

VITEX NEGUNDO EXTRACTS PROTECT HUMAN CORNEAL EPITHELIAL CELLS FROM ULTRAVIOLET C-INDUCED PHOTOTOXICITY

DEEKSHA K^{1,2}, CYNTHIA ARUNACHALAM^{2*}

¹Department of Ophthalmology Yenepoya Medical College, Yenepoya (Deemed to be University), Mangalore, Karnataka, India, ²Yenepoya Research Centre, Yenepoya (Deemed to be University), Mangalore, Karnataka, India. Email: cynthiaarunachalam@yahoo.com

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ABSTRACT

Objective: The objective of the study was to investigate the protective effects of *Vitex negundo* (VN) extracts against ultraviolet (UV) light C-induced oxidative stress in human corneal epithelial cells (HCEC) *in vitro*.

Methods: The VN extracts were checked for their antioxidant activities by biochemical tests. The preliminary phytochemical analysis was done to check the presence of secondary metabolites. To trigger oxidative stress, the HCEC were exposed to UVC irradiation (200 J/m²). Then, different concentration of VN extracts was administered after 1 h of UVC irradiation. The effects of UVC irradiation and the extracts on the HCEC viability, morphology, cell migration by scratch assay, and reactive oxygen species (ROS) production by dichlorofluorescein diacetate method were measured.

Results: VN ethanol (VNE) extract showed the highest antioxidant activity than the aqueous extract. Phytochemical screening shows the presence of alkaloids, carbohydrates, proteins, phenolics, and tannins. The extracts showed no cytotoxicity on the HCEC and also the VNE extract did not alter the morphology of the cells. Results suggest that the VNE extract enhanced the proliferation and migration of HCEC. The VNE extract significantly decreased UVC-induced oxidative toxicity by inhibiting the intracellular ROS production and maintained cell viability.

Conclusion: VN extracts with its strong antioxidant potential protects HCEC from UVC-induced oxidative stress. The results suggest that these extracts can be used as prophylactic agents or as a therapeutic agent in overcoming UV light-induced damage in various ocular surface diseases such as pterygium and photokeratitis.

Keywords: Ultraviolet rays, Antioxidant, Corneal epithelium, *Vitex negundo*.

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INTRODUCTION

Cornea acts as a primary and structural barrier of the eye. The corneal epithelium is frequently exposed to ultraviolet (UV) radiation such as UVA (315–400 nm), UVB (280–315 nm) from the sun, and UVC (200–280 nm) through occupational exposures [1]. The cornea is known to absorb 100% of UVC without allowing them to penetrate further into the interior of the eye [2]. Thus, corneal epithelium is susceptible to UVC-induced damages. Imbalance in the antioxidant defense mechanism takes place during UV exposure leading to injury in the cornea and other parts of the eye [3]. Excessive exposure to these radiations leads to oxidative stress in the ocular surface cells resulting in epithelial exfoliation followed by extreme pain, a condition known as photokeratitis [4].

Reactive oxygen species (ROS) are formed during aerobic metabolism as metabolic by-products in which their levels are maintained in healthy cells through intrinsic antioxidant mechanisms in healthy cells. Abnormal redox homeostasis which involves overproduction of ROS will induce oxidative stress leading to the damage of the normal physiological condition [5]. Various studies have shown that ROS overproduction of ROS damage the stability of genome; enhance cancer progression [6,7]. In the cornea, ROS directly cleavage the stromal glycosaminoglycans and alter physiological properties, which leads to oxidative stress and sometimes in severe conditions leads to vision loss [8]. Medicinal plants are a potential source of natural antioxidants, and they are known to protect the cells from ROS-induced oxidative damages. Various medicinal plants possess constituents such as flavonoids, flavones, anthocyanins, lignans, and isocatechins which contribute to their antioxidant activity and their therapeutic potential [9,10].

Vitex negundo (VN) is commonly known as a five-leaved chaste tree. It is used in different treatment systems such as Ayurveda, Unani, Siddha, Homeopathy, and Allopathy [11]. This plant possesses anti-inflammatory, antioxidant, hepatoprotective, neuroprotective, and cardioprotective functions which can be attributed to their therapeutic potential [12–15]. Various therapeutic uses have been reported, VN leaf extract is used as an external application for treatment of mumps, wounds, and cuts. It is also used an external application for treating tonsillitis, congestion, and scabies along with other plant mixtures [16]. Apart from these medicinal properties, they are also indicated for Netra roga by Acharya Sushruta in Uttartantra as Anjana in Timira roga and Kapha Vidhagdha Dristi [17]. Different nighantus described this plant as Netrahita and chakshusya [18,19].

Although there are many studies on the antioxidant activity of VN extracts, the potential for using these extracts in the treatment of eye disorders is not yet investigated. The effects of these extracts on UVC-induced injury in human corneal epithelial cells (HCEC) are yet to be evaluated. Hence, the present study aims to explore the protective effects of VN leaf extracts in preventing oxidative injury in HCEC and its antioxidant activity.

METHODS

Plant-source and extract preparation

The leaves of VN were collected from Yenepoya (Deemed to be University) Garden, Derlakatte, Mangalore and were identified by the botanist. The leaves were air-dried and grounded into a powder. Powdered samples were subjected to maceration using distilled water and ethanol for 24 h in a shaker incubator. The extracts were filtered using Whatman filter paper and concentrated using SpeedVac Concentrator. The dried extract was stored at 4°C until further use [20].

Phytochemical screening

The phytochemical evaluation was done for both aqueous and ethanol extracts using standard protocol [21].

Estimation of antioxidant activity

Total antioxidant capacity (TAC) assay by phosphomolybdate method

VN leaf extracts were evaluated for their antioxidant activity by phosphomolybdenum method [22]. This method determines the antioxidant activity through the formation of the phosphomolybdenum complex. The experiment was performed in triplicates against the standard ascorbic acid. The total antioxidant activity of the leaf extracts was expressed as ascorbic acid equivalents (AAE).

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

Free radical scavenging ability of extracts was measured by the DPPH assay [23]. This is based on the ability of antioxidants to reduce the stable dark violet radical DPPH to the yellow colored diphenyl-picrylhydrazine. Plant extract at concentrations (50–600 µg/ml) was used for the experiment. Ascorbic acid was used as a standard for the free radical scavenging activity. The percentage radical scavenging activity was calculated using the formula given below,

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

Where, Ac is the Absorbance of Control (DPPH), AE is the Absorbance of test.

Quantitative phytochemical screening

Total phenolic content (TPC)

The TPC of the VN extract was investigated according to the standard protocol [24] using gallic acid as a standard and samples were taken in triplicates. The total phenolic content of the VN extracts was expressed in terms of gallic acid equivalents (mg GAE/g extract).

Total flavonoid content (TFC)

TFC of the VN extracts was determined according to the standard protocol given Kumar and Karakumaran [25]. All samples were taken in triplicate using quercetin as a standard. The total flavonoid content was expressed as quercetin equivalents (mg QE/g extract).

HCEC culture

HCEC were cultured in Dulbecco's modified Eagle's medium DMEM/Hams F12 (1:1) (Himedia/Gibco) that was supplemented with 10% heat-inactivated FBS and antibiotic-antimycotic solution and maintained at 37°C in a humidified atmosphere with 5% CO₂.

Cell viability assay

For evaluating the cell viability and proliferation of HECE cells, MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide; Sigma-Aldrich) assay was used. Only viable cells can reduce MTT to insoluble purple formazan. The MTT assay was performed according to the manufacturer instructions. For MTT assay, cells were cultured in 96-well plate at a density of 5 × 10³ cells per for 24 h. Then, the medium was supplemented with different concentrations (12.5–200 µg/ml) of VN extracts and incubated for 48 h. After which, MTT solution was added and incubated for 4 h at 37°C in the dark. The formazan crystals that formed were solubilized with DMSO addition, and the absorbance was read 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 source and irradiation

UVP-2000 ultraviolet crosslinker was used for UVC irradiation. Cells were irradiated at a dose of 200 J/m² of wavelength 254 nm and post

1 h; cells were incubated with aqueous and ethanol extracts of VN leaves at a concentration of 12.5 µg/ml followed by MTT assay.

Wound healing assay

To evaluate the migration of HCEC, wound healing assay was performed. Briefly, HCEC were seeded onto 6-well plate and expanded them to reach up to 80% confluence. A scratch was made using 200 µl micropipette after discarding the media followed by phosphate buffered saline (PBS) wash. The media supplemented with different concentration (12.5 and 200 µg/ml) of VN extracts and cells were incubated at 37°C. Images were taken under an inverted microscope (Carl Zeiss, Germany) pre- and post-treatment. Scratch area was analyzed using ZEN software, and the data were represented as percentage wound closure. The experiment was performed using triplicates.

Measurement of ROS production

The levels of ROS were analyzed using 2',7'-dichlorodihydrofluorescein diacetate (DCF-DA). To analyze the ROS production, HCEC after UVC irradiation at a wavelength 254 nm and dose 200 J/m² and plant extract treatment (12.5 µg/ml) were washed twice with PBS and then incubated with 10 µM DCF-DA for 20 min. Post incubation cells were analyzed using flow cytometer, with an excitation wavelength of 488 nm and an emission wavelength of 530 nm. Data analysis was based on 5,000 detected events using Guava 2.6 software.

Statistical analysis

The experiment was done in triplicates and data were represented as mean±standard deviation (SD). Data were analyzed using one-way analysis of variance with Dunnnett *post hoc* test. Differences of p≤0.01 were considered significant.

RESULTS

Phytochemical screening of VN extracts

The phytochemical composition of the aqueous and ethanol extracts of VN is listed in Table 1. The result shows that both extracts contain alkaloids, phenol, and flavonoids in high amount. Phytosterols and saponins were found in moderate amount only in ethanol extract.

Table 1: Screening of phytochemicals of *Cassia fistula* leaf extracts

Tests	<i>Vitex negundo</i> extracts	
	Aqueous	Ethanol
Alkaloids		
Mayer's test	+	+
Wagners 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 phytocompound and (-) Indicates the absence of phytocompound

Total antioxidant activity

The TAC was done by phosphomolybdenum assay for VN extract in comparison with that of ascorbic acid as a standard reference. Overall, the ethanol extract showed a much higher antioxidant potential than

the aqueous extracts which was similar to standard ascorbic acid. There was a dose-dependent increase in the TAC in both ethanol and aqueous extracts (Fig. 1). The antioxidant capacity of the ethanol extract could be correlated to the proportion of phenolic compounds present in the extracts.

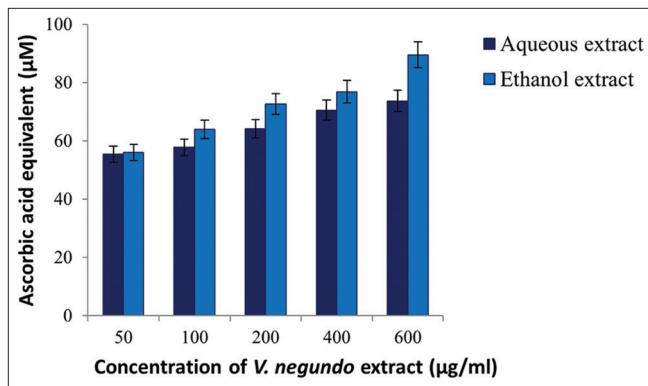


Fig. 1: Total antioxidant activity of *Vitex negundo* extracts. Ethanol and aqueous extracts were taken at different concentrations (50–600 µg/ml) and total antioxidant activity was expressed as AAE using ascorbic acid as a standard. Values are expressed as the mean±SD (n=3).

DPPH radical scavenging activity

ROS reducing the activity of VN extracts is given in Fig. 2. The study was done based on the ability of the extracts to reduce the stable radical DPPH. The radical scavenging effect of the extract was concentration dependent. We found that aqueous extract had less DPPH reducing activity than the ethanol extracts of VN. Ethanolic extract at 200 µg/ml showed significant activity with 90% activity followed by 400 and 600 µg/ml when compared to standard ascorbic acid.

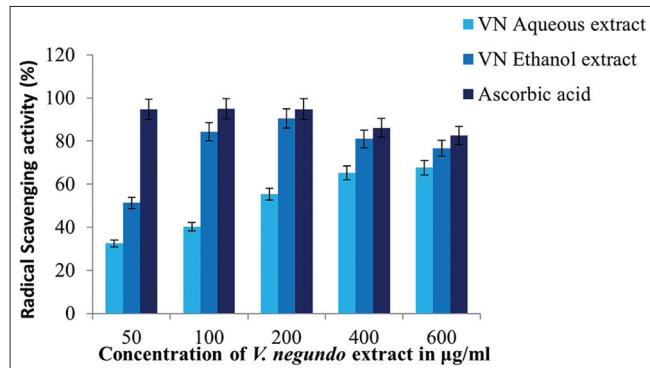


Fig. 2: 1,1-diphenyl-2-picrylhydrazyl radical scavenging activity of *Vitex negundo* extracts. Different concentrations of ethanol and aqueous extracts ranging from 50 to 600 µg/ml were taken for the experiment. Values are expressed as mean±SD (n=3). Ascorbic acid at concentrations (50–600 µg/ml) was used as a standard. p<0.01 is considered significant.

Total phenolic and flavonoid content of the VN extracts

Table 2: Total phenolic and flavonoid content of aqueous and ethanol extracts of *Vitex negundo* extracts

Test	<i>Vitex negundo</i> leaves	
	Aqueous extract	Ethanol extract
Total phenolic content (mg GAE/g extract)*	20.48±0.01	32.43±0.021
Total flavonoid content (mg QE/g extract)*	13.78±0.571	28.42±0.002

*All values are expressed as mean±SD, n=3

To evaluate the potential antioxidant capacity of the extracts from VN leaves, it was essential to determine the polyphenols content in aqueous extract and ethanol extract. The total phenols and flavonoids have been determined for both extracts by spectrophotometric methods, and the results are presented in Table 2.

Effect of VN extracts on the proliferation of HCEC

The proliferation of HCEC was evaluated by MTT assay after 48 h post-treatment with different concentrations (12.5–200 µg/ml) of VN ethanol (VNE) and aqueous extracts (VNA). The results indicated that aqueous and ethanol extracts at a concentration of 200 µg/ml had no adverse effect on the viability and proliferative ability of HCEC. There was no significant difference between the groups when compared to control in the aqueous group. In the ethanol group, the concentrations 12.5 and 25 µg/ml showed a significant difference compared to control as shown in Fig. 3. Considering the effect of VN extract on the proliferation of HCEC, the concentrations of 12.5 µg/ml of the extracts were used for further experiments.

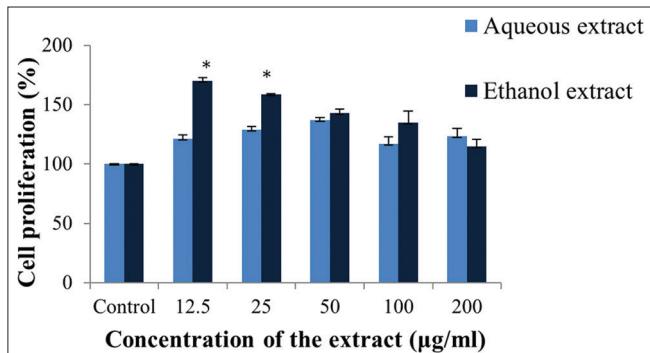


Fig. 3: Effect of *Vitex negundo* aqueous and ethanol extract on human corneal epithelial cells proliferation. HCEC were cultured with various concentrations of extracts and number of viable cells was quantified by MTT assay for 48 h. Data are represented as mean±SD; (n=3), p<0.01 is considered significant.

Effect of VN extracts against UVC-induced injury in HCEC

To investigate the protective effects of VN leaf extracts, the UVC irradiated 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 HCEC decreased the cell viability by 47% which was then regained to 73.22% and 82.07% by aqueous and ethanol extracts. When compared, the ethanol extract found to be more effective in protecting HCEC from UV-induced injury (Fig. 4).

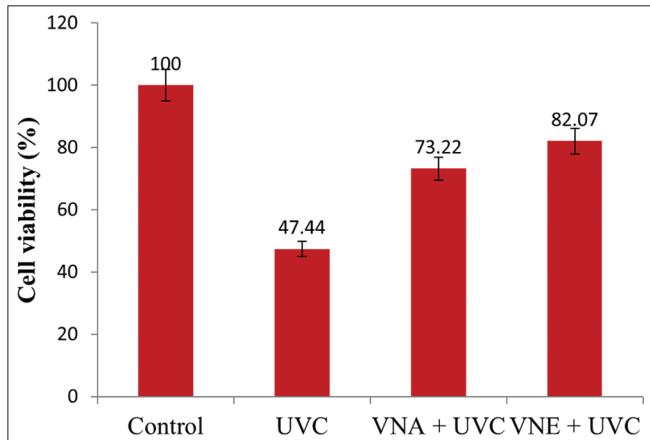


Fig. 4: Effect of *Vitex negundo* leaf extracts on ultraviolet light C irradiated human corneal epithelial cells. HCEC were irradiated with Ultraviolet C radiation at a dose of 200 J/m², and after 1 h, cells were incubated with aqueous and ethanol extracts of VN (12.5 µg/ml) extracts followed by MTT assay. Data are represented as mean±SD (n=3).

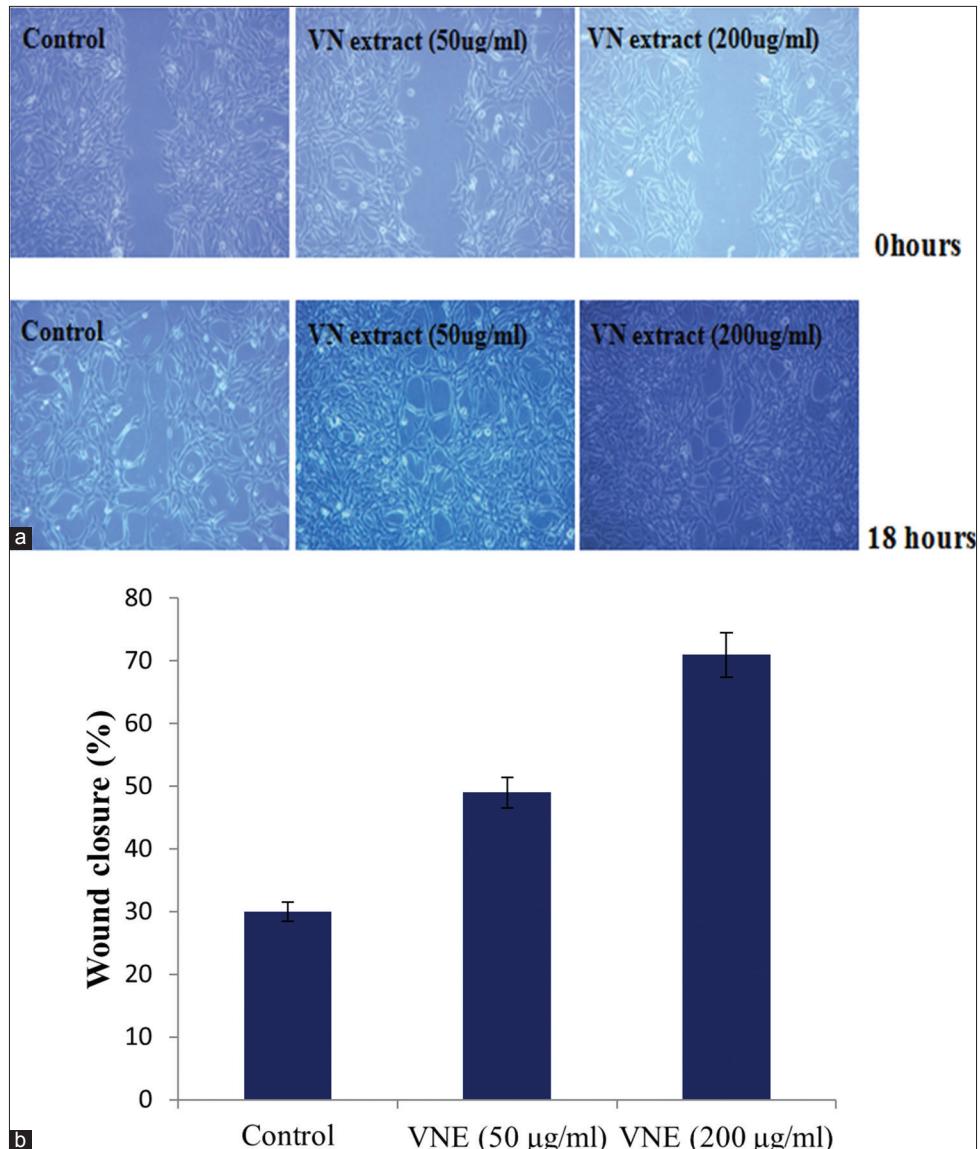


Fig. 5: (a) Representative micrographs pictures of human corneal epithelial cells scratch assays immediately after the scratches, and in time (18 h) course of the experiments. Scale bar, 100 µm.(b) Cellular proliferation response elicited by *Vitex negundo* (VN) ethanol extracts on human corneal epithelial cells (HCEC) was evaluated using *in vitro* cell migration assay and wound closure percentage was calculated. HCEC were seeded onto six-well plates, and after confluence, a scratch was created in the center of the well and cells were treated with VN ethanol extracts. Treatment with extracts promoted proliferation and migration of epithelial cells to the scratch area

VN promotes HCEC migration

Effect of VN extracts on the migration of HCEC was studied by wound healing assay in confluent culture. The migration rate of HCEC was evaluated for 18 h after creating a wound in the cultures with supplementation of VN extracts at concentrations 50 and 200 µg/ml along with control. The representative images of migration of the HCEC in the cultures supplemented with extracts and control culture at 0 h and 18 h after making wound are shown in Fig. 5. It was found that the wound area recovered faster in the culture supplemented with extracts than without the extracts due to the faster migration of HCEC. With the increase in the concentration of the extract (200 µg/ml), re-epithelialization of corneal epithelium was found to be enhanced during wound healing. The percentage wound closure at 18 h was found to be 40% and 70.99% for 50 and 200 µg/ml concentration of the extract.

Inhibition of ROS production in HCEC *in vitro* by VN extracts

The inhibition of intracellular ROS production in HCEC by VN extracts was analyzed using DCF-DA fluorescence. After UV irradiation at a wavelength of 254 nm and a dose of 200 J/m², plant extracts (12.5 µg/ml) were added to the cell cultures followed by incubation

for 30 min. The extracellular ROS was evaluated with flow cytometry, the mean intensities of fluorescence significantly increased after UV irradiation compared with the control. The extracts significantly inhibited the amount of extracellular ROS produced after UV irradiation. Ethanol extract showed higher inhibition rate than the aqueous extract as shown in Fig. 6a. The graphical representation of the data is given in Fig. 6b.

DISCUSSION

VN leaves are used in traditional medicine due to its anti-inflammatory, analgesic, and anti-itching properties for internal and external application [26]. It is used conventionally in the treatment of eye-disease, toothache, inflammation, leukoderma, skin-ulcers, in catarrhal fever, rheumatoid arthritis, gonorrhea, and bronchitis [27]. However, its potential to protect the HCEC from UV induced oxidative stress has not been studied, and very little is known about the cytotoxic and protective effects of VN extracts on HCEC. This is the first study to evaluate the use of VN extracts in protecting HCEC and its effects in enhancing cell migration *in vitro* and its application in corneal damage.

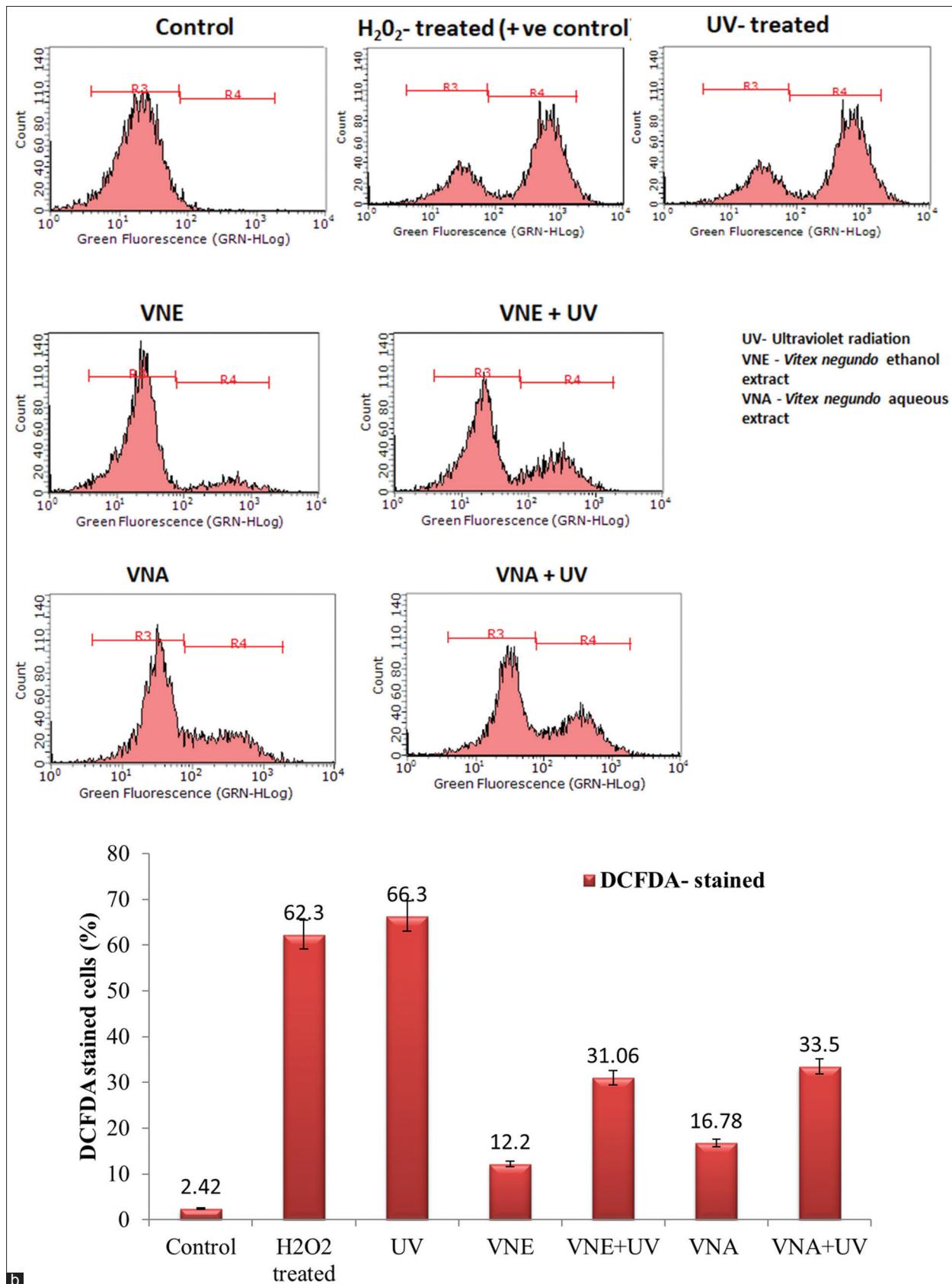


Fig. 6: (a) The levels of extracellular reactive oxygen species measured by dichlorodihydrofluorescein diacetate and flow cytometry. Representative figures showing the inhibition of reactive oxygen species production by the plant extracts with concentration (12.5 µg/ml) against irradiation of UV light (254 nm and 200 J/m²) in human corneal epithelial cells. The experiment was performed in triplicates (n=3).

(b) Graph showing the inhibition of reactive oxygen species production by the plant extracts at concentrations (12.5 µg/ml) against irradiation of Ultraviolet C (254 nm and 200 J/m²) in HCEC. Data represented as percentage dichlorofluorescein diacetate stained cell count versus treatment; mean±SD (n=3)

In the present study, the VN leaf extracts were screened for their phytochemicals and antioxidant activity. Our study reveals that the extracts have significant antioxidant potential with the presence of prominent phytochemical such as alkaloids, flavonoids, carbohydrates, and proteins. Antioxidant properties in the extracts can be exploited for their use in ayurvedic drug formulations and can be used for combating free radicals, eye diseases, and skin diseases. Total antioxidant activity, DPPH radical scavenging, total phenolics, and flavonoid content of the VNE and VNA leaves were determined. Ethanol extract showed significant antioxidant and free radical scavenging activity than aqueous extract. The total phenolic content was high in the ethanol extract than the aqueous extract, and this property is attributed to their antioxidant activity. This is mainly due to the ability of the extracts to absorb and neutralize the free radicals, to quench the singlet, or triplet oxygen species [28]. The total flavonoid content was also found to be high in ethanol extract than the aqueous extract. Flavonoids are phenolic compounds which are present naturally and are involved in various biological and chemical processes such as free radical scavenging and daily ingestion of polyphenolics have therapeutic effect during carcinogenesis and mutagenesis [29]. Previous reports have shown the presence of phenols, flavonoids, terpenoids, and carbohydrates in VN extracts also, high phenolic and flavonoid content were reported in VN [30,31].

In the present study, the effect of the VN extracts on the cell viability and cell migration was studied. Furthermore, the effect of these extracts on protecting the HCEC from ROS production during UV-induced damage was also checked. Both aqueous and ethanol extracts did not exert any cytotoxic effects on HCEC. This shows that the extract can be effectively and safely used for therapeutic or prophylactic purposes during ocular surface disorders. The effect of VN extracts was studied on the *in vitro* migration of HCEC to understand the potential use of these extract in corneal epithelial wound healing as a therapeutic agent. It was found that the supplementation the VN ethanol extracts at concentration 50 and 200 µg/ml significantly enhanced the migration of HCEC after 18 h of wounding. Faster re-epithelialization was seen with an increase in concentration. Previous studies have reported that VN aqueous and ethanol extracts exhibited strong wound healing properties in Wistar rats in experimentally induced excision and incision wound [32].

Overexposure to UV radiation is one of the most common causes of radiation injury to the eye which is an associated risk factor during human corneal damage [33]. UV-induced damages in the eye lead to clinical conditions such as photokeratitis, pterygium, UV keratitis, and clinical droplet keratopathy [34,35]. Therefore, studying a protective agent is important in developing better remedial strategies for corneal cell damage. Our findings suggest that UVC radiation at a dose 200 J/m² decreased the cell viability and induced production of ROS significantly which suggests that UV light especially UVC, can have a harmful impact on the corneal surface. Among the evaluated extracts, the ethanol extract showed protective activity on HCEC from oxidative stress induced by UV-irradiation by maintaining the cell viability. Interestingly, the ethanol extracts could effectively inhibit the ROS produced after UVC irradiation than the aqueous extracts. This correlates to the increased antioxidant activity due to the presence of antioxidant enzymes in the extracts. Similar studies showed that the mixture of medicinal plant extracts protected HCEC from blue light-induced oxidative stress by inhibition of ROS production [36]. Reports also confirm that VN has a role in the modulating cellular events such as cell cycle, apoptosis, motility of sperms, and polycystic ovary disease [37].

To sum up, the VN extracts have good antioxidant activity and contain phytocompounds that enhance HCEC migration and were effective in protecting HCEC from UV induced damage by inhibiting ROS production.

CONCLUSION

Taken together, our results indicate that UVC radiation (254 nm) can induce oxidative damage to the corneal epithelium and that VN extracts are effective in decreasing ROS production due to their high antioxidant activities. These findings can be extrapolated to elucidate

the pathogenesis and develop treatments for eye diseases. Several antioxidants having anti-inflammatory and antioxidant property are in use for topical or systemic application during clinical conditions of eye diseases. Since VN extracts exhibited potent efficacy in the treatment of UVC-induced oxidative stress in this study, it may be effective in the treatment of various oxidative stress-induced ocular surface diseases such as photokeratitis and dry eye. Further studies are needed to validate the anti-inflammatory and antioxidant markers involved during oxidative stress in HCEC.

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AUTHORS' CONTRIBUTIONS

Deeksha K conceived, designed and performed the experiments, analyzed the data and wrote the paper. Dr. Cynthia Arunachalam provided the nurturing environment and intellectual insights.

DECLARATION OF INTEREST

All authors have none to declare.

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