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BIOMOLECULAR PROTECTIVE EFFECT OF THE METHANOLIC EXTRACT OF THE FLOWERS OF *CAESALPINIA PULCHERRIMA*, SWARTZ. AGAINST OXIDATIVE DAMAGE

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Keywords:

Lipid peroxidation (LPO), DNA damage, *Caesalpinia pulcherrima*, Protein Carbonyl, Oxidative stress

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ABSTRACT: Free radicals and reactive oxygen species (ROS) production above basal levels cause irreversible damage to the cell membrane, DNA and other cellular structures by oxidizing lipids, proteins and nucleic acids resulting in dysfunction of biomolecules within cells and, finally, cell death. These free radical-induced reactions are ascertained to play multiple roles in degenerative or pathological events especially carcinogenesis. Apart from the radical scavenging activity, the antioxidant potential of a test compound or herbal preparation is also based on their protective effect against oxidant-induced damage to cellular biomolecules. In the present study, the protective effect of the methanolic extract of the three different flowers of *Caesalpinia pulcherrima* (yellow, pink and orange) against oxidative stress-induced damage to biomolecules like lipids, DNA and proteins were analyzed in both cell-free systems and intact cells. The results showed that the flowers of *C. pulcherrima* rendered significant biomolecular protection against oxidative stress, both in cell-free systems and in intact cells.

INTRODUCTION: A deleterious phenomenon called the oxidative stress is caused by excessive production of free radicals and oxidants beyond the antioxidant defense. Oxidative stress induces alterations in the cell membranes and other structures such as proteins, lipids, lipoproteins and DNA [1]. Such progressive adverse changes accumulate with age throughout the body. Genetics and environment factors influence these changes and modulate free radical damage, thereby causing various pathological conditions such as diabetes, cardiovascular disease, neurological disorders, ischemia, aging and cancer [2].

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Free radicals produce oxidized lipids from polyunsaturated lipids through the lipid peroxidation process and thereby causes cell membrane damage. Malondialdehyde (MDA) is one of the final products of peroxidation of unsaturated fatty acids in phospholipids and is responsible for cell membrane damage [3].

Modifications of metabolic and structural proteins cause alterations in the processing and trafficking of proteins and also cause protein dysfunction that leads to regenerating tissue damage. Protein carbonylation has been found to play a vital role in the pathogenesis of numerous diseases [4]. Reactive species can also modify DNA bases, induce inter- and intra-strand crosslinks, promote DNA-protein crosslinks, produce sugar moiety modifications and create strand break [5]. Accumulation of DNA damage induces mutagenesis that results in carcinogenesis [6].

Recent researches have shown that the antioxidants isolated from plants have gained importance as therapeutic agents in oxidative stress-related diseases. Different plant extracts and their phytoconstituents have been identified as effective radical scavengers and inhibitors of oxidative damage to biomolecules [7]. Many research studies focus on identifying such plants with significant antioxidant and biomolecular protective potential.

By this, the present study was formulated to investigate the biomolecular protective effects of the flowers of the candidate plant *Caesalpinia pulcherrima*, which blooms in three different colors (orange, pink and yellow) with unique long stamens. The three different flowers of *C. pulcherrima* have already been studied extensively in our laboratory and found that these flowers are rich in both enzymic and non-enzymic antioxidants [8]. They also have been found to significantly improve the antioxidant status of the goat liver slices challenged with oxidative stress *in-vitro* [9]. Apoptotic studies showed that these flowers increased the cell viability of untransformed cells subjected to oxidative stress and influenced the process of apoptosis induced *in-vitro* [10]. Oxidative damage to biomolecules causes detrimental 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 [11]. Hence, this study was conducted to determine the protection rendered by the methanolic extract of *C. pulcherrima* flowers against oxidative stress-induced damage to cellular biomolecules like membrane lipids, DNA and proteins in cell-free systems and intact cells.

MATERIALS AND METHODS:

Preparation of the Plant Extracts: Fresh flowers of *Caesalpinia pulcherrima* were collected from the local areas of Coimbatore. The plant was identified and certified by the Botanical Survey of India, Tamil Nadu Agricultural University, Coimbatore. The methanolic extract of the three different flowers of *C. pulcherrima* (yellow, pink and orange) was prepared using cold extraction method fresh flowers of *C. pulcherrima* (5 g) were collected. The petals were collected, washed with tap water to remove the surface contaminants, dried by gently blotting between folds of tissue paper and cut into fine strips using a knife. These pieces were taken in a flask covered with aluminum foil and filled with methanol. The flasks were stoppered, and the contents were extracted for 72 h at 4 °C with mild shaking. After 72 h, the methanolic extracts were filtered by passing through Whatmann filter paper using a Büchner funnel connected with a vacuum pump. The filtrate was then concentrated at low temperature (40 - 50 °C) and reduced pressure. The yields of the extracts were calculated, and the residues were re-dissolved in dimethyl sulfoxide (DMSO) [20 mg flower extract per 5 µl of DMSO]. The concentration of the flower extract used for each assay was 100 µg.

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

Evaluation of LPO in RBC Ghosts: Oxidative damage of lipids by reactive species can be measured from the extent of formation of thiobarbituric acid reactive substance (TBARS) from the damaged lipids. Erythrocyte ghost membranes were prepared by osmotic lysis using the method of Dodge *et al*., (1963) [12].

Estimation of LPO in Goat Liver Homogenate: LPO assay in goat liver homogenate was performed according to the method of Okhawa *et al.,* (1979) [13]. Fresh goat liver was procured from a slaughterhouse and washed thoroughly with Tris HCl buffer (40 mM, pH 7.0). The liver was cut into thin pieces, and a 20% liver homogenate was prepared in Tris HCl buffer using a motorized Teflon homogenizer, followed by low-speed centrifugation and the supernatant was used as the lipid source for the assay.

Estimation of LPO in Goat Liver Slices: The extent of inhibition of LPO in goat liver slices was estimated by the method proposed by Niehaus and Samuelsson (1968) [14].

Effect of *C. pulcherrima* Flower Extracts on Oxidant Induced DNA Damage: The effect of the flowers of the candidate plant on oxidant-induced DNA damage was assessed *in-vitro* in commercially available preparations of DNA. DNA from different hierarchies of evolutionary development were selected for the analysis, which included the commercially available preparations of viral DNA (λ DNA), the bacterial plasmid (pUC18) and DNA of animal origin (herring sperm DNA).

Estimation of the DNA Damage in λ DNA and pUC18 DNA: The extent of DNA damage in λ DNA and pUC18 DNA was determined by the method proposed by Chang *et al.,* (2002) [15].

Estimation of Damage in Herring Sperm DNA: The extent of DNA damage in herring sperm DNA and the effects of *C. pulcherrima* flower extracts were studied according to the method proposed by Aeschlach *et al*., (1994) [16].

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

Protein Carbonyl Assay: Protein carbonyls are the most widely used biomarkers for the measurement of protein oxidation and oxidative stress in aging and diseases [17]. The protein carbonyl formation was analyzed by the method outlined by Jean *et al*., (2010) [18].

Analysis of Protein Oxidation by 1-D Gel Electrophoresis: The ability to identify specific proteins that are most susceptible to oxidative modifications facilitates the development of methods for early diagnosis, assessment of new potential therapies and understanding the overall disease mechanisms.

However, it is difficult to identify specific proteins that are most susceptible to oxidative modifications. In the present study, the differences in the mobility of the protein subjected to oxidative stress *in-vitro* and the influence of the flower extracts on the electrophoretic mobility was visualized using polyacrylamide gel electrophoresis (PAGE).

Statistical Analysis: The experimental results were expressed as means ± SD of triplicates. The parameters analyzed were subjected to statistical analysis using SigmaStat (Version 3.1) statistical software. Statistical significance was determined by one-way ANOVA, followed by post-hoc Fischer analysis and the values with $P<0.05$ were considered to be significantly different.

RESULTS:

Effects of the Flower Extracts of *C. pulcherrima* against *in-vitro* Lipid Peroxidation: In three different membrane models namely, goat RBC ghosts (plasma membrane lipids), goat liver homogenate (plasma membrane and intracellular lipids) and liver slices (intact cells), the percent inhibition of *in-vitro* lipid peroxidation by the flower extracts in all the three membrane systems is presented in **Fig. 1**. 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.

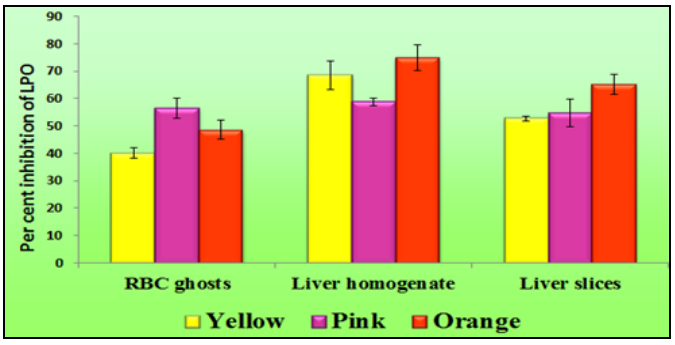
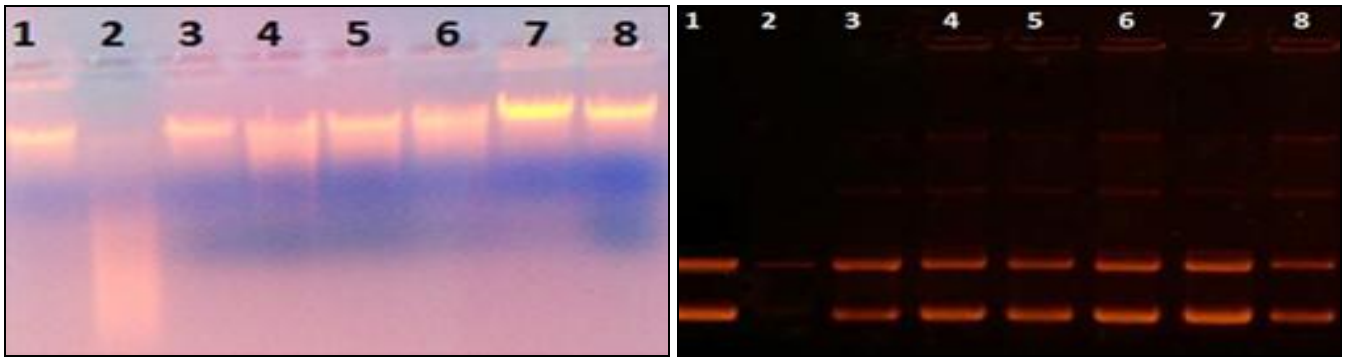


FIG. 1: INHIBITION OF LIPID PEROXIDATION BY THE FLOWER EXTRACTS OF *CAESALPINIA PULCHERRIMA* IN DIFFERENT MEMBRANE PREPARATIONS. The values are Mean ± S.D. of triplicates

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

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

analyzed using agarose gel electrophoresis in which the migration of DNA was observed. The results are presented in **Fig. 2**. 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).



LAMBDA DNA		pUC18 DNA
CPY – *C. pulcherrima* yellow flower	Lane 1: Untreated control	Lane 5: DNA + CPP
CPP – *C. pulcherrima* pink flower	Lane 2: DNA + H_2O_2	Lane 6: DNA + CPP + H_2O_2
CPO – *C. pulcherrima* orange flower	Lane 3: DNA + CPY	Lane 7: DNA + CPO
	Lane 4: DNA + CPY + H_2O_2	Lane 8: DNA + CPO + H_2O_2

FIG. 2: MIGRATION PATTERNS OF Λ DNA AND pUC18 DNA TREATED WITH H2O2 WITH AND WITHOUT *C. PULCHERRIMA* FLOWER EXTRACTS

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). The respective values are presented in **Table 1**.

TABLE 1: 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

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 **Fig. 3**. 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.

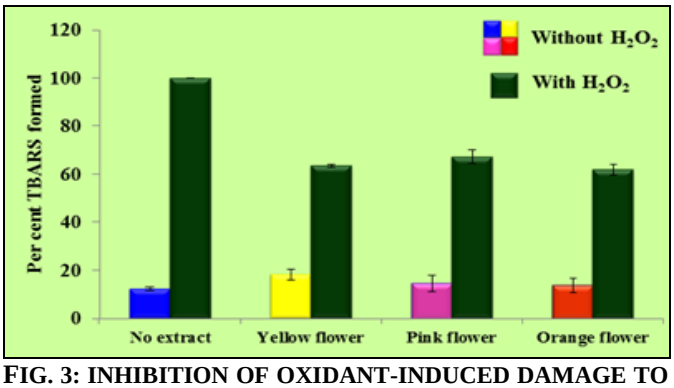


FIG. 3: INHIBITION OF OXIDANT-INDUCED DAMAGE TO HERRING SPERM DNA BY C. PULCHERRIMA FLOWER EXTRACTS. The values are Mean ± S.D. of triplicates. The value of H_2O_2-treated group was fixed as 100 percent and the relative values in percentage were calculated for the other groups.

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

Effect of *C. pulcherrima* Flower Extracts on Protein Carbonyl Formation: The effect of the flower extracts on protein oxidation is depicted in **Table 2**.

TABLE 2: 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

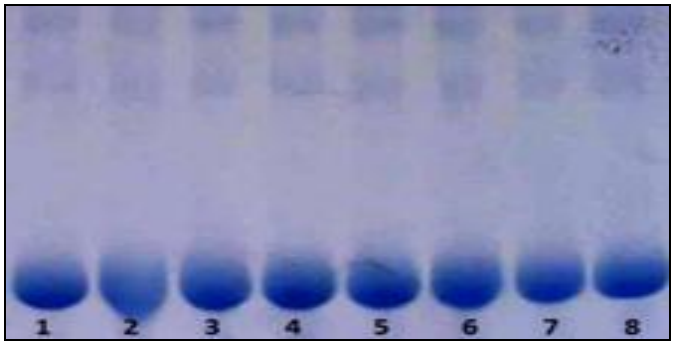
a – Statistically significant ($p<0.05$) compared to untreated control

b – Statistically significant ($p<0.05$) compared to H_2O_2 control

c – Statistically significant ($p<0.05$) compared to the respective plant control

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.

Effect of *C. pulcherrima* Flower Extracts on Protein Migration on 1D Gel: 1D gel probing of oxidized proteins evaluated the effect of the flower extracts on protein oxidation in-vitro. The differences in the electrophoretic mobility of the protein bovine serum albumin subjected to oxidative stress *in-vitro* were determined in the presence and absence of H_2O_2 and the flower extract. It is evident from the results of the SDS-PAGE depicted in **Fig. 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). The co-treatment counteracted this effect with the flower extracts (Lanes 4, 6 and 8).



CPY – *C. pulcherrima* yellow flower	Lane 1: Untreated control	Lane 5: BSA + CPP
CPP – *C. pulcherrima* pink flower	Lane 2: BSA + H_2O_2	Lane 6: BSA + CPP + H_2O_2
CPO – *C. pulcherrima* orange flower	Lane 3: BSA + CPY	Lane 7: BSA + CPO
	Lane 4: BSA + CPY + H_2O_2	Lane 8: BSA + CPO + H_2O_2

FIG. 4: EFFECT OF *C. PULCHERRIMA* FLOWER EXTRACTS ON THE MIGRATION OF PROTEINS SUBJECTED TO OXIDATIVE STRESS

TABLE 3: 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 showed that the methanolic extracts of the three different flowers of *C. pulcherrima* rendered significant biomolecular protection against oxidative stress, both in cell-free systems and in intact cells.

DISCUSSION: At pathological levels, free radicals and oxidants generate a deleterious process called oxidative stress, which, in turn, causes damage to structures like cell membranes and other macromolecules including lipids, proteins, lipo-

proteins and DNA [19]. Overproduced reactive species (ROS and RNS) react with cell membrane fatty acids and proteins, thereby impairing their function permanently and trigger some human diseases. Free radicals also induce DNA damage, resulting in mutations that will predispose to cancer and age-related disorders [20]. The antioxidant potential of a test compound or herbal preparation is also based on their protective effect against oxidant-induced damage to cellular biomolecules.

Oxygen radicals cause damage to cellular membranes through the initiation of a process known as lipid peroxidation. Lipid peroxidation occurs as a radical chain reaction that spreads rapidly, affecting a large number of lipid molecules, which leads to altered membrane integrity and permeability [21]. In recent years, many studies have focused on measuring lipid peroxidation products that can be used as potential biomarkers to assess the oxidative stress status *in-vivo* and to evaluate the effectiveness of antioxidants [22]. In the present study, in all the three membrane preparations, the extent of lipid peroxidation was substantially decreased by the three flower extracts.

A vast literature assessing the inhibitory effects of plants and herbs on LPO in different membrane lipid sources is available to support our findings. Significant inhibition of malondialdehyde formation by the aqueous extract of *Moringa oleifera* leaves in liver homogenate was reported in a study [23]. The same extract also significantly reduced the levels of lipid peroxides in goat liver slices under CCl_4-induced oxidative stress [24]. A high degree of inhibition of LPO was shown by the aqueous and ethanolic extract of *Phyllanthus fraternus* callus [25]. Crude extract and fractions of *Harpagophytum procumbens* inhibited LPO in brain homogenates in a concentration-dependent manner [26]. A dose-dependent reduction in LPO was observed in experimental rats treated with Amukamara choornam ethanolic extract [27].

These reports support the findings of the present study, wherein, all the three flower extracts rendered strong protection to intracellular lipids. Better protection by the flower extracts against lipid peroxidation was observed to liver slices, which implies that some bioactive component present in the extract is capable of penetrating through the cell membrane that renders the antioxidant property for inhibiting LPO.

Oxidative damage arises from endogenous and exogenous sources and affects both nuclear and mitochondrial DNA as well as RNA and proteins. DNA is constantly damaged by ROS and RNS directly. The lipid peroxidation (LPO) products also affect DNA, forming exocyclic adducts to DNA bases [28].

A wide variety of oxidatively-generated DNA lesions such as single-strand breaks to complex lesions like double-strand breaks, and other oxidatively generated clustered DNA lesions are present in living cells [29]. Accumulation of oxidative DNA lesions due to misrepair or incomplete repair causes mutagenesis, which consequently leads to carcinogenesis [30].

Many research studies have reported the protective effects of plant extracts and their isolated bioactive compounds against oxidative DNA damage. The protective effect of the natural product affinity isolated from the ethanol extract of *H. longipes* against norfloxacin-induced DNA damage has been reported [31]. The methanolic extract of *Koelreuteria paniculata* leaves showed strong genoprotective activity against H_2O_2-induced damage to pUC18 and calf thymus DNA [32]. Eugenol and isoeugenol rendered strong protection against H_2O_2-induced damage to pBR322 plasmid [33]. Aqueous extract of *Curcuma amada* (Roxb) showed a concentration-dependent protecting effect against H_2O_2-induced damage in herring sperm DNA [34].

The leaf and herbal extracts of *Withania somnifera* rendered significant protection against H_2O_2-induced oxidative damage to pUC18, lambda and herring sperm DNA [35]. A flavonoid called apigenin-8-C-α-L-rhamnopyranose-(1→2)- β- D-glucopyranoside isolated from the leaves of *Garcinia gracilis* prevented the pBR322 plasmid DNA damage induced by oxidative stress [36]. The results of the present study are in agreement with the above reports that the flowers of *C. pulcherrima* are very effective in protecting the DNA from oxidative damage. The variation found in the extent of protection with different hierarchical levels of DNA signifies that the

biomolecular protective effects of a substance is quantified by analyzing its effect on different forms and sources of DNA exposed to oxidative stress.

Oxidative modifications of protein by ROS/RNS include the formation of protein carbonyls, tyrosine, nitrated and chlorinated tyrosines, resulting in diverse functional consequences. Accumulation of oxidized proteins has been found in diseased tissues of patients with various diseases like inflammatory diseases, atherosclerosis, rheumatoid arthritis and cataractogenesis [37]. Protein carbonyls, a major form of protein oxidation can be used as markers for oxidative stress [38]. In the present study, the reduction in the oxidant-induced protein carbonyl formation observed in the flower extract treated groups indicated the protective effect of the flowers of *C. pulcherrima* against protein oxidation.

These results corroborated with a study, in which the pigment rubropunctatin isolated from the hexane extract of fermented *Monascus purpureus* CFR 410-11 significantly inhibited protein carbonyl formation and oxidation as assayed by SDS-PAGE [39]. In a similar study by Razack *et al.,* (2015), using BSA, a dose-dependent inhibitory effect of the ethanolic extract of *Nardostachys jatamansi* against protein oxidation was reported by measuring the protein carbonyl formation [40].

It was observed that a mixture of aqueous extract of *Allium sativum* and methanolic extract of *Lagerstroemia speciosa* decreased the hepatic protein carbonyl levels significantly in the type-II diabetic rats [41]. Administration of polyphenolic-rich extract prepared from *Sorghum bicolor* grains decreased the level of protein carbonyl in DEN-treated rat microsomes [42]. Kędzierska *et al.,* (2013) suggested that protein carbonyls can be used as markers for hemostasis changes in breast cancer patients. They also showed that in an *in-vitro* system, treatment with a commercial extract prepared from *A. melanocarpa* berries reduced the levels of protein carbonyls in plasma from breast cancer patients, after surgery and different phases of chemotherapy [43]. The inhibitory activity of *Mesona chinensis* extract on fructose-mediated protein glycation was confirmed from the reduced level of carbonyl content of BSA treated with *M. chinensis* extract [44].

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

Makri *et al.,* (2013) confirmed the protective effect *Crocus sativus* stigmas (saffron) extract against selenium-induced crystalline proteolysis of rat lens proteins using SDS-PAGE [45]. Densitometric and quantified gel image analysis of protein bands showed that the aqueous extracts of *Hertia cheirifolia* significantly protected BSA against oxidative stress which is evident from the restored high protein band intensity [46]. Using SDS-PAGE analysis, it was found that glutathione and ascorbate partially protected the riboflavin-induced photo-oxidation of human αA-crystallin protein [47].

Our results also showed that the methanolic extracts of the flowers of *C. pulcherrima,* blooming in all the three different colors, were very efficient in protecting proteins against oxidative damage. From the results, it is evident that the flowers exhibited a significant biomolecular protective effect against oxidative stress.

CONCLUSION: Oxidative stress-induced biomolecular damage is the key mechanism underlying various steps involved in the development of the malignant phenotype such as evasion of apoptosis, uncontrolled proliferation, angiogenesis, tissue invasion, and metastasis. The study on free radical-induced damage has become a major thrust of carcinogenesis research. The results of this study thus signify that a strong anticancer potential to be associated with the protection rendered by the flowers of *Caesalpinia pulcherrima* against oxidative damage. Further, research needs to be carried out to determine the anticancer activity of the flowers of *Caesalpinia pulcherrima* using both *in vitro* and *in vivo* models.

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CONFLICT OF INTEREST: The authors declare that there is no conflict of interests.

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