

PROTECTIVE EFFECT OF *CASSIA FISTULA* EXTRACTS AGAINST ULTRAVIOLET C INJURY IN HUMAN CORNEAL EPITHELIAL CELLS

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ABSTRACT

Objective: The objective of this study was to investigate the antioxidant activity of Indian laburnum (*Cassia fistula* L. [CF]) leaf extracts and their impact on ultraviolet C (UVC) radiation-induced damage on human corneal epithelial (HCE) cells.

Methods: The antioxidant activity and free radical scavenging ability of CF leaf extracts were determined by *in vitro* methods such as 1,1-diphenyl-2-picrylhydrazyl radical scavenging capacity, Total antioxidant capacity (TAC), and reducing power. The total phenolic content (TPC), total flavonoid content (TFC), and preliminary phytochemical screening were done to ensure the pharmacological effects of the extracts. The UVC radiation at wavelength 254 nm was used to irradiate HCE cells and cell viability was assessed by methyl thiazolyl tetrazolium assay.

Results: Extracts at the concentration of 200 µg/ml did not affect the cell viability of HCE cells. Almost 50% cell death was observed after UVC irradiation at a dose of 200 J/m². Both extracts showed a protective effect by increasing the cell viability of irradiated cells up to 57.28% and 62.39%. A dose-dependent increase in the TAC and reducing power of the extract was observed. The TPC in aqueous and ethanol extracts of CF leaves was 18.8 and 27.80 mg gallic acid equivalent/g sample while TFC was 8.47 and 16.5 mg quercetin equivalent per/g sample, respectively.

Conclusions: CF leaves are a potent source of bioactive compounds with good antioxidant potential. Exposure to UVC radiation cause harmful effects on HCE cells and the extracts have shown to have potent protective effects on UV light-induced oxidative stress in HCE cells.

Keywords: Corneal epithelium, Ultraviolet rays, Antioxidant, *Cassia fistula*.

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INTRODUCTION

Photochemical damage to ocular tissues is caused by solar radiation and other ultraviolet (UV) sources. Since ocular tissues do not develop tolerance to UV exposure, excessive exposure of UV radiation to the ocular surface is damaging to the eye. The corneal epithelium is the external layer of the eye which is directly exposed to the UV radiation of the sun. Wavelengths from 400 to 100 nm are within the UV spectrum which is further categorized as UVA 400–315 nm, UVB 315–280 nm, UVC 280–200 nm, and UV vacuum 200–100 nm [1]. The shorter and more toxic wavelengths of UVC and UV vacuum are blocked from reaching the earth by ozone in the stratosphere. However, the eye is susceptible to UVC radiation during occupational exposures by artificial sources such as welding arcs, tanning lamps, therapeutic high-intensity UV for skin conditions or seasonal affective disorder, germicidal UV lamps in hospital and research purpose, and also from the sun through reflection from snow and water. Cornea absorbs 100% of UVC radiation; hence, cornea is more susceptible to UVC-induced oxidative damages than the other parts of the eye such as lens and retina [2].

Antioxidants are free radical scavengers which inhibit protein denaturation and lipid peroxidation, by protecting the human body from several diseases caused due to the triggering of reactive oxygen species (ROS). Oxidative stress occurs due to the imbalance between the generation of ROS and cellular antioxidant enzymes present in the body [3]. Medicinal plants are known to possess a number of antioxidants. These natural antioxidants are shown to reduce oxidative damage and the development of major diseases. Various studies have been carried out on numerous plants and are a potential source of antioxidants.

Cassia fistula L. is used in phytochemical and pharmacological research due to their excellent medicinal values and this potent herb is used as an alternative medicine for centuries. The plant is also used for adenopathy, burning sensations, leprosy, skin diseases, syphilis, and tubercular glands. It has mildly laxative, constricting, and anthelmintics property [4] and used as a treatment for burns, convulsions, constipation, cancer, hematuria, delirium, epilepsy, diarrhea, hematuria, pimples, and glandular tumors. The leaves have been used since ancient times for treating malaria, ulcers, rheumatism, fever, and constipation, skin disease such as leprosy, abdominal pain, and heart disease [5]. Yunani uses the leaves for inflammation, antipyretic, abortifacient, demulcent, purgative, chest complaints, eye ailments, flu, and rheumatism [6]. In Ayurvedic classics, this plant is mainly used as laxative during fever (*Mrudurechaka*) and many conditions such as skin diseases (*Kushta*), rheumatic diseases (*Amavaata*), cervical lymphadenitis (*Gandamala*), cardiac diseases (*Hrudroga*), worm infestations (*Krimi*), abdominal pain (*Shoola*), abdominal disorders (*Udararoga*), polyuria (*Prameha*), dysuria (*Mutrakrichra*), and bloating of abdomen (*Gulma*). It is also said to be the best for the elimination of different *dosha* of the gastrointestinal tract [7]. *Acharya Sushruta* has indicated the use of *Aragvada* in *netrapaka* in the form of *raskriya* [8].

Since it is important to prevent the UV-induced oxidative damage, we thought that medicinal plants may protect the cornea from UV-induced oxidative stress, and also, CF is known to possess various pharmacological activities. We thought that medicinal plants may protect the cornea from UV-induced oxidative stress, and also, CF is known to possess various pharmacological activities. Hence, we chose this plant as a powerful candidate for our study. Till date, the effect of

CF extracts on UV-induced damages in human corneal epithelial (HCE) cells is not studied.

Therefore, in the present study, we aimed to investigate the antioxidant activities of the aqueous and ethanol extracts of CF leaves and check the protective effect of these extracts on UVC-induced damage in HCE cells.

METHODS

Plant material

The leaves of CF L. were collected from Yenepoya (Deemed to be University) garden, Derlakatte, Mangalore, and were identified by the botanist.

Extracts preparation

The air-dried plant powder was subjected to maceration using solvents such as aqueous and ethanol extracts for 24 h in a shaker incubator. After 24 h, the extract was centrifuged and the supernatant was filtered using Whatman filter paper, and then, the extracts were concentrated using a Speedvac Concentrator and the dry residue was kept in a refrigerator at 4°C for further analysis [5].

Phytochemical screening

The condensed extracts was used for preliminary screening of phytochemicals such as alkaloids (Wagner and Mayer's tests), flavonoids (alkaline reagent test), carbohydrates (Molisch's test, Fehling's test, and Barfoed's test), glycosides (Legal's test), saponins (foam test), protein's (Biuret test), amino acids (Ninhydrin test), sterols (Liebermann-Burchard test), phenolic compounds, and tannins (gelatin test, ferric chloride test, and lead acetate test). All the tests were carried out using standard protocol [9-12].

Cell line and culture

HCE cells were cultured in Dulbecco's modified Eagle's medium/Hams F12 (1:1) supplemented with 10% heat-inactivated fetal bovine serum and antibiotic-antimycotic solution and maintained at 37°C and 5% CO₂ humidity.

Cytotoxicity assay

HCE cells were treated with different concentrations of extracts and incubated for 48 h and the cell viability was assessed by standard spectrophotometric methyl thiazolyl tetrazolium (MTT) assay. Briefly, cells were plated at a density of 5000 cells/well in 96-well microplates. After incubation for 24 h, the selected plant extracts were added to the culture medium at different concentrations (12.5–200 µg/ml) and incubated for 48 h. Then, MTT solution was added, followed by the addition of dimethyl sulfoxide after 4 h to solubilize the formazan crystals. The absorbance was taken at 570 nm using multimode microplate reader (FluoSTAR Omega, BMG Labtech, Ortenberg, Germany). The cell viability was expressed as the percentage of the control.

UVC irradiation

Cells were irradiated with UVC radiation (254 nm) at a dose of 200 J/m² using UVP-2000 UV crosslinker, and after 1 h, cells were incubated with aqueous and ethanol extracts of CF leaves (12.5 µg/ml) followed by MTT assay.

ESTIMATION OF ANTIOXIDANT ACTIVITY

Total antioxidant capacity (TAC) assay by phosphomolybdate method

The total antioxidant capacity of the CF leaf extracts was evaluated by the phosphomolybdenum method [13]. Ascorbic acid (AA) was used as a standard antioxidant. Different concentration of plant extracts ranging from 50 to 600 µg/ml was taken for the experiment. The experiment was performed in triplicates (n=3). The antioxidant activity of the extracts was expressed in terms of AA equivalents AAE.

1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity

The antioxidant activities of plant extracts and the standard were assessed on the basis of the free radical scavenging ability of DPPH by modified method [14]. Plant extract at concentrations (50–600 µg/ml)

was taken for the experiments. The L-AA (100 µg/ml) was used as standard. The experiment was performed in triplicates (n=3) and the percentage radical scavenging activity was calculated using the formula:

$$\% \text{ Radical scavenging ability} = (\text{Ac-AE}) \times 100 / \text{Ac}$$

Where,

Ac is the absorbance of control (DPPH), AE is the absorbance of test. The decrease in the absorbance of DPPH solution indicates an increase in the DPPH radical scavenging activity.

Determination of reducing power

Ferric reducing antioxidant power (FRAP)

Reducing ability of the plant extracts was investigated based on the procedure developed by Oyaizu [15]. Different concentrations of the plant extracts (250, 500, and 1000 µg/ml) were used for the experiments. The experiments were performed in triplicate (n=3). L-AA was used as a standard

Quantitative phytochemical screening

Total phenolic content (TPC)

The TPC of the plant extract was investigated according to the standard protocol [16]. Gallic acid was used as a standard. The samples were taken in triplicates (n=3). The TPC was expressed as gallic acid equivalents (mg GAE/g extract).

Total flavonoid content (TFC)

TFC of the plant extracts was determined according to standard protocol [17]. Quercetin was used as a standard. All samples were taken in triplicate (n=3). The TFC was expressed as quercetin equivalents (mg QE/g extract).

Statistical analysis

Data are represented as mean±standard deviation from three experiments. Data were analyzed using one-way analysis of variance with Dennett *post hoc* test. Differences of p≤0.01 were considered statistically significant.

RESULTS

Effect of CF extracts on HCE cells

The cell viability analysis of HCE cells was performed by MTT assay after 48 h incubation in aqueous and ethanol extracts of CF leaves at different concentrations (12.5–200 µg/ml). The cell proliferation was expressed as a percentage of control. Both aqueous and ethanol extract did not affect the viability and proliferative activity of the cells even at concentration of 200 µg/ml (Fig. 1).

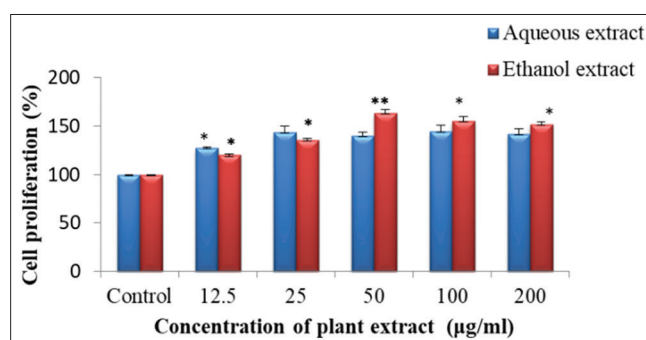


Fig. 1: Effect of aqueous and ethanol extracts of CF on human corneal epithelial cells. Cells were treated with different concentrations (12.5–200 µg/ml) of extracts and incubated for 48 h and the cell proliferation was determined by methyl thiazolyl tetrazolium assay. Data are represented as mean±standard deviation (n=3), p<0.01 is considered statistically significant. CF: *Cassia fistula*

Effect of CF extracts against UVC-induced injury in HCE cells

To investigate the protective effects of CF leaf extracts, the HCE cells were irradiated with UVC radiation (254 nm) at 200 J/m² using UVP-2000 UV crosslinker, and after 1h, cells were incubated with aqueous and ethanol extracts at a concentration of 12.5 µg/ml followed by MTT assay. The results showed that UVC irradiation on HCE cells reduced the cell viability and caused cell death by 47%. The cell death decreased after they were treated with CF aqueous and ethanol extracts. Both aqueous and ethanol extract showed high protective effect about 57.28% and 62.39% cell viability, respectively (Fig. 2).

Phytochemical screening of extracts

The aqueous and ethanol extracts of CF leaves were screened for phytochemical constituents. The aqueous extracts showed the presence of alkaloids, carbohydrates, protein, amino acids, flavonoids, phenolic compounds, and tannins. The ethanol extracts showed the presence of alkaloids, protein, amino acids, flavonoids, phenolic compounds, and tannins. These results confirmed that the extracts had a significant amount of bioactive compounds. The results are summarized in Table 1.

Total antioxidant activity

The total antioxidant capacity of aqueous and ethanol extracts of CF leaves was calculated using the standard curve of AA ($y=0.012x-0.971$; $R^2=0.991$) and expressed as an AAE/g of extract. Both aqueous and ethanol extracts exhibited a dose-dependent increase in antioxidant activity shown in Fig. 3. Aqueous extract at concentrations of 50 and 600 µg/ml showed the antioxidant activity of 51.18 and 86.22 µM AAE, respectively. Ethanol extract at concentrations of 50 and 600 µg/ml showed the antioxidant activity of 65.86 and 88.11 µM AAE, respectively.

DPPH radical scavenging activity

Aqueous and ethanol extracts of CF leaves exhibited a significant DPPH scavenging activity as shown in Fig. 4. Aqueous extract exhibited 10.25%, 21.31%, 25.42%, 31.11%, and 33.24% of scavenging activity at 50, 100, 200, 400, and 600 µg/ml concentrations, respectively, whereas ethanol extract exhibited 32.85%, 60.94%, 70.46%, 71.24%, and 54.43% with the same concentrations. The standard AA showed 94.73%, 94.92%, 94.81%, 86.17%, and 82.58% of the activity with the same concentrations. Ethanolic extracts showed the highest activity when compared to aqueous extracts. Percentage of scavenging activity or percentage inhibition was calculated by the linear regression method.

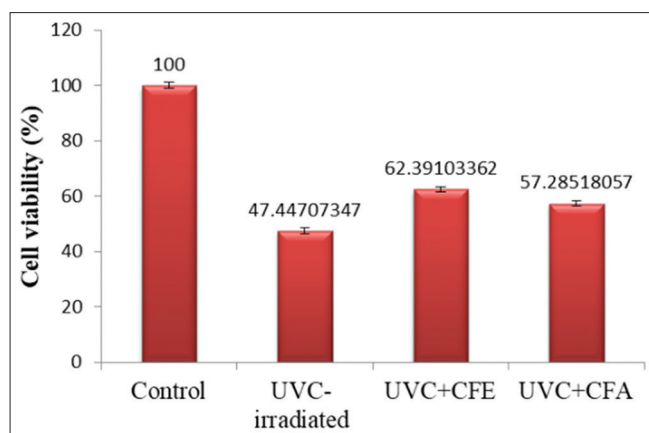


Fig. 2: Effect of aqueous and ethanol extracts of CF on UVC irradiated human corneal epithelial cells. Cells were irradiated with UVC radiation at a dose of 200 J/m², and after 1 h, cells were incubated with aqueous and ethanol extracts of CF leaves (12.5 µg/ml) followed by methyl thiazolyl tetrazolium assay. Data are represented as mean±standard deviation (n=3). CFA: Aqueous extracts of *Cassia fistula* leaves; CFE: Ethanol extract of *Cassia fistula* leaves; UVC: Ultraviolet C, CF: *Cassia fistula*

Reducing power ability

The reducing power ability of aqueous and ethanol extracts of the leaves of CF was tested as shown in Fig. 5. Among the two extracts, the highest percentage of reducing power ability was seen in ethanol extract than the aqueous extract when compared to standard AA. There was a dose-dependent increase in the reducing power ability in both aqueous and ethanol extracts of CF leaves.

Total phenolic and flavonoid content of the CF leaves

The TPC in aqueous and ethanol extract of CF leaves was found to be 18.8 and 27.80 mg GAE/g extract, respectively. The TFC in aqueous and ethanol extracts of CF leaves was found to be 8.47 and 16.5 mg QE/g extract, respectively. The results are shown in Table 2.

Table 1: Screening of the phytochemicals of CF leaf extracts

Tests	CF extracts	
	Aqueous	Ethanol
Alkaloids		
Mayer's test	+	+
Wagner's test	-	-
Carbohydrates and glycosides		
Molisch's test	-	-
Fehling's test	+	-
Legal's test for glycosides	-	-
Saponins		
Foam test	-	-
Protein and amino acids		
Biuret test	+	+
Ninhydrin test	+	+
Phytosterols		
Liebermann-Burchard's test	-	-
Phenolics and tannins		
FeCl ₂ test	+	-
Gelatin test	-	-
Lead acetate test	+	+
Flavonoids		
Alkaline reagent test	+	+

(+) indicates the presence of the phytochemical and (-) indicates the absence of phytochemical. CF: *Cassia fistula*

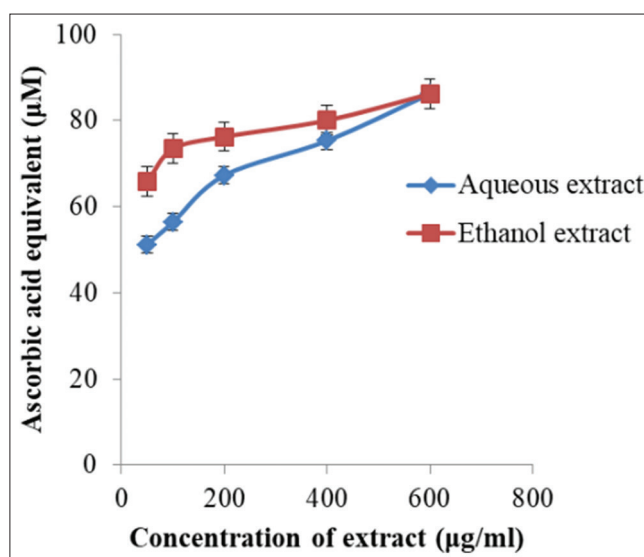


Fig. 3: Total antioxidant activity of aqueous and ethanol extracts of CF leaves. Different concentrations (50–600 µg/ml) of extracts were taken and evaluated for their TAC. Values are expressed as the mean±standard deviation (n=3). Ascorbic acid (AA) was used as a standard. The antioxidant activity is expressed as equivalents of ascorbic acid

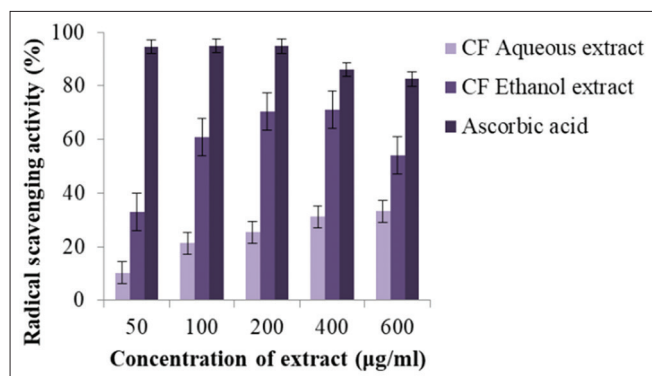


Fig. 4: 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity of aqueous and ethanol extracts of CF leaves. Different concentrations (50–600 µg/ml) of extracts were taken and evaluated for their DPPH scavenging ability. Values are expressed as the mean±standard deviation (n=3). Ascorbic acid at concentrations (50–600 µg/ml) was used as a standard. $p < 0.01$ is considered statistically significant. CF: *Cassia fistula*

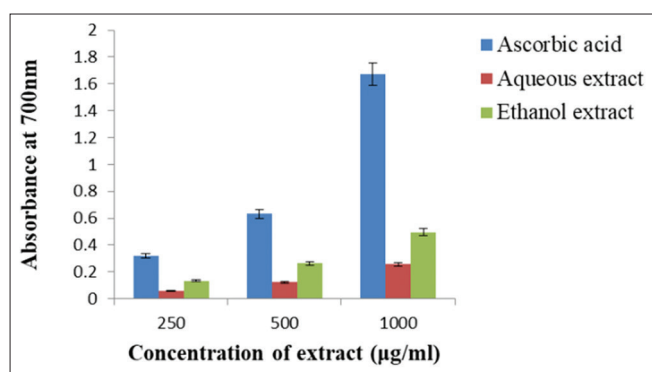


Fig. 5: Reducing power ability of aqueous and ethanol extracts of CF leaves. Different concentrations (250, 500, and 1000 µg/ml) of extracts were taken and evaluated by ferric reducing antioxidant power method. Values are expressed as the mean±standard deviation (n=3). Ascorbic acid was used as a standard. $p < 0.01$ is considered statistically significant. CF: *Cassia fistula*

DISCUSSION

Medicinal plants are known to possess various bioactive compounds with potential health benefits and medicinal properties. Since the plant compounds also contain toxic compounds, it is necessary to standardize the dose and check the cytotoxic effect on the cells. One of the ways to check the cytotoxicity of the extracts is by the *in vitro* cell culture method [18]. This study is done so that CF extracts can be used in the formulation of eye drops in future.

In the current study, the toxicity and antiproliferative activity of the aqueous and ethanol extracts of CF leaves was tested on HCE cells. The extracts even at high concentrations (12.5–200 µg/ml) did not show any cytotoxic effect on the cells. Furthermore, it did not interfere with the proliferative activity of the HCE cells. Since this plant extracts did not show any cytotoxic effect even at high concentrations on HCE cells, this extract proves to be less harmful to be used as a therapeutic agent for eye-related disorders and prophylaxis. Medicinal plant extracts with reduced cytotoxicity are needed to discover a potential drug.

Plants are known to possess potent antioxidant and free radical scavenging activities. The amount of phenolic compound directly correlates to the antioxidant activity of the plant extracts [19]. The scavenging ability is due to the presence of the hydroxyl group of the phenolic compounds. In the present study, the antioxidant activity of

Table 2: Total phenolic and flavonoid content of aqueous and ethanol extracts of CF leaves

Test	CF leaves	
	Aqueous extract	Ethanol extract
TPC (mg GAE/g extract)*	18.8±0.019	27.80±0.003
TFC (mg QE/g extract)*	8.47±0.142	16.5±0.003

TPC: Total phenolic content, TFC: Total flavonoid content, GAE: Gallic acid equivalent, CF: *Cassia fistula*, QE: Quercetin equivalents

the extracts was checked by TAC assay, DPPH assay, and FRAP assay. The TPC and TFC were also quantified.

The *in vitro* antioxidant activity of aqueous and ethanol extracts of CF leaves was tested and is discussed below. The DPPH scavenging ability of the extracts was evaluated against standard AA and it was found that ethanol extract at a concentration of 400 µg/ml showed the maximum DPPH scavenging activity of 71.24% than the aqueous extract which was similar to that of standard AA. The DPPH reduction is directly proportional to the antioxidant activity of the extract and due to the stabilization of the free radicals by the proton-donating ability of the extracts [20]. The proton radical scavenging action is an important mechanism for measuring antioxidant activity. This assay determines the scavenging of stable radical species DPPH by antioxidants compounds present in the extracts. The results indicate greater DPPH scavenging activity of ethanolic extracts as compared to the aqueous extracts which are probably due to the presence of high phenolic compounds. Our study clearly shows that the ethanolic extract of CF leaves exhibits high phenolic compounds which significantly correlate to the DPPH radical scavenging activity of the extracts ($p < 0.01$). The findings of the study suggest that the extracts of CF leaf contain numerous bioactive phytochemicals which are capable of donating hydrogen to a free radical. The presence of alkaloids, carbohydrates, protein, amino acids, flavonoids, phenolic compounds, and tannins in the extracts is closely associated with medicinal properties such as the anti-inflammatory, antidiabetic, and analgesic activity of CF leaves [21].

The total antioxidant capacity of ethanol extract was found to be higher than the aqueous extract. There was a concentration-dependent increase in the total antioxidant activity in both ethanol and aqueous extract of CF leaves. The total antioxidant activity of the CF extracts corresponds to the capability to counteract ROS, resist the oxidative damage, and combat diseases caused due to oxidative stress [20].

The reducing power assay is done to evaluate the extracts ability to donate an electron [22]. The ethanol and aqueous extract of CF leaves showed good activity in reducing Fe^{+3} to ferrous ion. The extracts reveal their antioxidant activities by donating a hydrogen atom through breakage of the free radical chain and they convert the radicals into stable and non-toxic products. The reducing power sequence of the samples is as follows: AA>ethanol extract>aqueous extract. The reducing power of CF ethanol extracts was higher than the aqueous extract of CF leaves. Dose-dependent increase in the reducing power ability was observed in both ethanol and aqueous extracts. The reducing power assay is mostly used to evaluate the antioxidant ability to donate an electron which is an important mechanism of phenolic antioxidant action [23]. Previous studies have shown that there is a direct correlation between antioxidant activities and reducing the power of plant extracts [24,25].

Plant phenol compounds possess potent antioxidant, antimicrobial, and anti-inflammatory activity [26-28]. The TFC in aqueous and ethanol extract was 8.47 and 16.5 mg QE/g sample, respectively. High flavonoid content in ethanol extract contributes to the antioxidant, antimicrobial, and anti-inflammatory properties in various studies [29,30].

UV rays act as a significant oxidative stimulus for the human eye, leading to the development of several eye disorders such as cataracts,

photokeratitis, several retinopathies, as well as neoplasms. UV-induced pathologic changes in the eye are due to the generation of ROS, leading to protein, lipid, and DNA damage following epithelial cell viability loss [31]. In our study, we checked the efficacy of the CF leaf extracts in protecting HCE cells from UVC-induced injury by MTT assay. When the HCE cells were irradiated with UVC at 200 J/m², almost 50% cell death was observed which was found to be decreased after extract treatment. CF ethanol extracts at a concentration of 12.5 µg/ml exhibited a protective effect by increasing the cell viability up to 62.39%. These results indicate that the ocular surface could be damaged by the exposure to UVC radiation (254 nm), and the application of medicinal plant extracts will be effective in decreasing the damage by their antioxidant activity. Among the two extracts evaluated, the ethanol extract proved to have high antioxidant activity and reducing power which is due to the presence of phenolic and flavonoid content which might be related to the reduction of ROS and an increase in the antioxidant enzymes. Previous studies have shown that the use of natural antioxidants has been helpful in reducing the oxidative stress in various parts of the eye. Antioxidants such as omega-3 fatty acids and green tea polyphenols are known to have anti-inflammatory and antioxidant effects on HCE cells, the topical or systemic application of these antioxidants is known to decrease inflammatory markers in the tear film, ocular surface, and lacrimal gland of dry eye [32,33]. A mixture of ethanol extracts of four selected plants protected HCE cells from oxidative stress induced by light-emitting diode irradiation of shorter wavelengths through their antioxidant enzymatic defense system [34]. Abid *et al.* have reported that the CF extracts showed remarkable antioxidant and protective activity against induced oxidative stress in erythrocytes [35]. Methanolic and ethyl acetate extract of CF leaves are also known for its antihyperlipidemic and antiatherosclerotic activity as reported by Reddy *et al.* [36].

Taken together our results suggest that the CF leaves have good antioxidant and reducing power, and non-cytotoxic, and also, they are capable of protecting the HCE cells from UVC damage. Hence, they might also be effective in the treatment of oxidative stress-induced ocular surface diseases such as pterygium and dry eye diseases where the failure of antioxidant defense plays a pivotal role.

CONCLUSIONS

CF leaf extracts at low concentrations showed no toxicity to human corneal cells and protected the cells from UVC damage which might be due to their antioxidant potential. This *in vitro* study shows that the CF leaf has the therapeutic potential in the treatment of ocular surface inflammatory diseases. The present study was carried out only on aqueous and ethanol extracts of CF leaves. Additional studies can be done with other extracts with fractionates to evaluate the components in the plant extract. The study needs further validation by checking the level of antioxidant enzymes, ROS production at the cellular and molecular level. This validation will help further in elucidating the pathogenesis and treatment of oxidative stress-induced ocular surface diseases.

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AUTHORS' CONTRIBUTIONS

Deeksha K conceived, designed and performed the experiments, and analyzed the data. Dr. Cynthia Arunachalam provided the nurturing environment and intellectual insights. Both were involved in writing the paper.

CONFLICTS OF INTEREST

All authors have none to declare.

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