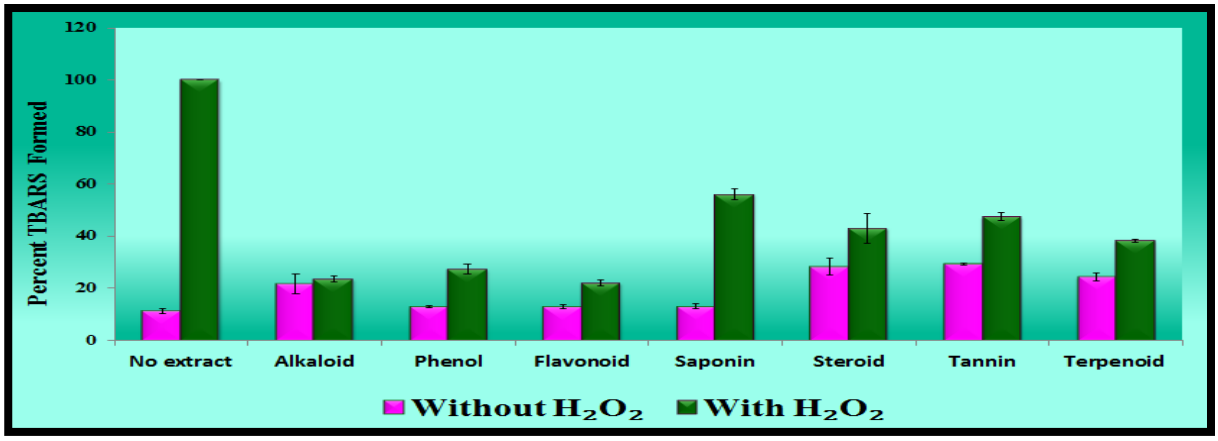
The values are mean ± SD of triplicates

FIGURE 4.22 (a)
Hydroxyl Scavenging Effects of the Phytochemical Fractions of *C. pulcherrima* Yellow Flower



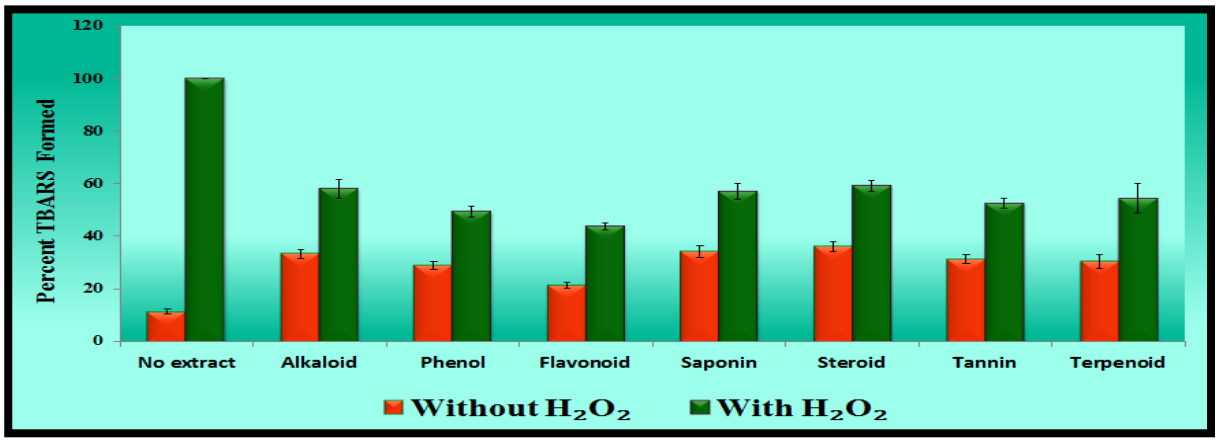
The values are mean ± SD of triplicates

FIGURE 4.22 (b)
Hydroxyl Scavenging Effects of the Phytochemical Fractions of *C. pulcherrima* Pink Flower



The values are mean ± SD of triplicates

FIGURE 4.22 (c)
Hydroxyl Scavenging Effects of the Phytochemical Fractions of *C. pulcherrima* Orange Flower



The values are mean ± SD of triplicates

4.10. UV ABSORPTION SPECTRUM OF THE FLOWERS OF *C. pulcherrima*

The UV absorption spectrum of the methanolic extract of the three flowers of *C. pulcherrima* was evaluated in the wavelength ranging from 190 nm to 420 nm. The specific absorption spectrum obtained for the three flower extracts are shown in Figures 4.23 – 4.25.

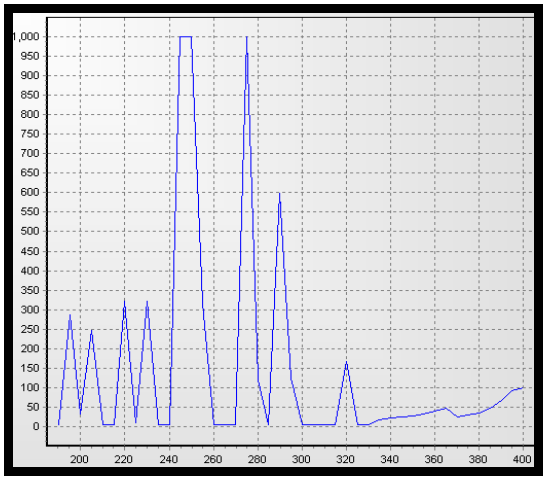


FIGURE 4.23

UV Absorption Spectrum of the Methanolic Extract of *C. pulcherrima* Yellow Flower

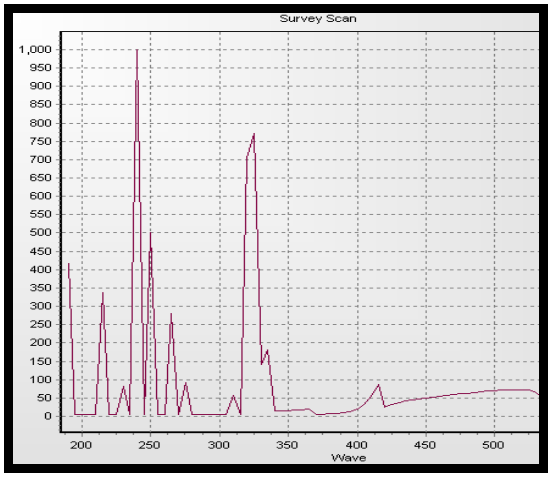


FIGURE 4.24

UV Absorption Spectrum of the Methanolic Extract of *C. pulcherrima* Pink Flower

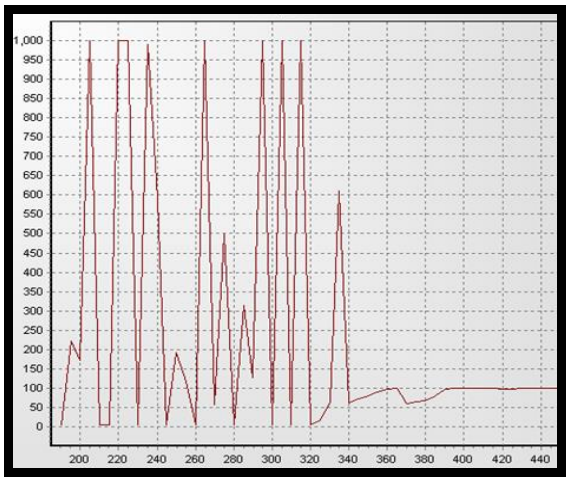


FIGURE 4.25

UV Absorption Spectrum of the Methanolic Extract of *C. pulcherrima* Orange Flower

The absorption spectrum of the methanolic extract of the yellow flowers of *C. pulcherrima* (Figure 4.23) showed the presence of three major and four minor peaks between 190 nm to 300 nm, followed by an another minor peak at 320 nm. In the absorption spectrum of the methanolic extract of the pink flowers of *C. pulcherrima* (Figure 4.24), five major peaks and four minor peaks between 190 to 350 nm were found. The absorption spectrum of the methanolic extract of the orange flowers of *C. pulcherrima* (Figure 4.25) revealed the presence seven major peaks and four minor peaks between 190 nm to 320 nm.

4.11. TLC ANALYSIS OF THE OF THE FLOWERS OF *C. pulcherrima*

The TLC phytochemical profiles of the phytochemical fractions of the three flowers of *C. pulcherrima* are presented in Plate 4.15. The chromatogram of the TLC plate detected with the alkaloid-specific Dragendroff's spraying reagent, showed six major bands with R_f vales 0.32, 0.45, 0.54, 0.61, 0.72 and 0.78 for all three flowers of *C. pulcherrima* (Plate 4.17a). The phenolic compounds were detected using Folin-Ciocalteau reagent (Plate 4.17b), in which five major bands with R_f vales 0.36, 0.49, 0.59, 0.78 and 0.82 were found in all the three flower extracts. The chromatogram of all the three flowers sprayed with 1% ethanolic aluminium chloride for the presence of flavonoids, showed five major bands with R_f values 0.44, 0.50, 0.57, 0.69 and 0.82 (Plate 4.17c). TLC chromatogram for saponins showed four major bands with R_f values 0.46, 0.53, 0.64 and 0.83 (Plate 4.17d) for all the three flowers.

The TLC profile of the steroids (Plate 4.17e) showed eight major bands with R_f values 0.14, 0.17, 0.26, 0.35, 0.48, 0.60, 0.68 and 0.77 for the yellow flower extract, whereas seven major bands with R_f values 0.13, 0.16, 0.24, 0.48, 0.60, 0.68 and 0.77 were observed for the pink and the orange flower extracts. The presence of tannins in the TLC chromatogram was detected using 5% ferric chloride reagent, wherein ten major bands with R_f values 0.20, 0.29, 0.40, 0.50, 0.57, 0.65, 0.72, 0.78, 0.85 and 0.90 were observed for all the three flower extracts (Plate 4.17f). The terpenoid profile of the three flower extracts using TLC showed the presence of fourteen major bands with R_f values 0.11, 0.15, 0.19, 0.23, 0.29, 0.34, 0.37, 0.43, 0.49, 0.54, 0.64, 0.68, 0.78 and 0.83 (Plate 4.17g).

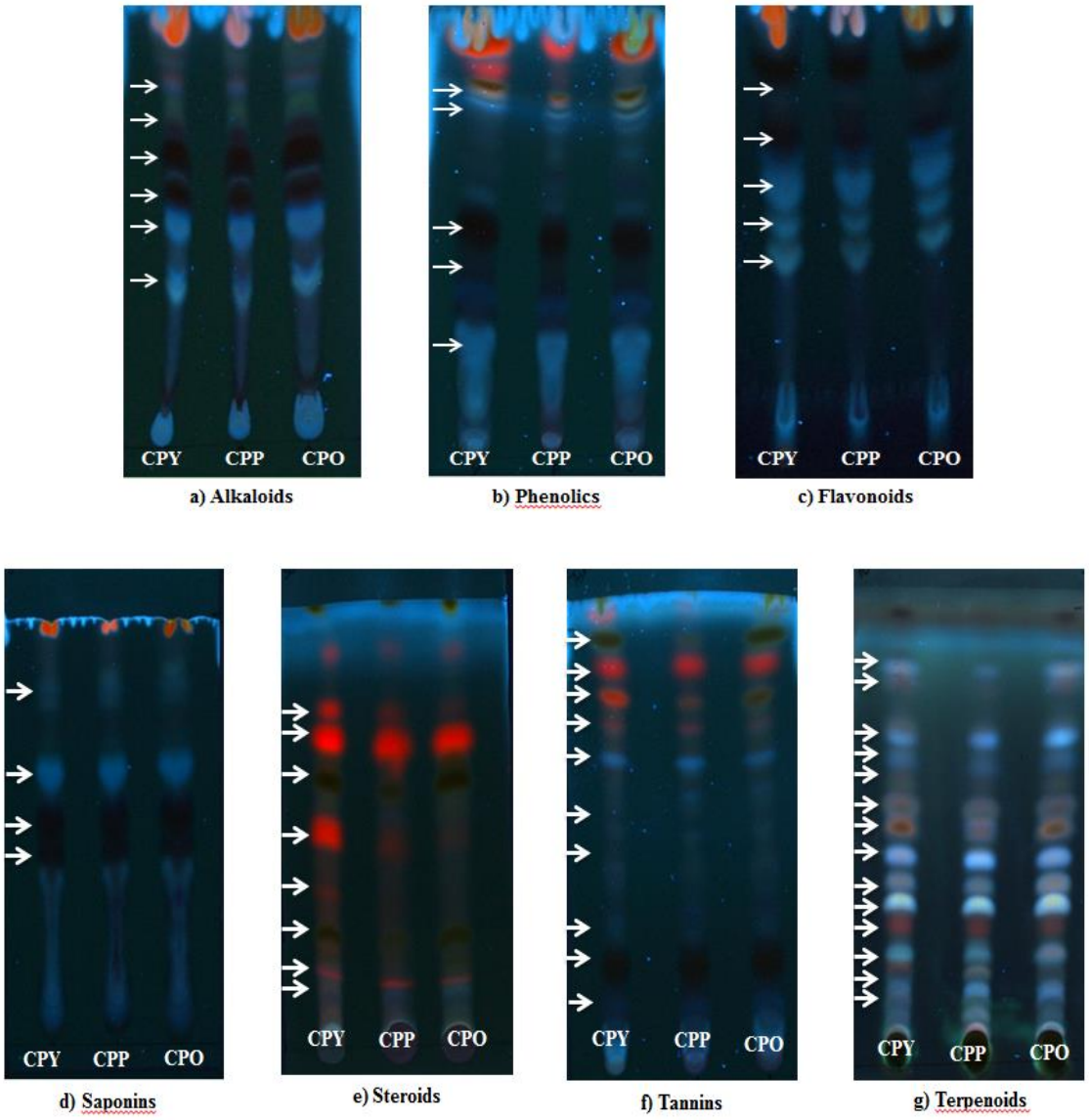


PLATE 4.18

TLC Analysis of the Flowers of *C. pulcherrima*

4.12. HPTLC ANALYSIS OF THE FLOWERS OF *C. pulcherrima*

The methanolic extract of all the three flowers of *C. pulcherrima* were subjected to HPTLC analysis for the presence of alkaloids, phenols, flavonoids, saponins, steroids, tannins and terpenoids. The alkaloid profile (R_f values, height and area of the peaks) of the methanolic extract of the three flowers of *C. pulcherrima* are shown in Table 4.39. In the chromatogram after derivatization, the yellowish brown coloured zones in the day light mode found in the respective standard and sample tracks (Plate 4.18) showed the presence of alkaloid compounds. The extracts were run along with the standard alkaloid compound colchicine. The R_f value of the colchicine standard was

0.47. In the methanolic extract of the yellow flower, five peaks (3, 5, 6, 7 and 8) with the R_f values 0.13, 0.34, 0.50, 0.56 and 0.66 showed the presence of five alkaloid compounds. Similarly, five peaks showed the presence of alkaloid compounds in the methanolic extract of the pink flower (peaks 2, 3, 4, 5 and 6 with R_f values 0.14, 0.35, 0.50, 0.57 and 0.66) and the orange flower (peaks 2, 3, 4, 5 and 6 with R_f values 0.13, 0.36, 0.52, 0.58 and 0.68).

The phenolic profile (R_f values, height and area of the peaks) of the methanolic extract of the three flowers of *C. pulcherrima* are shown in Table 4.40. Blue coloured zones at day light mode after derivatization in the sample tracks (Plate 4.19) confirmed the presence of phenolic compounds. The standard phenolic compound quercetin was used as the reference compound. The R_f value of the quercetin standard was 0.71. As seen in the peak densitogram (Figure 4.27), in the methanolic extract of all the three flowers, eight peaks (1 to 8) with R_f values 0.07, 0.14, 0.19, 0.29, 0.42, 0.53, 0.67 and 0.70 showed the presence of phenolic compounds, among which the peak 8 with R_f value 0.70 closely matched with the standard compound quercetin.

The flavonoid profile (R_f values, height and area of the peaks) of the methanolic extract of the three flowers of *C. pulcherrima* are shown in Table 4.41. Yellowish blue coloured fluorescent zones in the UV 366 mode after derivatization in the sample tracks (Plate 4.20) confirmed the presence of flavonoid compounds. The standard flavonoid compound rutin was used as the reference compound. The R_f value of the rutin standard was 0.44. In the methanolic extract of all the three flowers, four peaks (peak 2, 6, 7 and 8 for yellow and orange flower and peak 2, 7, 8 and 9 for the pink flower) with R_f values 0.26, 0.69, 0.81 and 0.85 showed the presence of phenolic compounds. The peak densitogram of the flavonoid profile of the three flowers are shown in Figure 4.28.

Plate 4.21 confirmed the presence of saponins in the methanolic extract of the three flowers of *C. pulcherrima* where the standard saponin was used as the reference compound. Blue, yellowish brown coloured zones in the visible light mode confirmed the presence of saponins in the given samples (Figure 4.29; Table 4.42). Three peaks with R_f values 0.33, 0.47 and 0.56 were found for the standard compound saponin. As shown in Table 4.35, in the methanolic extract of the yellow flower, six peaks (1, 4, 5, 6, 7 and 8) with R_f values 0.01, 0.26, 0.40, 0.62, 0.70 and 0.78 showed the presence of six saponins. Similarly, six peaks showed the presence of saponins in the methanolic extract of the pink flower (peaks 2, 4, 5, 6, 7 and 8 with R_f values 0.29, 0.45, 0.64, 0.72,

0.80 and 0.96) whereas seven peaks (peaks 5, 6, 7, 8, 9 and 10 with R_f values 0.29, 0.41, 0.42, 0.67, 0.74, 0.81 and 0.97) showed the presence of saponins in the methanolic extract of the orange flower.

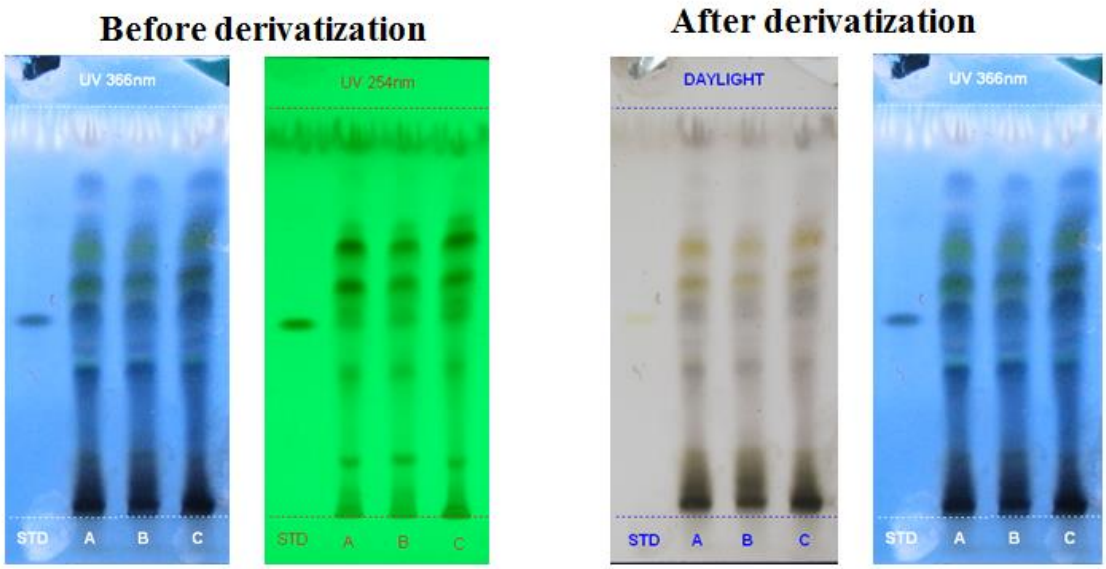
The steroid profile of the methanolic extract of the three flowers of *C. pulcherrima* was analysed using solasodine as the standard. Blue-violet coloured zones in the day light mode present in the given standard and sample tracks observed in the chromatogram after derivatization confirmed the presence of sterols in the three flowers of *C. pulcherrima* (Plate 4.22; Figure 4.30; Table 4.43). The R_f value of the solasodine standard was 0.19. In the methanolic extract of the yellow flower, seven peaks (peak 3, 4, 5, 6, 7, 9 and 11) with R_f values 0.06, 0.09, 0.17, 0.22, 0.26, 0.46 and 0.59 showed the presence of streoids. Five peaks showed the presence of steroids in the methanolic extract of the pink flower (peaks 3 to 7 with R_f values 0.06, 0.10, 0.17, 0.23 and 0.25) whereas six peaks (peaks 2 to 6 with R_f values 0.06, 0.10, 0.18, 0.23, 0.27 and 0.61) showed the presence of steroids in the methanolic extract of the orange flower.

The tannin profile of the methanolic extract of the three flowers of *C. pulcherrima* was analysed using tannic acid as the reference standard and the developed plate was sprayed with 5% ferric chloride. Bluish brown coloured zones in the day-light mode confirmed the presence of tannins (Plate 4.23). The R_f value of the standard tannic acid was 0.45. The peak table (Table 4.44) and peak densitogram (Figure 4.31) showed five peaks in the methanolic extract of all the three flowers (for the yellow flower, peaks 1, 2, 4, 5 and 7 with R_f values 0.12, 0.23, 0.38, 0.43 and 0.65; for the pink flower, peaks 2, 3, 5, 6 and 8 with R_f values 0.09, 0.13, 0.24, 0.23 and 0.65; for the orange flower, peaks 1 to 5 with R_f values 0.13, 0.25, 0.41, 0.61, 0.68) showed the presence of tannin compounds.

In the terpenoid profile of the methanolic extract of the three flowers of *C. pulcherrima*, bluish violet coloured zones at day-light mode confirmed the presence of terpenoids (Plate 4.24). The R_f value of the standard was 0.78. The peak table (Table 4.45) and peak densitogram (Figure 4.32) showed eight peaks in the methanolic extract of the yellow flower (peaks 2 to 6, 8, 9 and 12 with R_f values 0.15, 0.26, 0.36, 0.41, 0.46, 0.55, 0.65 and 0.84), six peaks in the methanolic extract of the pink flower (peaks 3, 5 to 8 and 10 with R_f values 0.15, 0.26, 0.35, 0.42, 0.46 and 0.65) and nine peaks in the methanolic extract of the orange flower (peaks 3 to 10 and 13 with R_f values 0.16, 0.23, 0.27, 0.37, 0.43, 0.47, 0.58, 0.66 and 0.84) showed the presence of terpenoids.

PLATE 4.19

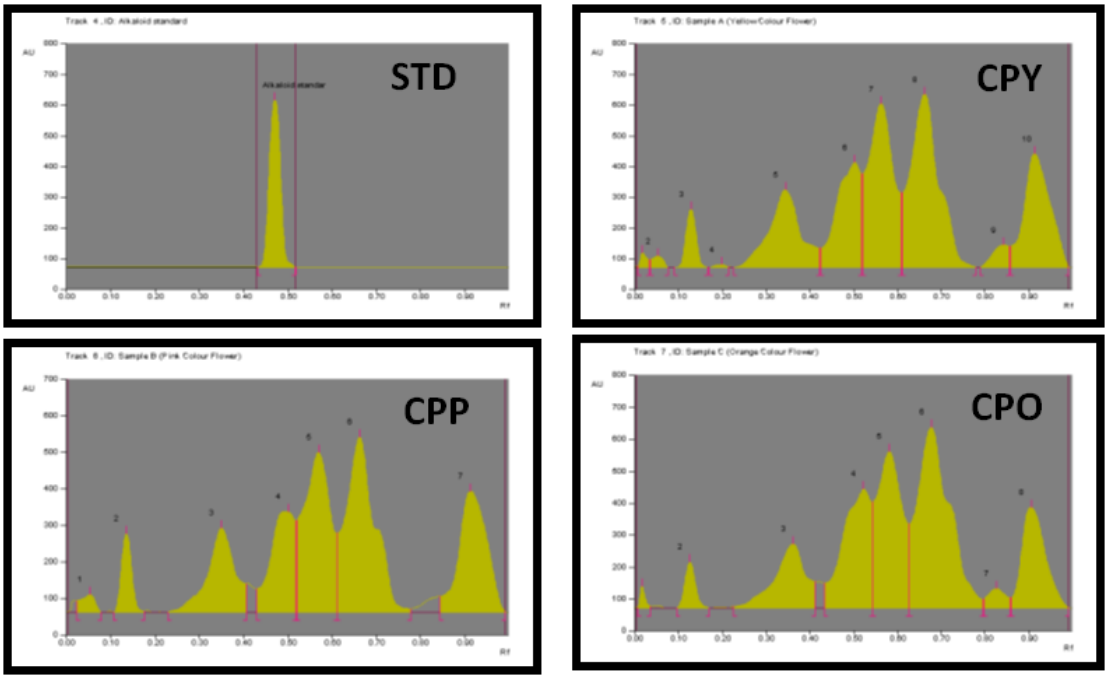
HPTLC of Alkaloids



Lane A – *C. pulcherrima* yellow flower
Lane B – *C. pulcherrima* pink flower
Lane C– *C. pulcherrima* orange flower

FIGURE 4.26

HPTLC Peak Densitogram of Alkaloids in the Flowers of *C. pulcherrima*



STD – Standard
CPY – *C. pulcherrima* yellow flower
CPP – *C. pulcherrima* pink flower
CPO – *C. pulcherrima* orange flower

TABLE 4.39

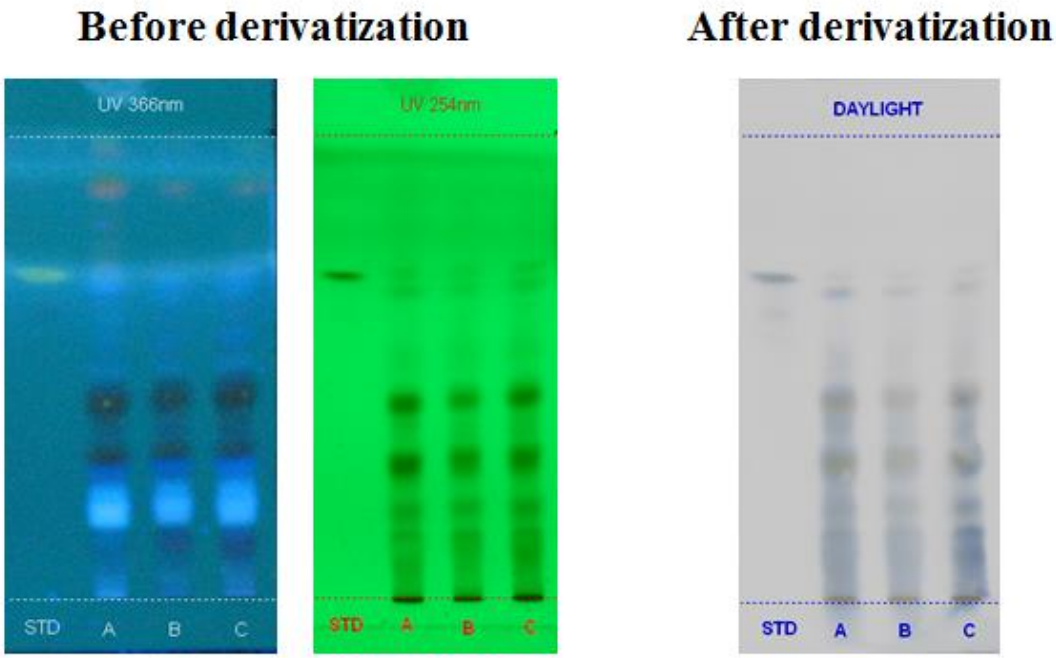
HPTLC PEAK TABLE FOR ALKALOIDS IN THE FLOWERS OF *C. pulcherrima*

Track	Peak	R_f	Height	Area	Assigned substance
STD	1	0.47	552.9	13482.7	Alkaloid standard
CPY	1	0.02	46.2	638.2	Unknown
	2	0.05	37.2	869.9	Unknown
	3	0.13	189.5	4057.7	Alkaloid 1
	4	0.20	10.2	206.3	Unknown
	5	0.34	253.4	16711.5	Alkaloid 2
	6	0.50	343.5	17414.8	Alkaloid 3
	7	0.56	534.9	28137.8	Alkaloid 4
	8	0.66	566.2	34144.9	Alkaloid 5
	9	0.84	77.5	2760.5	Unknown
	10	0.91	375.8	20865.1	Unknown
CPP	1	0.05	49.0	1493.6	Unknown
	2	0.14	215.4	4607.7	Alkaloid 1
	3	0.35	231.3	13112.1	Alkaloid 2
	4	0.50	277.7	14031.4	Alkaloid 3
	5	0.57	439.9	24160.6	Alkaloid 4
	6	0.66	481.2	29212.0	Alkaloid 5
	7	0.91	334.8	19745.3	Unknown
CPO	1	0.02	68.1	710.3	Unknown
	2	0.13	143.5	3209.7	Alkaloid 1
	3	0.36	201.4	13028.6	Alkaloid 2
	4	0.52	374.3	21872.9	Alkaloid 3
	5	0.58	492.1	25983.9	Alkaloid 4
	6	0.68	568.3	39142.6	Alkaloid 5
	7	0.82	64.4	2498.6	Unknown
	8	0.91	319.8	16277.9	Unknown

STD – Standard
CPY – *C. pulcherrima* yellow flower
CPP – *C. pulcherrima* pink flower
CPO – *C. pulcherrima* orange flower

PLATE 4.20

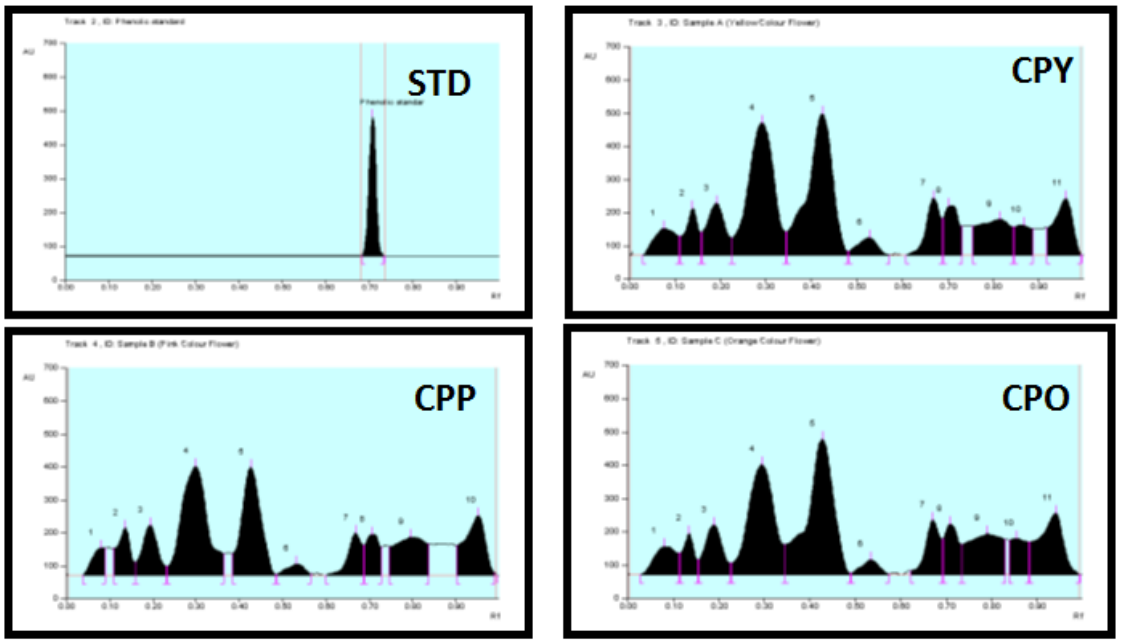
HPTLC of Phenolics



Lane A – *C. pulcherrima* yellow flower
Lane B – *C. pulcherrima* pink flower
Lane C– *C. pulcherrima* orange flower

FIGURE 4.27

HPTLC Peak Densitogram of Phenolics in the Flowers of *C. pulcherrima*



STD – Standard
CPY – *C. pulcherrima* yellow flower
CPP – *C. pulcherrima* pink flower
CPO – *C. pulcherrima* orange flower

TABLE 4.40

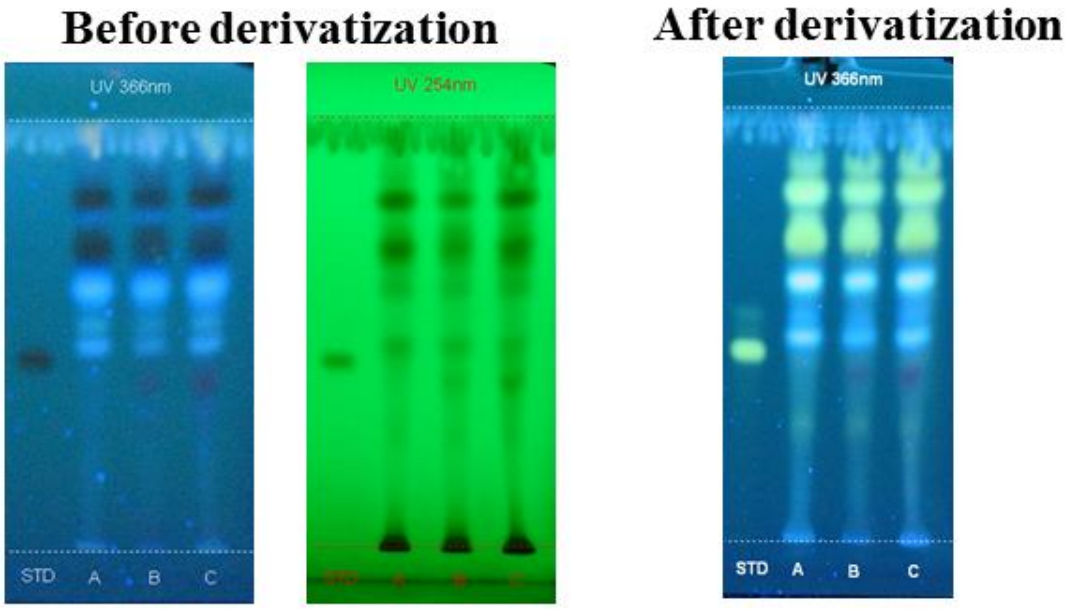
HPTLC PEAK TABLE FOR PHENOLICS IN THE FLOWERS OF *C. pulcherrima*

Track	Peak	R_f	Height	Area	Assigned substance
STD	1	0.71	486.1	10826.7	Phenolic standard
CPY	1	0.07	81.7	3664.7	Phenolic 1
	2	0.14	141.7	3570.3	Phenolic 2
	3	0.19	157.4	5775.9	Phenolic 3
	4	0.29	400.3	20964.1	Phenolic 4
	5	0.43	427.0	21945.6	Phenolic 5
	6	0.53	53.5	2137.4	Phenolic 6
	7	0.67	173.4	4947.5	Phenolic 7
	8	0.70	150.7	4426.9	Phenolic 8
	9	0.81	111.3	7227.2	Unknown
	10	0.87	93.5	3034.1	Unknown
	11	0.96	175.5	6673.7	Unknown
CPP	1	0.08	83.8	2202.2	Phenolic 1
	2	0.14	145.0	3981.3	Phenolic 2
	3	0.19	152.8	5140.3	Phenolic 3
	4	0.30	330.5	18220.7	Phenolic 4
	5	0.43	327.4	13333.2	Phenolic 5
	6	0.53	34.2	1316.6	Phenolic 6
	7	0.67	129.6	3826.7	Phenolic 7
	8	0.70	125.6	3661.0	Phenolic 8
	9	0.80	117.9	7806.5	Unknown
	10	0.95	183.9	8114.9	Unknown
CPO	1	0.08	85.5	3833.8	Phenolic 1
	2	0.14	124.0	2802.3	Phenolic 2
	3	0.19	150.3	5375.6	Phenolic 3
	4	0.29	331.0	17628.4	Phenolic 4
	5	0.43	407.2	21649.4	Phenolic 5
	6	0.53	44.7	1631.6	Phenolic 6
	7	0.67	164.9	4850.7	Phenolic 7
	8	0.70	152.4	4318.3	Phenolic 8
	9	0.79	124.8	8430.3	Unknown
	10	0.85	109.5	3675.4	Unknown
	11	0.94	184.5	9131.5	Unknown

STD – Standard
CPY – *C. pulcherrima* yellow flower
CPP – *C. pulcherrima* pink flower
CPO – *C. pulcherrima* orange flower

PLATE 4.21

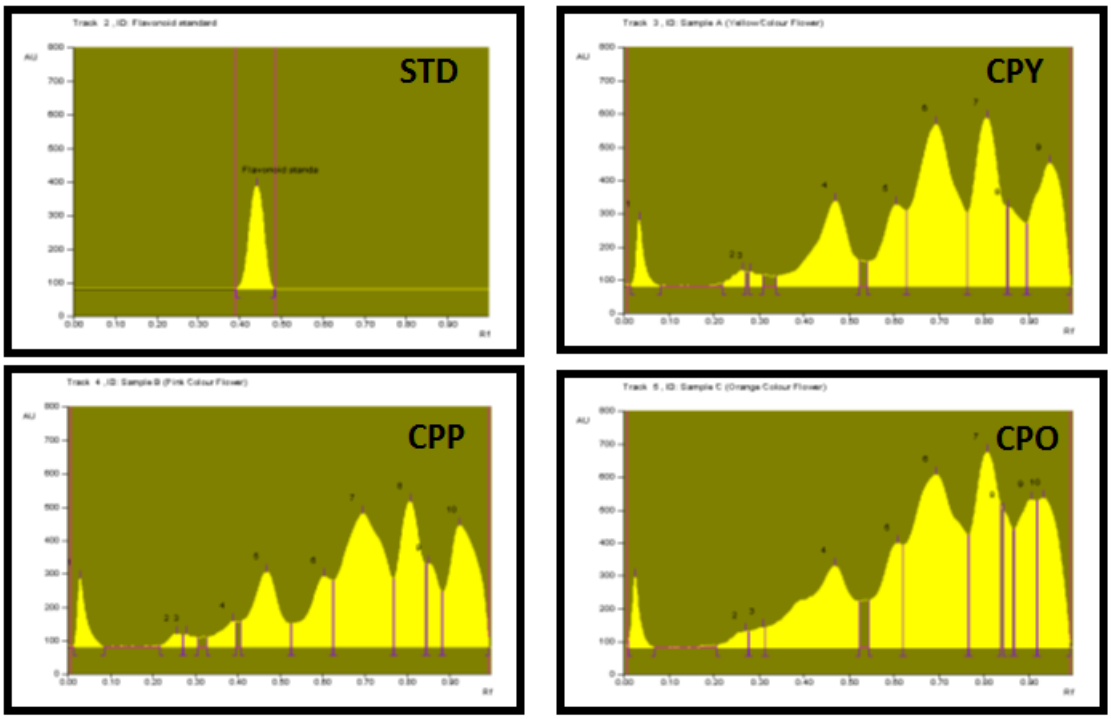
HPTLC of Flavonoids



Lane A – *C. pulcherrima* yellow flower
Lane B – *C. pulcherrima* pink flower
Lane C– *C. pulcherrima* orange flower

FIGURE 4.28

HPTLC Peak Densitogram of Flavonoids in the Flowers of *C. pulcherrima*



STD – Standard
CPY – *C. pulcherrima* yellow flower
CPP – *C. pulcherrima* pink flower
CPO – *C. pulcherrima* orange flower

TABLE 4.41

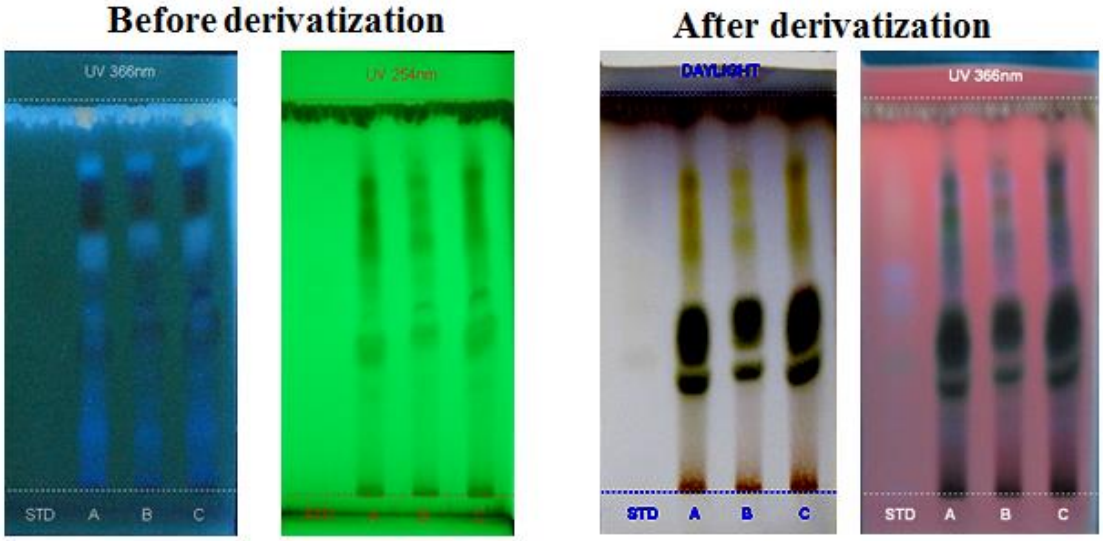
HPTLC PEAK TABLE FOR FLAVONOIDS IN THE FLOWERS OF *C. pulcherrima*

Track	Peak	R_f	Height	Area	Assigned substance
STD	1	0.44	338.9	12543.6	Flavonoid standard
CPY	1	0.03	200.6	2908.4	Unknown
	2	0.26	47.8	1318.3	Flavonoid 1
	3	0.28	44.5	988.5	Unknown
	4	0.47	256.4	17746.2	Unknown
	5	0.60	245.5	11773.2	Unknown
	6	0.69	487.0	37660.3	Flavonoid 2
	7	0.81	506.3	26002.5	Flavonoid 3
	8	0.85	236.0	6996.4	Flavonoid 4
	9	0.95	370.9	21011.7	Unknown
CPP	1	0.03	205.0	3721.8	Unknown
	2	0.26	39.3	1129.0	Flavonoid 1
	3	0.28	38.2	908.5	Unknown
	4	0.39	76.3	2832.8	Unknown
	5	0.47	224.2	13060.0	Unknown
	6	0.60	210.8	10909.0	Unknown
	7	0.69	399.4	35098.8	Flavonoid 2
	8	0.81	435.4	20265.5	Flavonoid 3
	9	0.85	249.0	6087.0	Flavonoid 4
	10	0.92	363.8	22522.9	Unknown
CPO	1	0.03	216.6	3243.6	Unknown
	2	0.27	50.6	1720.4	Flavonoid 1
	3	0.31	62.3	1697.6	Unknown
	4	0.47	248.6	25326.4	Unknown
	5	0.61	318.7	14394.8	Unknown
	6	0.69	525.9	49255.1	Flavonoid 2
	7	0.81	594.3	28733.4	Flavonoid 3
	8	0.85	416.3	7011.1	Flavonoid 4
	9	0.91	449.2	17094.5	Unknown
	10	0.93	454.0	19929.4	Unknown

STD – Standard
CPY – *C. pulcherrima* yellow flower
CPP – *C. pulcherrima* pink flower
CPO – *C. pulcherrima* orange flower

PLATE 4.22

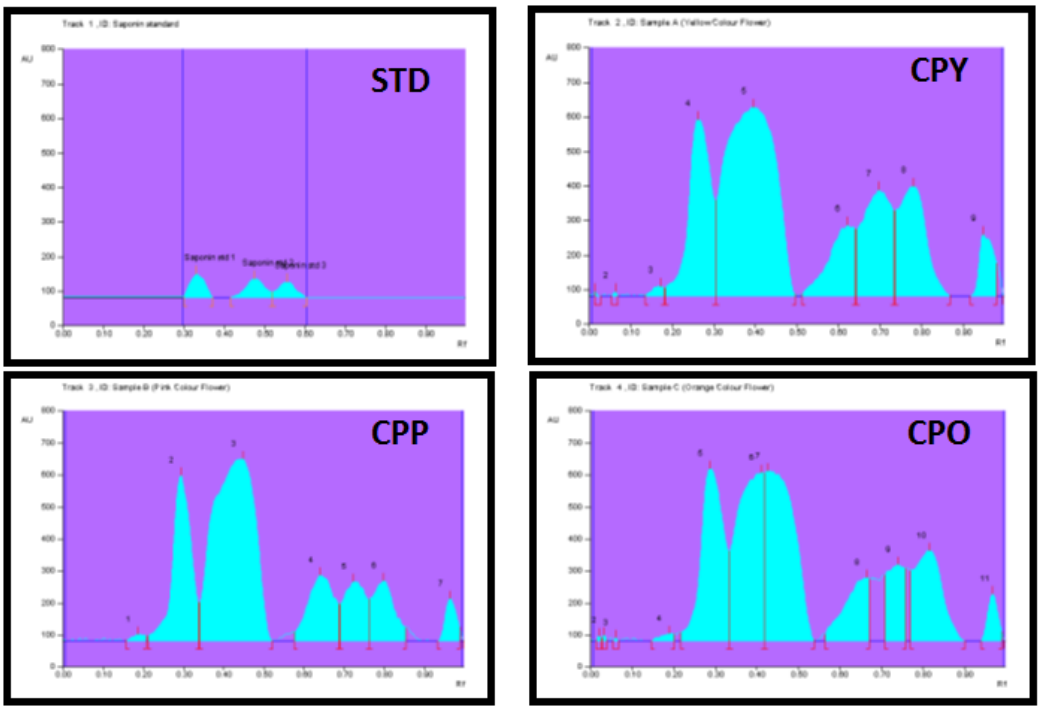
HPTLC of Saponins



Lane A – *C. pulcherrima* yellow flower
Lane B – *C. pulcherrima* pink flower
Lane C– *C. pulcherrima* orange flower

FIGURE 4.29

HPTLC Peak Densitogram of Saponins in the Flowers of *C. pulcherrima*



STD – Standard
CPY – *C. pulcherrima* yellow flower
CPP – *C. pulcherrima* pink flower
CPO – *C. pulcherrima* orange flower

TABLE 4.42

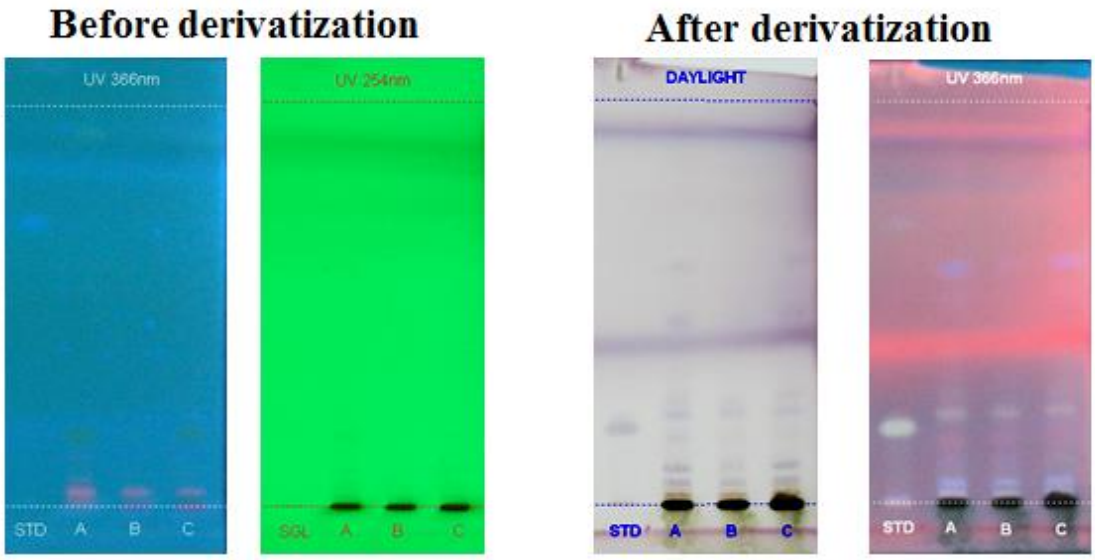
HPTLC PEAK TABLE FOR SAPONINS IN THE FLOWERS OF *C. pulcherrima*

Track	Peak	R_f	Height	Area	Assigned substance
STD	1	0.33	69.1	2356.9	Saponin standard 1
	2	0.47	53.1	2339.2	Saponin standard 2
	3	0.56	44.6	1764.5	Saponin standard 3
CPY	1	0.01	13.0	60.3	Saponin 1
	2	0.06	12.5	83.2	Unknown
	3	0.17	28.4	727.7	Unknown
	4	0.26	510.7	24895.8	Saponin 2
	5	0.40	545.7	61673.4	Saponin 3
	6	0.62	205.0	11657.3	Saponin 4
	7	0.70	306.4	19622.8	Saponin 5
	8	0.78	317.2	19476.4	Saponin 6
	9	0.95	188.2	6027.3	Unknown
CPP	1	0.19	21.5	570.8	Unknown
	2	0.29	517.1	21934.6	Saponin 1
	3	0.45	569.4	55268.6	Saponin 2
	4	0.64	205.1	12159.7	Saponin 3
	5	0.72	185.8	9241.5	Saponin 4
	6	0.80	189.2	9364.4	Saponin 5
	7	0.96	136.9	3520.9	Saponin 6
CPO	1	0.02	16.0	114.6	Unknown
	2	0.03	18.3	131.6	Unknown
	3	0.06	10.1	52.3	Unknown
	4	0.19	23.2	686.3	Unknown
	5	0.29	537.6	27138.1	Saponin 1
	6	0.41	525.8	30358.5	Saponin 2
	7	0.42	530.5	34728.8	Saponin 3
	8	0.67	198.5	10608.9	Saponin 4
	9	0.74	239.6	9272.8	Saponin 5
	10	0.81	283.0	17557.5	Saponin 6
	11	0.97	150.1	3405.3	Saponin 7

STD – Standard
CPY – *C. pulcherrima* yellow flower
CPP – *C. pulcherrima* pink flower
CPO – *C. pulcherrima* orange flower

PLATE 4.23

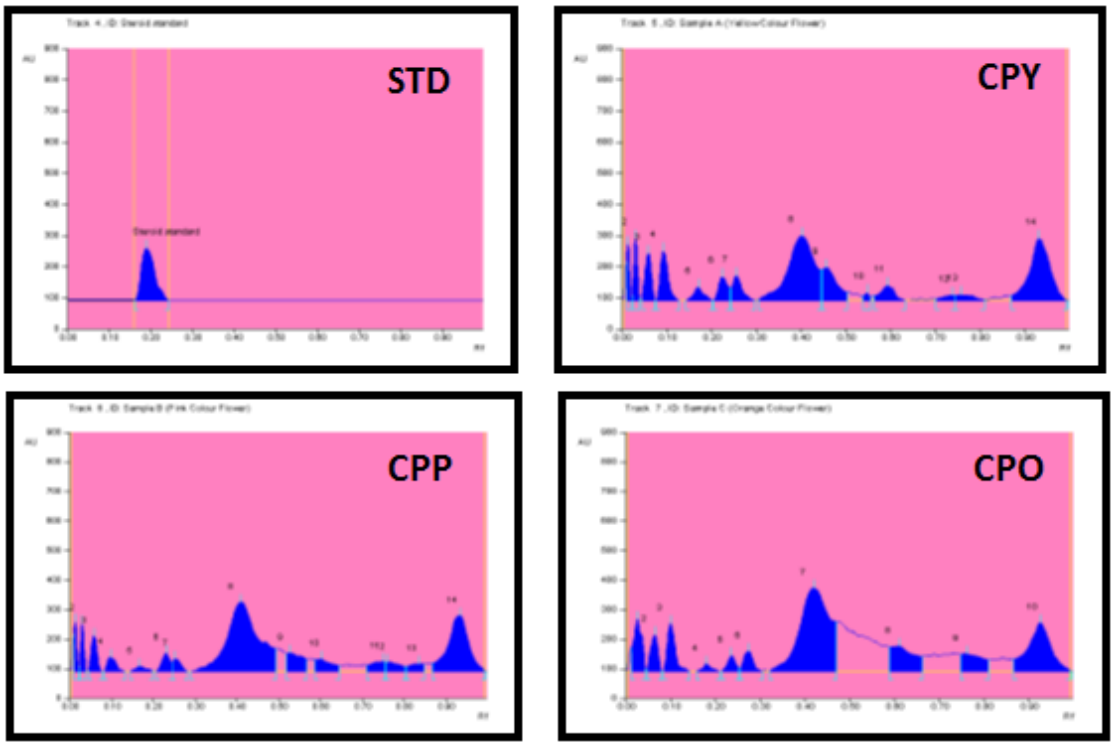
HPTLC of Steroids



Lane A – *C. pulcherrima* yellow flower
Lane B – *C. pulcherrima* pink flower
Lane C– *C. pulcherrima* orange flower

FIGURE 4.30

HPTLC Peak Densitogram of Steroids in the Flowers of *C. pulcherrima*



STD – Standard
CPY – *C. pulcherrima* yellow flower
CPP – *C. pulcherrima* pink flower
CPO – *C. pulcherrima* orange flower

TABLE 4.43

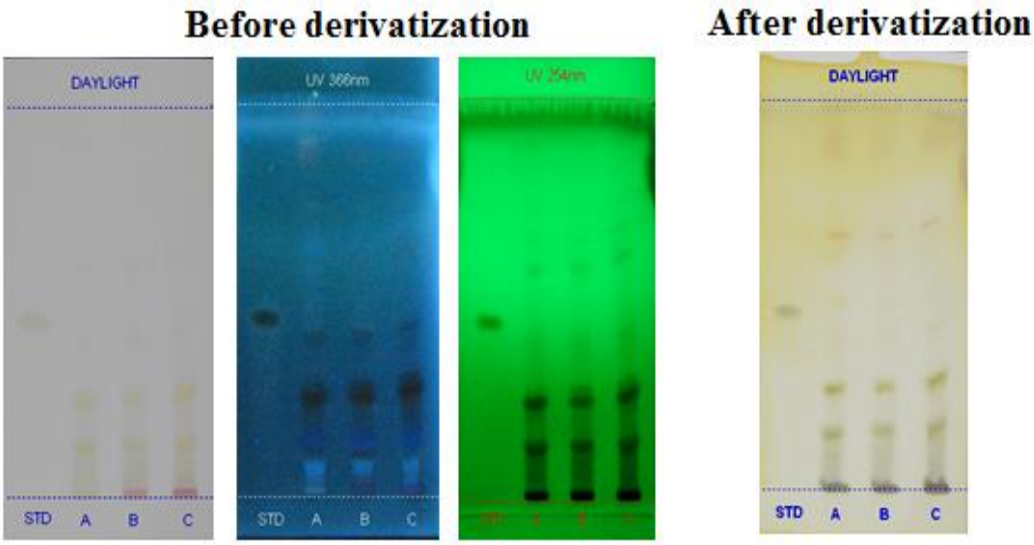
HPTLC PEAK TABLE FOR STEROIDS IN THE FLOWERS OF *C. pulcherrima*

Track	Peak	R_f	Height	Area	Assigned substance
STD	1	0.19	173.1	5342.8	Steroid standard
CPY	1	0.01	179.7	1177.4	Unknown
	2	0.03	200.5	1543.8	Unknown
	3	0.06	152.6	2007.7	Steroid 1
	4	0.09	159.0	2608.9	Steroid 2
	5	0.17	44.2	912.0	Steroid 3
	6	0.22	75.9	1433.1	Steroid 4
	7	0.26	80.2	1593.6	Steroid 5
	8	0.40	207.3	11421.5	Unknown
	9	0.46	106.8	3191.8	Steroid 6
	10	0.55	24.6	283.9	Unknown
	11	0.59	48.4	1408.1	Steroid 7
	12	0.74	17.1	424.3	Unknown
	13	0.76	18.5	686.2	Unknown
	14	0.93	198.2	8416.3	Unknown
CPP	1	0.01	168.7	1072.2	Unknown
	2	0.03	164.0	1200.2	Unknown
	3	0.06	120.8	1589.4	Steroid 1
	4	0.10	47.4	930.7	Steroid 2
	5	0.17	17.0	408.7	Steroid 3
	6	0.23	61.5	1092.2	Steroid 4
	7	0.25	42.3	763.2	Steroid 5
	8	0.41	234.5	16329.5	Unknown
	9	0.53	62.4	2106.1	Unknown
	10	0.60	43.0	1466.7	Unknown
	11	0.75	32.7	1030.7	Unknown
	12	0.76	30.4	953.0	Unknown
	13	0.84	25.4	799.0	Unknown
	14	0.93	188.6	8531.4	Unknown
CPO	1	0.03	177.9	3000.6	Unknown
	2	0.06	124.9	1873.4	Steroid 1
	3	0.10	161.8	2645.3	Steroid 2
	4	0.18	25.2	440.7	Steroid 3
	5	0.23	53.5	896.8	Steroid 4
	6	0.27	68.3	1353.5	Steroid 5
	7	0.42	281.8	17348.7	Unknown
	8	0.61	86.5	4129.0	Steroid 6
	9	0.76	58.2	2586.8	Unknown
	10	0.93	164.7	7605.7	Unknown

STD – Standard
CPY – *C. pulcherrima* yellow flower
CPP – *C. pulcherrima* pink flower
CPO – *C. pulcherrima* orange flower

PLATE 4.24

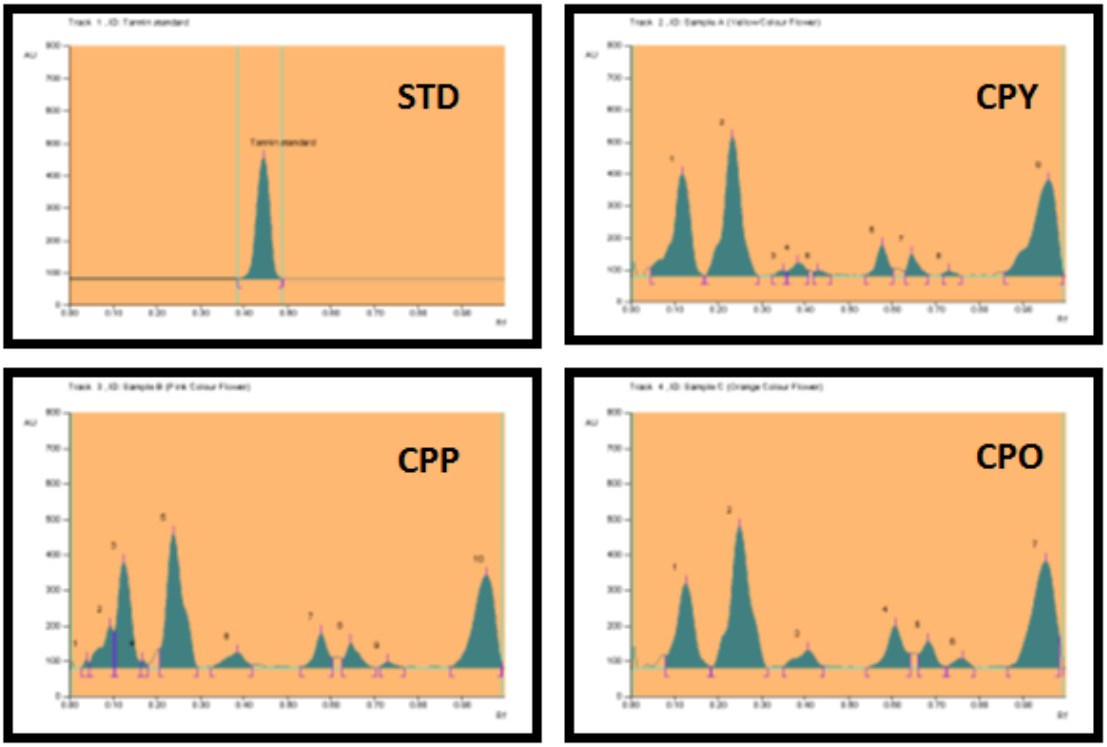
HPTLC of Tannins



Lane A – *C. pulcherrima* yellow flower
Lane B – *C. pulcherrima* pink flower
Lane C– *C. pulcherrima* orange flower

FIGURE 4.31

HPTLC Peak Densitogram of Tannins in the Flowers of *C. pulcherrima*



STD – Standard
CPY – *C. pulcherrima* yellow flower
CPP – *C. pulcherrima* pink flower
CPO – *C. pulcherrima* orange flower

TABLE 4.44

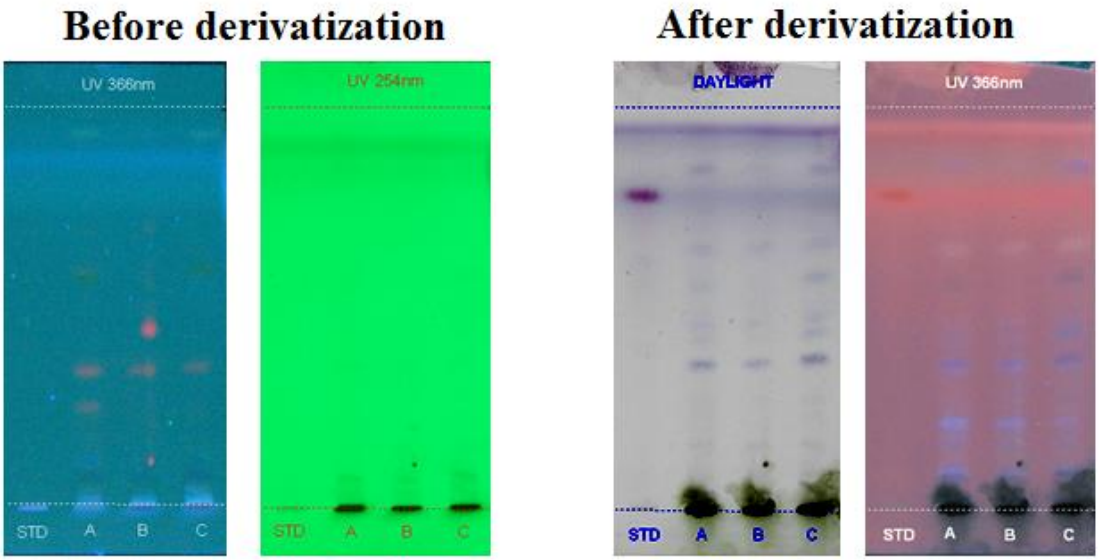
HPTLC PEAK TABLE FOR TANNINS IN THE FLOWERS OF *C. pulcherrima*

Track	Peak	R_f	Height	Area	Assigned substance
STD	1	0.45	379.7	10879.3	Tannin standard
CPY	1	0.12	317.3	11689.9	Tannin 1
	2	0.23	432.4	15394.2	Tannin 2
	3	0.35	16.40	303.9	Unknown
	4	0.38	39.80	1117.3	Tannin 3
	5	0.43	15.90	365.5	Tannin 4
	6	0.58	95.80	2206.8	Unknown
	7	0.65	67.40	1454.6	Tannin 5
	8	0.73	14.60	313.0	Unknown
	9	0.96	322.4	16883.4	Unknown
CPP	1	0.04	20.50	198.5	Unknown
	2	0.09	116.9	3074.4	Tannin 1
	3	0.13	296.0	7988.4	Tannin 2
	4	0.17	21.6	204.3	Unknown
	5	0.24	376.2	12602.3	Tannin 3
	6	0.39	41.8	1678.3	Tannin 4
	7	0.58	95.7	2404.6	Unknown
	8	0.65	69.9	1873.3	Tannin 5
	9	0.73	15.0	369.0	Unknown
	10	0.96	284.1	13026.8	Unknown
CPO	1	0.13	236.5	8458.2	Tannin 1
	2	0.25	396.4	16059.9	Tannin 2
	3	0.41	48.6	1923.2	Tannin 3
	4	0.61	117.3	3925.4	Tannin 4
	5	0.68	73.2	2071.1	Tannin 5
	6	0.76	26.6	774.3	Unknown
	7	0.95	333.4	16444.3	Unknown

STD – Standard
CPY – *C. pulcherrima* yellow flower
CPP – *C. pulcherrima* pink flower
CPO – *C. pulcherrima* orange flower

PLATE 4.25

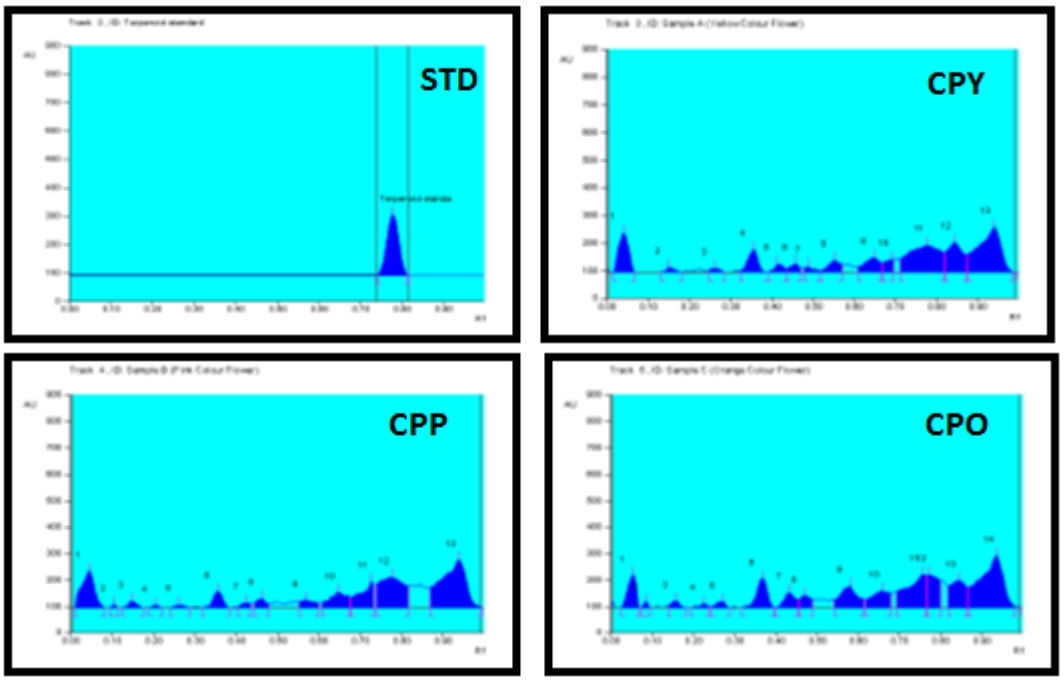
HPTLC of Terpenoids



Lane A – *C. pulcherrima* yellow flower
Lane B – *C. pulcherrima* pink flower
Lane C– *C. pulcherrima* orange flower

FIGURE 4.32

HPTLC Peak Densitogram of Terpenoids in the Flowers of *C. pulcherrima*



STD – Standard
CPY – *C. pulcherrima* yellow flower
CPP – *C. pulcherrima* pink flower
CPO – *C. pulcherrima* orange flower

TABLE 4.45

HPTLC PEAK TABLE FOR TERPENOIDS IN THE FLOWERS OF *C. pulcherrima*

Track	Peak	R_f	Height	Area	Assigned substance
STD	1	0.78	294.6	14650.8	Terpenoid standard
CPY	1	0.04	142.8	3238.0	Unknown
	2	0.15	17.5	321.2	Terpenoid 1
	3	0.26	12.0	283.5	Terpenoid 2
	4	0.36	82.2	1871.8	Terpenoid 3
	5	0.41	29.4	603.5	Terpenoid 4
	6	0.46	30.3	616.9	Terpenoid 5
	7	0.49	18.5	400.1	Unknown
	8	0.55	41.1	1146.4	Terpenoid 6
	9	0.65	51.9	1788.4	Terpenoid 7
	10	0.69	46.1	940.0	Unknown
	11	0.79	96.4	6851.1	Unknown
	12	0.84	107.0	3760.2	Terpenoid 8
	13	0.94	162.8	8059.5	Unknown
CPP	1	0.04	141.2	4050.1	Unknown
	2	0.11	12.7	110.0	Unknown
	3	0.15	24.9	470.3	Terpenoid 1
	4	0.20	10.4	139.2	Unknown
	5	0.26	11.5	246.1	Terpenoid 2
	6	0.35	62.0	1363.0	Terpenoid 3
	7	0.42	18.3	373.9	Terpenoid 4
	8	0.46	34.0	739.6	Terpenoid 5
	9	0.57	28.9	897.5	Unknown
	10	0.65	59.4	2291.3	Terpenoid 6
	11	0.73	101.2	2858.0	Unknown
	12	0.79	115.1	6370.6	Unknown
	13	0.94	182.2	9727.3	Unknown
CPO	1	0.05	123.7	2324.7	Unknown
	2	0.09	22.9	230.2	Unknown
	3	0.16	25.5	496.2	Terpenoid 1
	4	0.23	15.2	291.8	Terpenoid 2
	5	0.27	23.8	499.6	Terpenoid 3
	6	0.37	111.7	2705.3	Terpenoid 4
	7	0.43	57.0	1432.2	Terpenoid 5
	8	0.47	46.3	1091.2	Terpenoid 6
	9	0.58	84.2	3124.4	Terpenoid 7
	10	0.66	62.8	2523.1	Terpenoid 8
	11	0.76	124.2	5236.1	Unknown
	12	0.77	124.0	3122.1	Unknown
	13	0.84	103.3	3564.1	Terpenoid 9
	14	0.94	196.1	9741.9	Unknown

STD – Standard
CPY – *C. pulcherrima* yellow flower
CPP – *C. pulcherrima* pink flower
CPO – *C. pulcherrima* orange flower

4.13. HPLC ANALYSIS OF THE FLOWERS OF *C. pulcherrima*

The HPLC analysis of the methanolic extract of the three flowers of *C. pulcherrima* flowers was carried out using Supelco analytical-Discovery HS C18 reverse phase column (Sigma-Aldrich equipped with UV detector). The results obtained are presented in Figure 4.33 to 4.35. The HPLC spectrum of the methanolic extract of all the three flowers showed fifteen peaks, including major and minor ones. The retention time of the major and minor peaks, together with the respective peak area of all the three flowers, are represented in Table 4.46.

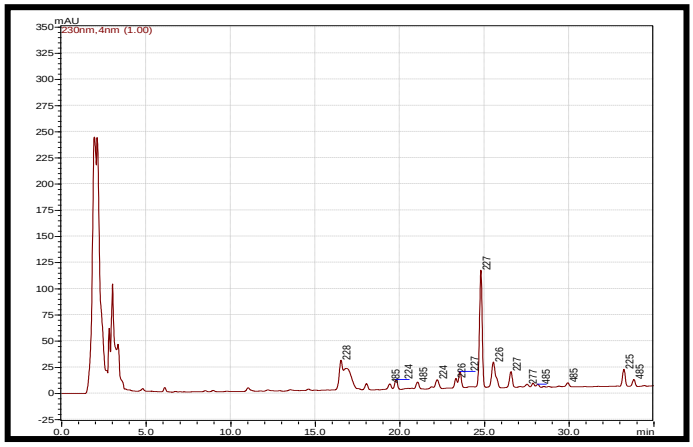


FIGURE 4.33

HPLC Analysis of the Methanolic Extract of *C. pulcherrima* Yellow Flower

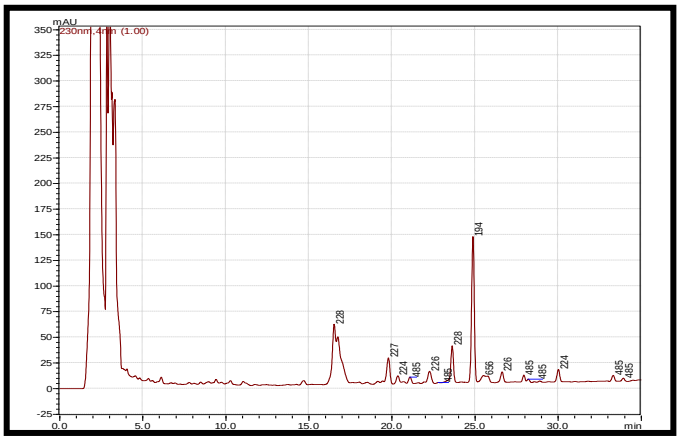


FIGURE 4.34

HPLC Analysis of the Methanolic Extract of *C. pulcherrima* Pink Flower

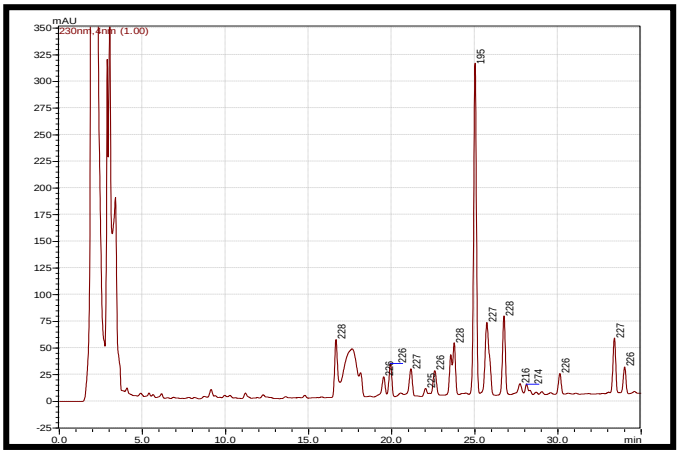


FIGURE 4.35

HPLC Analysis of the Methanolic Extract of *C. pulcherrima* Orange Flower

TABLE 4.46

HPLC PEAK TABLE OF THE METHANOLIC EXTRACT OF THE FLOWERS OF *C. pulcherrima*

Scanning at 230nm (UV Long range)					
Yellow		**Pink**		**Orange**	
Retention time (R_t) in minutes	**Peak area**	**Retention time (R_t) in minutes**	**Peak area**	**Retention time (R_t) in minutes**	**Peak area**
16.503	967206.1	16.508	1775312.9	16.639	482665.0
19.391	48372.9	19.777	302481.2	19.511	217558.1
19.765	97643.1	20.346	79330.3	19.919	322870.3
21.034	72775.2	21.081	72929.6	21.148	310198.9
22.191	100220.3	22.253	151281.8	22.030	61663.6
23.300	43722.9	22.792	7354.0	22.583	291385.6
23.541	103074.2	23.617	355609.1	23.753	905138.5
24.775	1215821.3	24.865	1575696.1	25.007	3551068.8
25.512	391413.0	25.494	165192.5	25.717	1157877.3
26.552	169561.8	26.620	110213.0	26.749	868908.2
27.504	23742.1	27.929	55150.5	27.712	111223.5
28.136	20691.2	28.199	18888.7	28.104	109046.2
29.911	39284.8	30.011	134170.1	30.113	224292.9
33.229	180410.8	33.311	60483.7	33.392	577016.8
33.817	71025.6	33.913	30790.5	34.008	268123.1

4.14. FT-IR ANALYSIS OF THE METHANOLIC EXTRACT OF THE FLOWERS OF *C. pulcherrima*

The IR spectrum of the methanolic extract of all the three flowers of *C. pulcherrima* (Figure 4.36 to 4.38) exhibited a broad band at 3421 cm^{-1}, along with a moderately intense band at 1096.33 cm^{-1} confirming the presence of hydroxyl group. A moderate band at 1642.09 cm^{-1} confirmed the presence of –C=O group. Thus, the IR spectral analysis shows that the major bioactive compounds present in the three flowers of *C. pulcherrima* may belong to phenolics and flavonoids groups of compounds.

FIGURE 4.36

IR Spectrum of the Methanolic Extract of the Yellow Flowers of *C. pulcherrima*

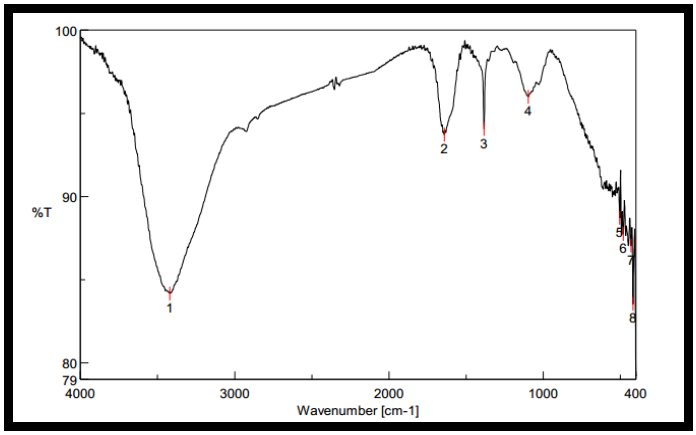


FIGURE 4.37

IR Spectrum of the Methanolic Extract of the Pink Flowers of *C. pulcherrima*

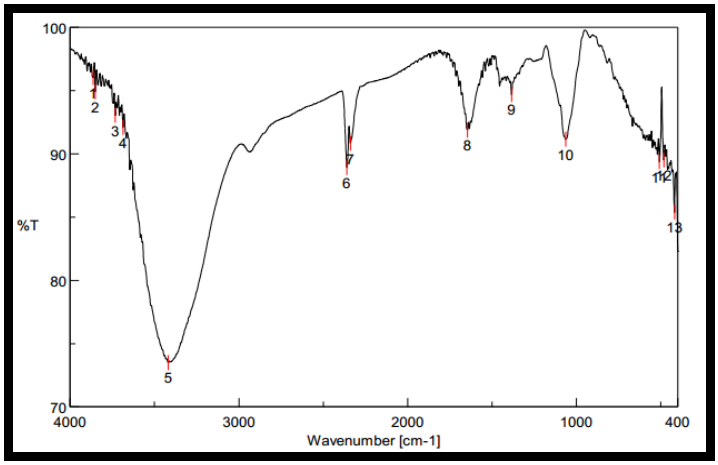
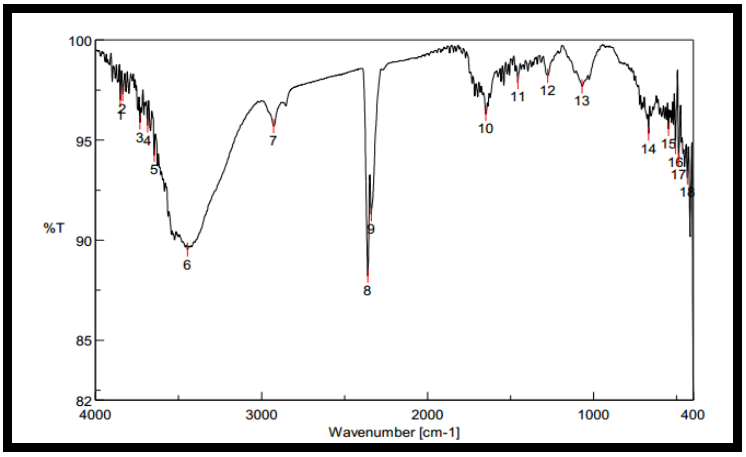


FIGURE 4.38

IR Spectrum of the Methanolic Extract of the Orange Flowers of *C. pulcherrima*



4.15. GC-MS ANALYSIS OF THE METHANOLIC EXTRACT OF THE FLOWERS OF *C. pulcherrima*

The GC-MS analysis of the methanolic extract of the three flowers of *C. pulcherrima* was carried out to identify the nature of the components present. The GC-MS output of the methanolic extract of the yellow flower showed three major peaks at retention times 28.55, 37.71 and 44.84 minutes (Figure 4.39).

The mass spectrum of methanolic extract of *C. pulcherrima* yellow flower at retention time 28.55 minutes (Figure 4.40) showed eight characteristic (M-14), (M-14), (M-14), (M-14), (M-14), (M-28), (M-28) and (M-27) peaks, m/e 69.1, 83.1, 97.1, 111.1, 125.1 and 152.2, indicating the presence of -CH_2, HCN and -C=O group.

The mass spectrum of methanolic extract of *C. pulcherrima* yellow flower at retention time 37.71 minutes (Figure 4.41) showed two characteristic (M-28) and (M-28) peaks, m/e 78.8 and 296.0 indicating the presence of -C=O group. The mass spectrum showed two more characteristic peaks (M-44) and (M-17) at m/e 296 and 162.1, indicating the presence of –COOH and –OH group. The mass spectrum of methanolic extract of *C. pulcherrima* yellow flower at retention time 44.84 minutes (Figure 4.42) showed one characteristic (M-45) peak, m/e 282.1 indicating the presence of COOH group.

The GC-MS output of pink flower showed seven major peaks at retention times 14.45, 15.73, 28.53, 30.19, 36.93, 41.56 and 43.60 minutes as shown in Figure 4.43.

The mass spectrum of methanolic extract of *C. pulcherrima* pink flower at retention time 14.45 minutes (Figure 4.44) showed one characteristic (M-28) peak, m/e 119.1, indicating the presence of -C=O group. The mass spectrum of methanolic extract of *C. pulcherrima* pink flower at retention time 15.73 minutes (Figure 4.45) showed five characteristic (M-14), (M-14), (M-14), (M-14) and (M-28) peaks, m/e 91.1, 105.1, 161.1, 218.1 and 105.1, indicating the presence of -CH2 and -C=O group. The mass spectrum of methanolic extract of *C. pulcherrima* pink flower at retention time 28.83 minutes (Figure 4.46) showed one characteristic (M-28) peak, m/e 97.1 indicating the presence of -C=O group.

The mass spectrum of methanolic extract of *C. pulcherrima* pink flower at retention time 30.19 minutes (Figure 4.47) showed two characteristic (M-45) and (M-29) peaks, m/e 255.1 and 284.1, indicating the presence of COOH and a nitrogen group.

The mass spectrum of methanolic extract of *C. pulcherrima* pink flower at retention time 36.93 minutes (Figure 4.48) showed five characteristic (M-14), (M-14), (M-14), (M-14) and (M-28) peaks, m/e 57.1, 71.1, 85.1, 183.1 and 168.9, indicating the presence of -CH_2 and -C=O group.

The mass spectrum of methanolic extract of *C. pulcherrima* pink flower at retention time 41.56 minutes (Figure 4.49) showed four characteristic (M-29), (M-27), (M-27) and (M-14) peaks, m/e 74.4, 104.1, 324.1 and 368.2, indicating the presence of nitrogen, HCN and -CH_2 group. The mass spectrum of methanolic extract of *C. pulcherrima* pink flower at retention time 43.60 minutes (Figure 4.50) showed four characteristic (M-14), (M-14), (M-28) and (M-28) peaks, m/e 74.4, 104.1, 324.1 and 368.2, indicating the presence of -CH_2 and -C=O group.

The GC-MS output of the methanolic extract of the orange flower showed eight major peaks at retention times 14.45, 15.71, 17.78, 28.53, 30.19, 36.97, 41.52 and 42.55 minutes (Figure 4.51). The mass spectrum of the peak at retention time 14.45 minutes (Figure 4.52) showed three characteristic (M-27), (M-27) and (M-29) peaks, m/e 134.1, 161.2 and 204.1, indicating the presence of HCN and nitrogen group.

The mass spectrum of the peak at retention time 15.71 minutes (Figure 4.53) showed three characteristic (M-14), (M-28) and (M-28) peaks, m/e 105, 133 and 161.1, indicating the presence of -CH_2 and -C=O group. The mass spectrum of the peak at retention time 17.78 minutes (Figure 4.54) showed two characteristic (M-14) and (M-28) peaks, m/e 93.1 and 149.0, indicating the presence of -CH_2 and -C=O group. The mass spectrum of the peak at retention time 28.53 minutes (Figure 4.55) showed one characteristic (M-29) peak, m/e 149.2, indicating the presence of nitrogen group.

The mass spectrum of the peak at retention time 30.19 minutes (Figure 4.56) showed three characteristic (M-29), (M-17) and (M-29) peaks, m/e 208.2, 255.2 and 284.1, indicating the presence of nitrogen and -OH group. The mass spectrum of the peak at retention time 36.97 minutes (Figure 4.57) showed five characteristic (M-17), (M-17), (M-17), (M-27) and (M-17) peaks, m/e 120, 167.2, 197.1, 224 and 298, indicating the presence of -OH and HCN group. Since, three (M-17) peaks were observed in the spectrum, the methanolic extract of the orange flower may contain polyphenols.

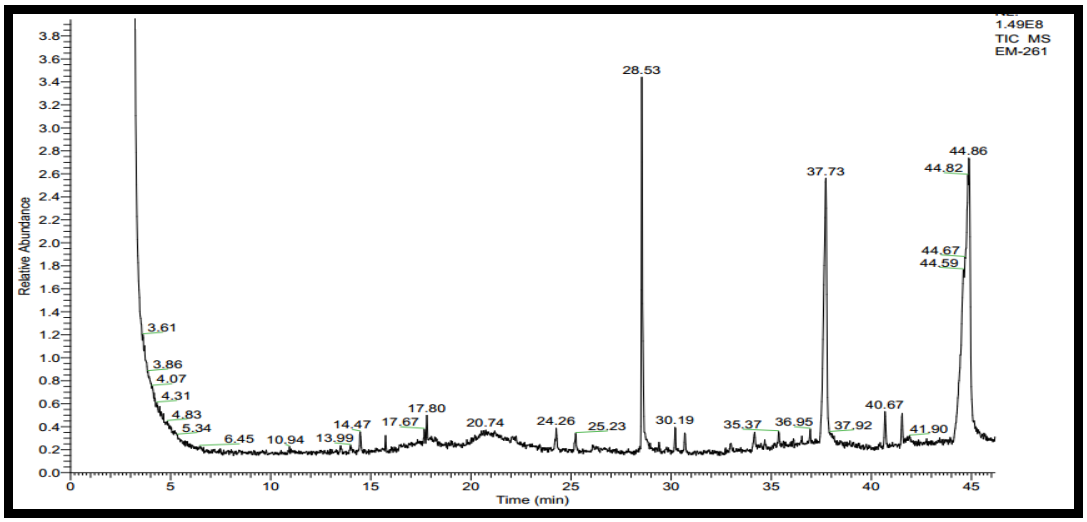


FIGURE 4.39

GC-MS Profile of the Methanolic Extract of the Yellow Flowers of *C. pulcherrima*

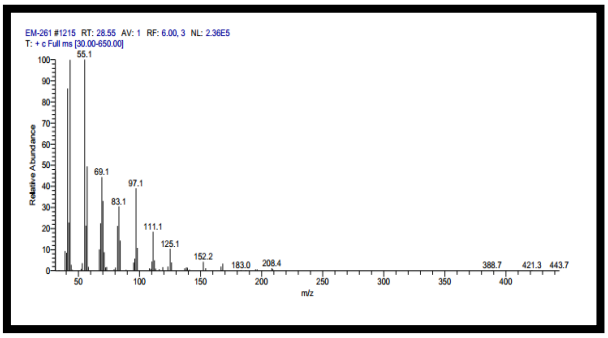
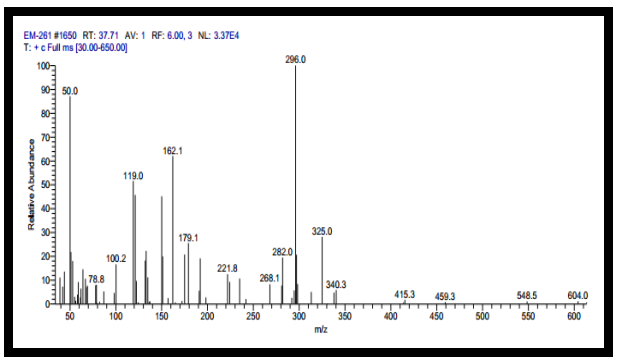


FIGURE 4.40

Peak Fragmentation of GC-MS Spectrum (28.55) of Yellow Flowers

FIGURE 4.41

Peak Fragmentation of GC-MS Spectrum (37.71) of Yellow Flowers



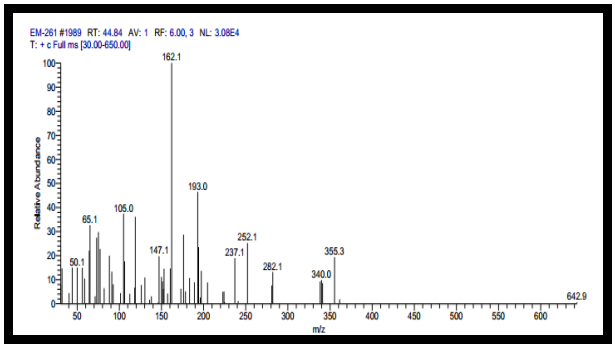


FIGURE 4.42

Peak Fragmentation of GC-MS Spectrum (44.84) of Yellow Flowers

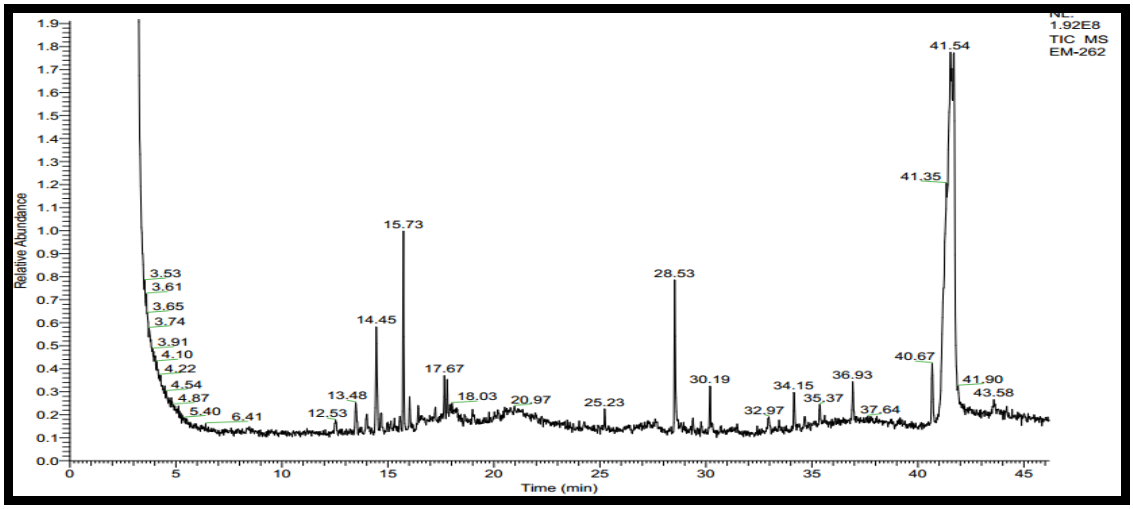


FIGURE 4.43

GC-MS Profile of the Methanolic Extract of the Pink Flowers of *C. pulcherrima*

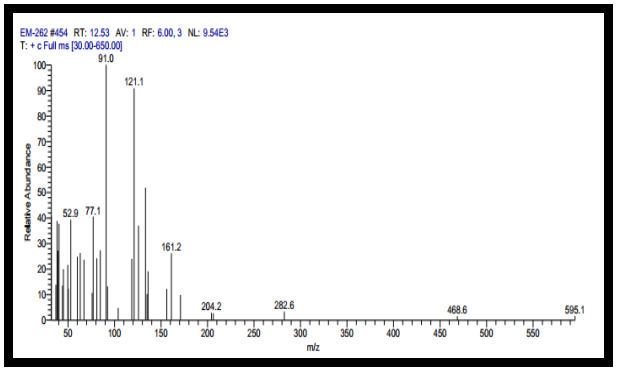
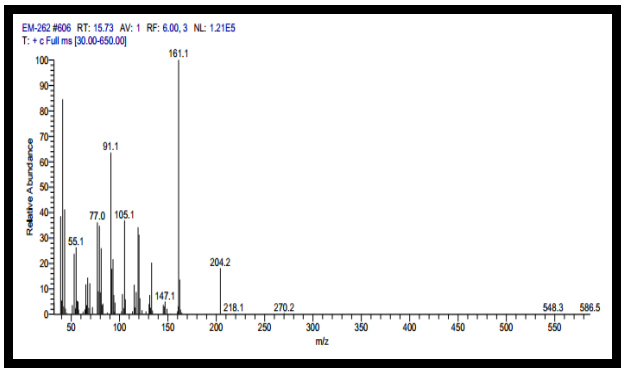


FIGURE 4.44

Peak Fragmentation of GC-MS Spectrum (12.53) of Pink Flowers

FIGURE 4.45

Peak Fragmentation of GC-MS Spectrum (15.73) of Pink Flowers



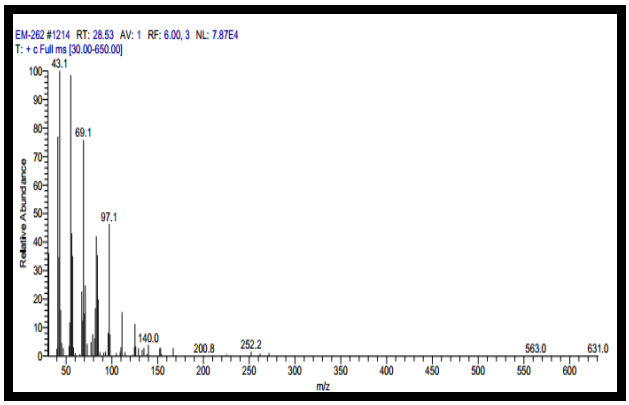


FIGURE 4.46

Peak Fragmentation of GC-MS Spectrum (28.53) of Pink Flowers

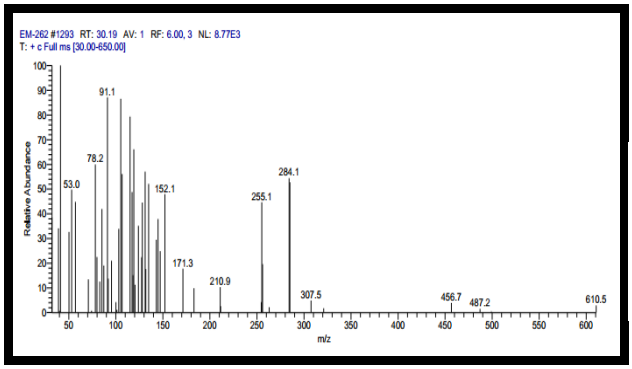


FIGURE 4.47

Peak Fragmentation of GC-MS Spectrum (30.19) of Pink Flowers

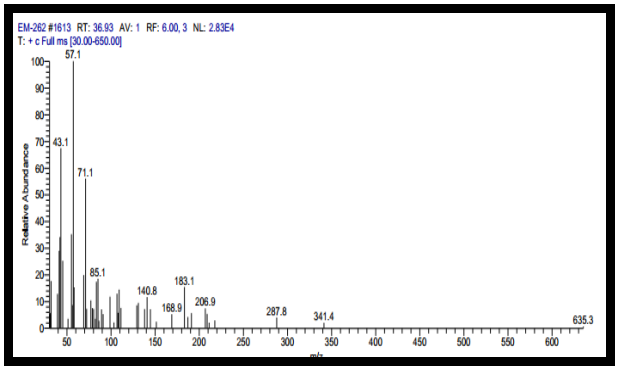


FIGURE 4.48

Peak Fragmentation of GC-MS Spectrum (36.93) of Pink Flowers

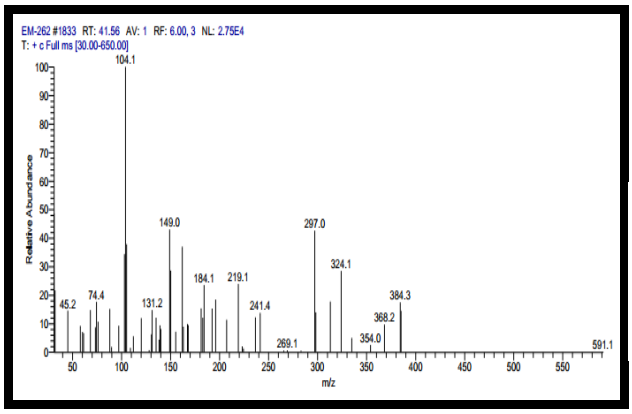


FIGURE 4.49

Peak Fragmentation of GC-MS Spectrum (41.56) of Pink Flowers

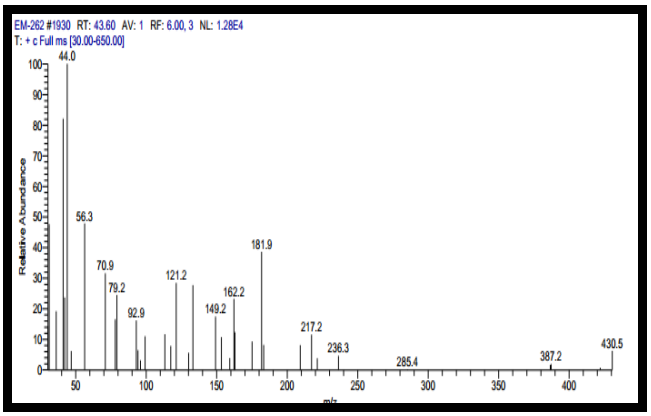


FIGURE 4.50

Peak Fragmentation of GC-MS Spectrum (43.60) of Pink Flowers

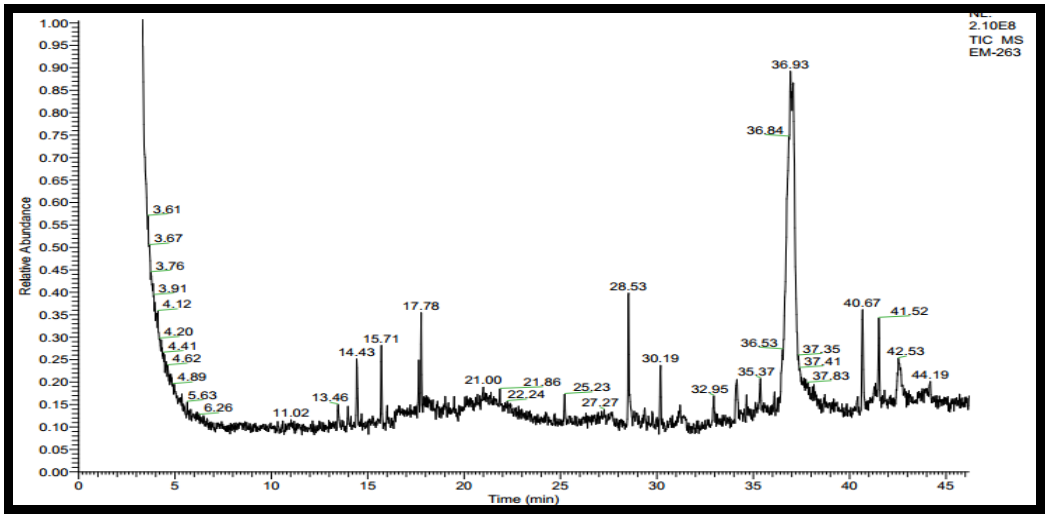


FIGURE 4.51

GC-MS Profile of the Methanolic Extract of the Orange Flowers of *C. pulcherrima*

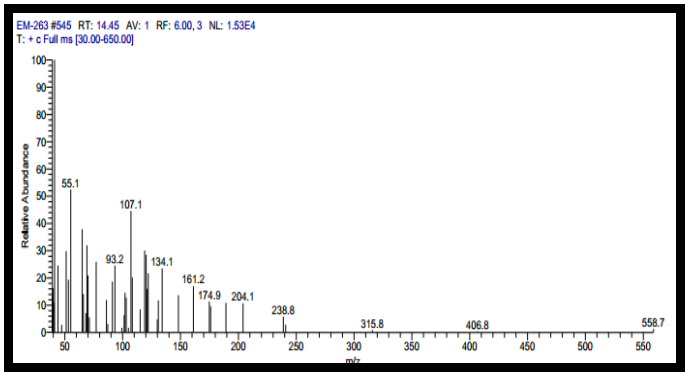


FIGURE 4.52

Peak Fragmentation of GC-MS Spectrum (14.45) of Orange Flowers

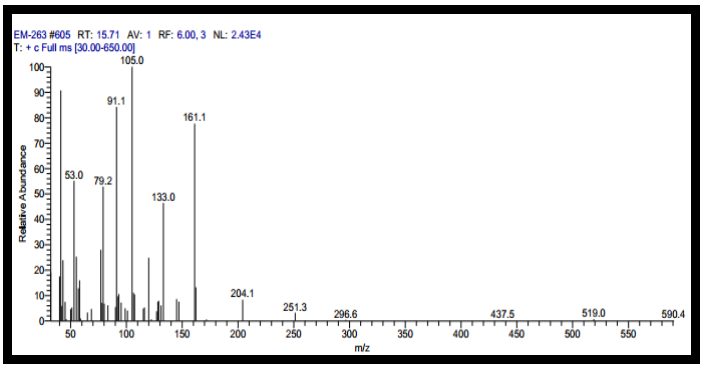


FIGURE 4.53

Peak Fragmentation of GC-MS Spectrum (15.71) of Orange Flowers

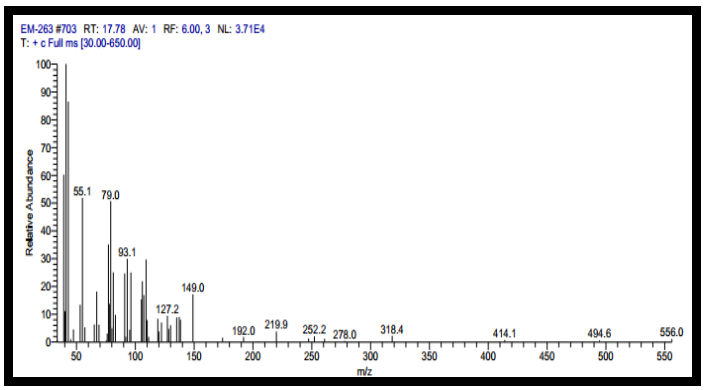


FIGURE 4.54

Peak Fragmentation of GC-MS Spectrum (17.78) of Orange Flowers

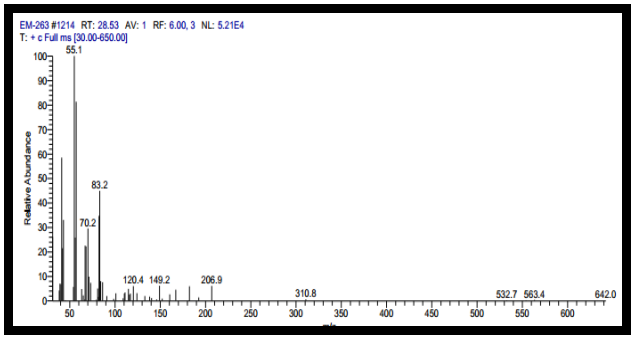
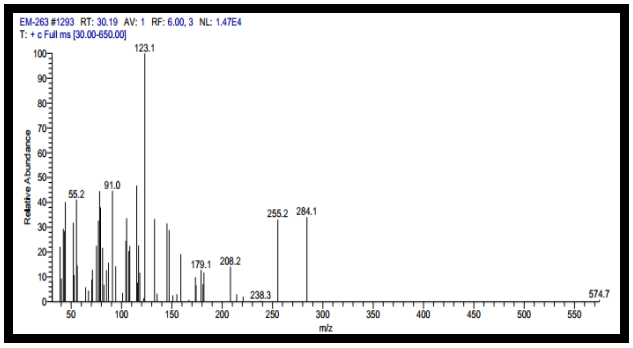


FIGURE 4.55

Peak Fragmentation of GC-MS Spectrum (26.53) of Orange Flowers

FIGURE 4.56

Peak Fragmentation of GC-MS Spectrum (30.19) of Orange Flowers



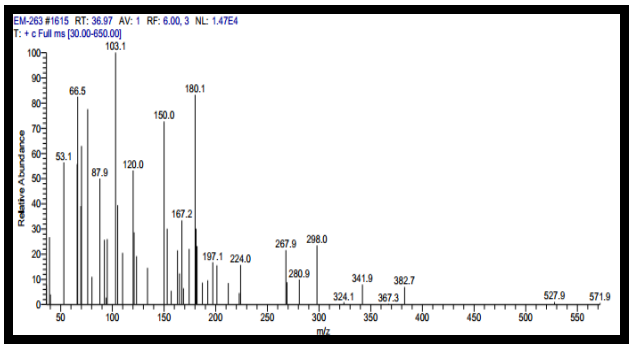
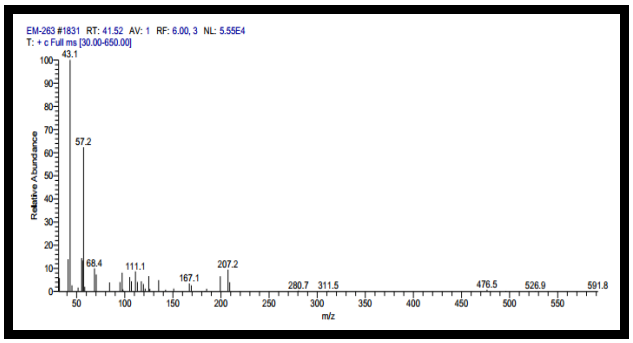


FIGURE 4.57

Peak Fragmentation of GC-MS Spectrum (36.97) of Orange Flowers

FIGURE 4.58

Peak Fragmentation of GC-MS Spectrum (41.52) of Orange Flowers



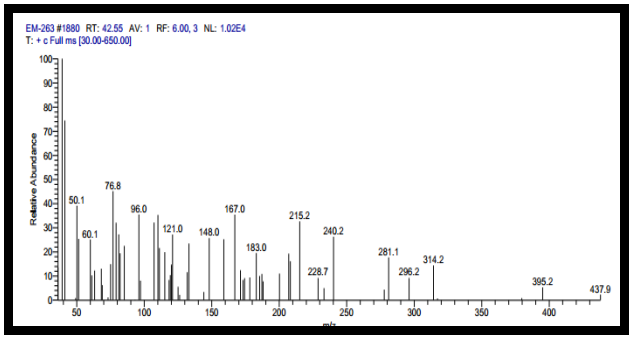


FIGURE 4.59

Peak Fragmentation of GC-MS Spectrum (42.55) of Orange Flowers

The mass spectrum of the peak at retention time 41.52 minutes (Figure 4.58) showed one characteristic (M-14) peak, m/e 68.4, indicating the presence of -CH_2 group. The mass spectrum of the peak at retention time 42.55 minutes (Figure 4.59) showed two characteristic (M-27), (M-17) and (M-44) peaks, m/e 50.1, 60.8 and 76.8, indicating the presence of HCN, -OH and COOH group.

The results obtained in phase IV of this study revealed that 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, all of which exhibited significant radical scavenging activity. The flavonoid fraction exhibited the maximum radical quenching potential, followed by the phenolic fraction. TLC analysis showed the presence of several bands in all three flowers, which was further confirmed by HPTLC. Spectral analysis showed that the major active constituents present in the flowers belong to phenolic and flavonoid type of compounds whose structure is yet to be elucidated.

Thus, the observations from the present study showed that all the three flowers of *C. pulcherrima* possess strong antioxidant, antiradical properties and protect biomolecules from oxidative damage. The antioxidant status of the liver against oxidative stress induced *in vitro* was significantly improved by the flower extracts. The flower extracts protected the non-cancerous cells from oxidant induced apoptosis, whereas, in the case of cancerous cells, they increased the number of cells undergoing apoptosis. These apoptosis modulating effects of the flowers of *C. pulcherrima* showed that they exhibit a differential response towards non-cancerous and cancerous cells. All 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.

The results of all the parameters analysed and the statistical evaluation of the results obtained clearly show that the null hypothesis can be rejected and the alternate hypothesis implicating that the flowers of *Caesalpinia pulcherrima* possess strong antioxidant and anticancer properties can be accepted.

The results obtained and observations made in the present study are discussed with reference to the available and relevant literature reports in the next chapter.