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Assessment of the cytoprotective effect of *Vitex negundo* ethanol extract on ultraviolet-C induced oxidative stress in human corneal epithelial cells

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Abstract

Human cornea is more susceptible to ultraviolet-C radiation during occupational injuries, and artificial sources such as welding arc, germicidal lamps, etc and continuous exposure leads to various ocular surface diseases. The cytoprotective role of plant extracts against oxidative damages induced by UVC irradiation is less studied in corneal epithelial cells.To investigate the protective of *Vitex negundo* ethanol (VNE) extract on ultraviolet-C (UVC) radiation induced oxidative stress in human corneal epithelial (HCE) cells. UVC radiation of wavelength (254 nm) and energy density 200 J/m^2 was used to irradiate HCE, and cell viability was assessed by trypan blue assay. The reactive oxygen species (ROS) production was analyzed using 2’,7’-dichlorodihydrofluorescein diacetate (DCF-DA) method. Cell cycle analysis was done to check the effects of UVC irradiation and VN extract on the cell growth cycle of HCE cells. The mitochondrial membrane potential (Δψm) was checked by 5,5′,6,6'-tetrachloro-1,1',3,3′-tetrathylbenzimidazol carbocyanine iodide (JC-1) staining method. The HCE cell viability was diminished after UVC irradiation which was improved after VNE extract treatment. The morphological analysis revealed that UVC irradiated HCE cells lost membrane permeability which led to cell death thereby having decreased confluency. VNE extract effectively decreased UVC- induced ROS production and reduced the G1 phase arrest of the cell cycle analysis in HCE cells. The changes or loss in Δψm after UVC treatment was restored after VNE extract treated cells.VNE extract showed strong protective effect against UVC light induced injury in HCE cells which suggests the therapeutic use of these extracts in ocular surface injuries.

Keywords: Corneal epithelium; Ultraviolet radiation; Oxidative stress; *Vitex negundo*; Antioxidant

1. Introduction

Prolonged unprotected exposure to Ultraviolet (UV) radiation from the sun and artificial sources (welder's arc, sun tanning beds, photographic flood lamps, lightning, electric sparks, and halogen desk lamps) cause corneal epithelial damage. This leads to ocular surface disorders like pinguecula, pterygium, climatic droplet keratopathy, OSSNs, carcinoma, UV keratitis and UV kerato conjunctivitis [1,2]. Based on the wavelength UV radiation is divided into UVA (400–315 nm), UVB (315–280 nm), and UVC (280–100 nm). UVC is generated from artificial sources as it is filtered by the ozone layer [3]. Due to its shorter wavelength it is fully absorbed by the cornea as of which cornea becomes susceptible to UVC induced damages. Longer wavelength passes through the cornea which reaches interior to lens and retina [4]. UV radiation leads to the generation of free radicals, including reactive oxygen species (ROS), which causes lipid peroxidation, DNA and protein damage. Increased ROS levels in the cells will disrupt the redox homeostasis in cells which expose the cells to oxidative stress [5]. Oxidative stress is an important mechanism involved in the UV mediated

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injury of the corneal epithelium which is due to the localized ROS production in the HCE cells. Mitochondria are highly dependent on the redox homeostasis for their functioning. In the cells, they exist as active network for modulating cell growth, death and differentiation. UV radiation is reported to cause mitochondrial dysfunction, causes cell death, and affects ocular structures, together with the cornea, lens and retina [6]. To understand the molecular basis of UVC induced oxidative stress in HCE cells, it is necessary to determine the ROS levels and mitochondrial membrane potential which will further help in characterizing the oxidative related events caused by UVC injury in HCE cells.

Vitex negundo(VN) commonly known as a five-leaved chaste tree is known to possess anti-inflammatory, antioxidant, hepatoprotective, neuroprotective, and cardioprotective functions which are attributed to their therapeutic potential [7-10].*Vitex negundo* ethanol (VNE) extract exhibited antioxidant activity due to the presence of flavonoids, ascorbic acid and carotene in the extracts [11]. Fresh leaves of VN possesses anti-inflammatory activity against acute, as well as sub-acute inflammation which might be due to the inhibition of prostaglandins and decrease of oxidative stress in carrageenan induced hind paw edema albino rats [12]. The extracts were also beneficial in decreasing superoxide dismutase, catalase and glutathione peroxidase levels in Freund's adjuvant induced arthritic-rats [13]. The extracts also possess the ability to combat oxidative stress by reducing lipid peroxidation owing to the presence of flavones, vitamin C and carotene [14]. Studies have evaluated the antioxidant and therapeutic potential of flavonoids present in VN extracts in controlling selenite-induced cataract and found it effective [15]. VN extract treated rats show an elevation in the renal antioxidant marker (CAT, GPx and SOD) levels which suggests that these enzymes protects against renal oxidative stress and scavenged free radicals [16]. The ethanolic extract of VN leaves exhibited hepatoprotective property in thioacetamide-induced liver fibrosis in male rats [17]. *Vitex negundo* ethanol (VNE) extract was effective in reducing the deleterious and myocardial infarction related effects induced by isoprotenol in wistar rats which suggests its use as a potent cardioprotective therapeutic agent [8].

There are no coherent and reliable strategies with regard to the ideal treatment of ocular pathological conditions resulting from UV light-induced oxidative stress. In addition in the past decade, there is increasing evidence of the potential health benefits of polyphenols derived from plants, due to their biological functions as antioxidant and anti-inflammatory compounds. The protective effect of VNE extract on the UVC induced injury in HCE cells is not yet investigated. Hence the present study is aimed to assess the potential role of VNE extract as an agent which alleviates the ROS levels, reduce cell growth phase arrest and evaluate the variations in the mitochondrial membrane potential induced by UVC radiation in HCE cells.

2. Material and methods

2.1. Plant-material and preparation of extract

The *Vitex negundo* leaves were collected from Yenepoya (Deemed to be University) Garden, Derlakatte, Mangalore and was authenticated by the botanist. The air-dried leaves were grounded into a powder. The powdered samples were subjected to maceration using ethanol for 24 h in a shaker incubator at 37˚C. After 24 h, the extract was centrifuged and the supernatant was filtered using Whatman filter paper No. 1. The extracts obtained were concentrated using a Speedvac concentrator and the dry residue was kept in a refrigerator at 4°C until further [18].

2.2. Cell line and culture

Human corneal epithelial (HCE) cells procured from ATCC, India, were used for the study. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) /Hams F12 (1:1) which was supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% antibiotic-antimycotic solution and was maintained at 37°C and 5% CO_2 humidity. The cells were grown until confluency in T25 or 10 cm cell culture dishes, and further trypsinized using Trypsin-EDTA solution and used for further studies.

2.3. Ultraviolet- C (UVC) irradiation source

UVC radiation was used to induce oxidative stress in the study model. UVP-2000 UV crosslinker was used as the source of UVC radiation with wavelength of 254 nm.

2.4. Cell viability by trypan blue assay

To check the protective effect of *Vitex negundo* ethanol (VNE) extract on HCE cells against UVC induced stress, the cells were irradiated with UVC radiation (254 nm) at a dose of 200 J/m^2 using UVP-2000 UV crosslinker, and after 1 h of incubation, cells were treated with VNE extract, at 12.5 μg/mL concentration, and after 24 h of incubation, the cells were harvested using tyrpsin – EDTA and incubated for 5 min at 37°C, and the resulting cell suspension in the media were

triturated uniformly and the cell counting was done using haemocytometer and represented as cell number ($x10^6$) /mL of media. The experiments were performed in triplicates (n=3) and data was expressed as mean ± SD.

2.5. Analysis of cell morphology

HCE cells were seeded into 35mm dishes and then treated with VNE extract at 12.5 μg/mL concentration and irradiated with UVC radiation (254 nm) at a dose of 200 J/m^2 using UVP-2000 UV crosslinker. The morphology of cells post treatment was observed by an inverted phase contrast microscope (ZOE fluorescent cell imager, Bio-rad) and images were captured at 10× magnification.

2.6. Measurement of intracellular reactive oxygen species (ROS)

The intracellular ROS was measured by 2', 7' –dichlorofluorescindiacetate (DCFDA) method using standard protocol using flow cytometry[19]. For ROS estimation, HCE cells irradiated with UVC (wavelength 254 nm, dose 200 J/m^2) and treated with VNE extract (concentration 12.5 μg/mL) were washed with PBS twice and then incubated for 20 min with 10 μM DCF-DA dye. Post incubation, HCE cells were analyzed using flow cytometer, with an excitation wavelength (488 nm) and an emission wavelength (530 nm) spectra. Data analysis was based on 5,000 detected events using Guava 2.6 software.

2.7. Cell cycle analysis

Cell cycle analysis of the HCE cells was done by hypotonic method using standard protocol [20]. In brief, HCE cells were cultured with or without supplementation of VNE extract at concentration of 12.5 μg/mL and UVC irradiation (200 J/m^2). After treatment, cells were trypsinized and centrifuged at 4500 rpm for 5 min. Cell pellet was resuspended in 1 mL of the buffer solution and 5×10^5 cells per sample were used and then stained with propidium iodide (PI). PI-stained single nuclei suspensions were analyzed using flow cytometer and raw data were collected using CELL Quest software. A minimum of 5×10^4cells was analyzed for one cycle of test for one sample and was repeated 3 times for each sample. Data analysis was performed using In-Cyte software.

2.8. Mitochondrial membrane potential (ΔΨm) assay

HCE cells were seeded at a density of 0.35x 10^6 in 12.5 cm^2 tissue culture flasks and incubated for overnight at 37°C. Following 24 h of UVC irradiated (wavelength 254 nm, dose 200 J/m^2) HCE cells treated with and without plant extract (concentration 12.5 μg/mL), the cells were harvested assessment of change in the ΔΨm was done using JC-1 dye. JC-1 a cationic, fluorescent, carbocyanine dye that can be used as a ratiometric indicator of mitochondrial potential ΔΨm in cells. The cells treated with H_2O_2 are taken for positive gating. The cellular fluorescence intensity of both JC-1 green monomers and red JC-1 aggregates (JC- 1 fluorescence intensity falls λex = 580 nm, λem=595) was recorded using flowcytometry system [21].

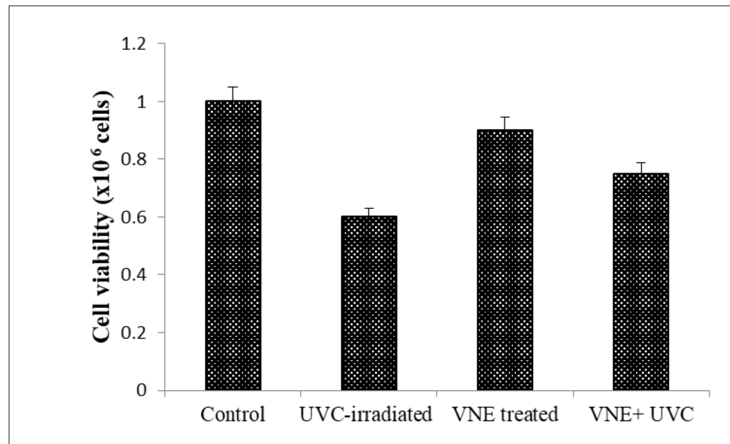
2.9. Statistical analysis

Results are presented as mean ± SD from three experiments. Data were analyzed using one-way analysis of variance (ANOVA) with Dennett's post hoc test and Tukey's multiple comparisons test using SPSS software 22.0. Differences of $p\leq0.01$ were considered significant.

3. Results

3.1. Cytoprotective effect of VNE extracts on UVC induced stress in HCE cells

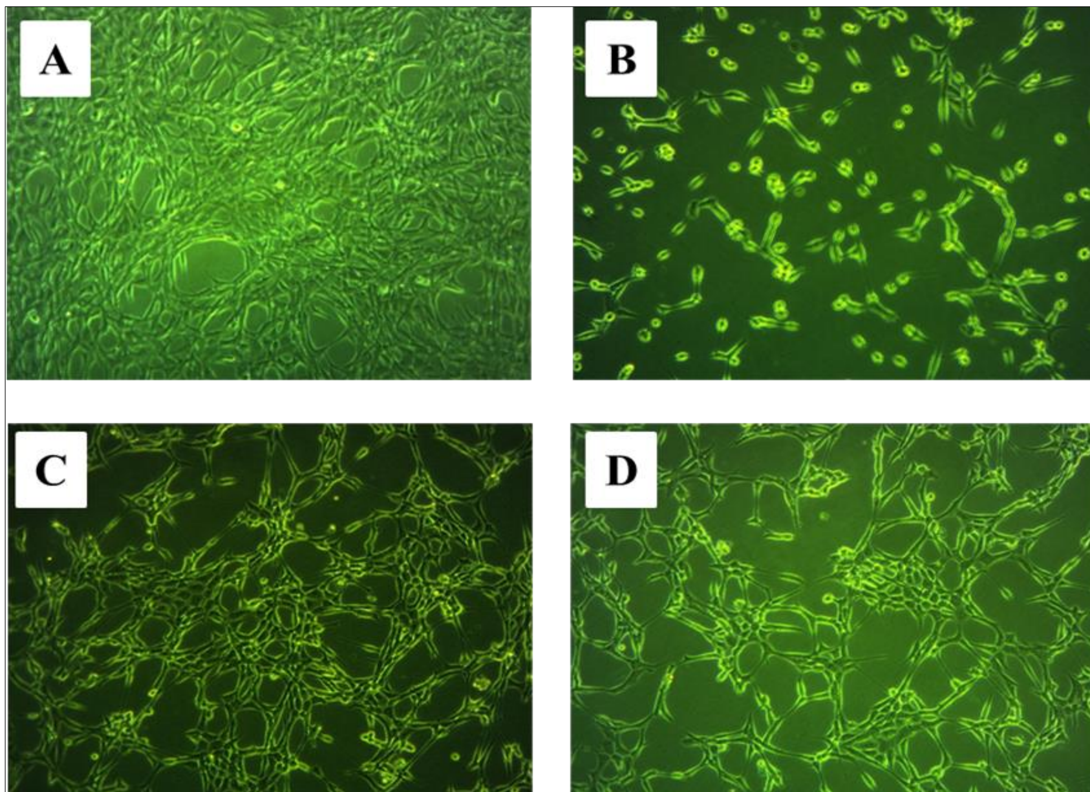
VNE extract at 12.5 μg/mL concentration was used to the check the protective effect on UVC treated HCE cells as it did not show toxic effect even at the lowest concentration. The results showed that UVC irradiation reduced the cell viability and caused cell death in HCE cells and it was found that the cell viability in VNE extract treated HCE cells exhibited strongest protection against UVC-induced cell death (Fig. 1).



HCE cells were ir-radiated with UVC at wavelength 254nm and energy density 200 J/m² and post 1 h of ir-radiation they were treated with VNE extract to check the protective effect by trypan blue assay Data are represented as mean± SD, n=3; p<0.0. VNE- Ethanol extract of *Vitex negundo*

Figure 1 Effect of VNE extract on the cell viability of UVC- irradiated HCE cells

3.2. Effect of VNE extract and UVC on the cell morphology of HCE cells



Representative images of HCE cells treated with UVC and VNE extract. A. Control, B. UVC irradiated at 254 nm at a dose of 200 J/m² ,C. HCE cell treated with VNE extract at 12.5 µg/mL concentration. D. VNE and UVC treated cells.The Images were taken using the ZOE fluorescent cell imager at 10× magnification.

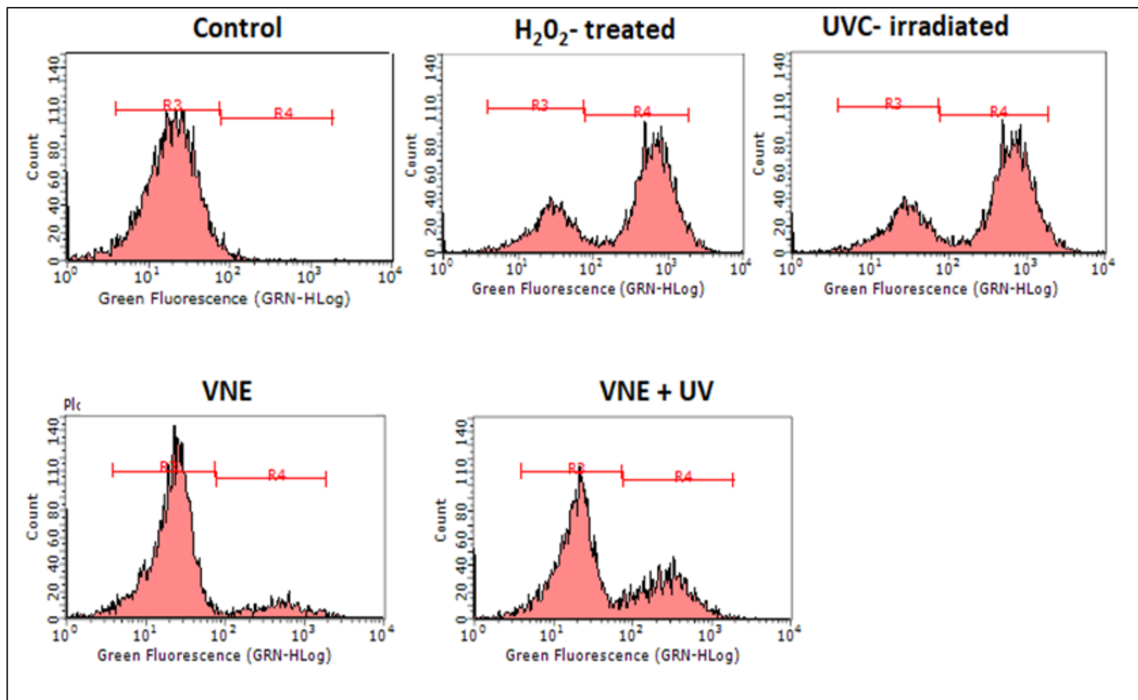
Figure 2 Morphological changes of HCE cells upon exposure to UVC and VNE extract

The alterations in the morphology of HCE cells after VNE extract and UVC irradiation was assessed by ZOE fluorescent cell imager.The exposure to UVC irradiation had a significant effect on the confluency of the HCE cells but it was found to be less affected on the VNE extract treated cells. UVC radiation induced alterations on the shape and morphology of HCE cells such as distortion of the cellular membrane which caused shrinkage of cells which further led to detachment of cells from the plates. There were no much changes in the VNE extract treated cells when compared to control. The control HCE cells exhibited slightly elongated cytoplasmic projections with an intact nucleus. The UVC irradiated cells

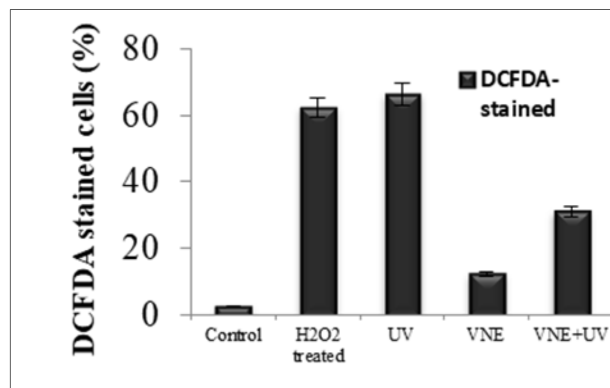
displayed necrotic and apoptotic features due to the loss of plasmatic permeability of the membrane, loss of cytoplasmic volume and, changes in symmetry of the cell membrane along with viable cells as shown in Fig. 2.

3.3. Assessment of the UVC induced ROS scavenging activity of VNE extracts

The inhibition of intracellular ROS production in HCE cells by VNE extracts was analyzed using DCF-DA fluorescence. After UVC irradiation (wavelength of 254 nm and a dose of 200 J/m^2), the HCE cells were treated with VNE extracts at 12.5 µg/mL concentrations, and incubated for 30 min. The intracellular ROS was evaluated with flow cytometry, the mean intensities of fluorescence significantly increased after UV irradiation compared to control. The extracts significantly inhibited the amount of intracellular ROS produced after UVC irradiation. VNE extract showed higher inhibition rate. The graphical representation of the data is given in Fig. 3.



A



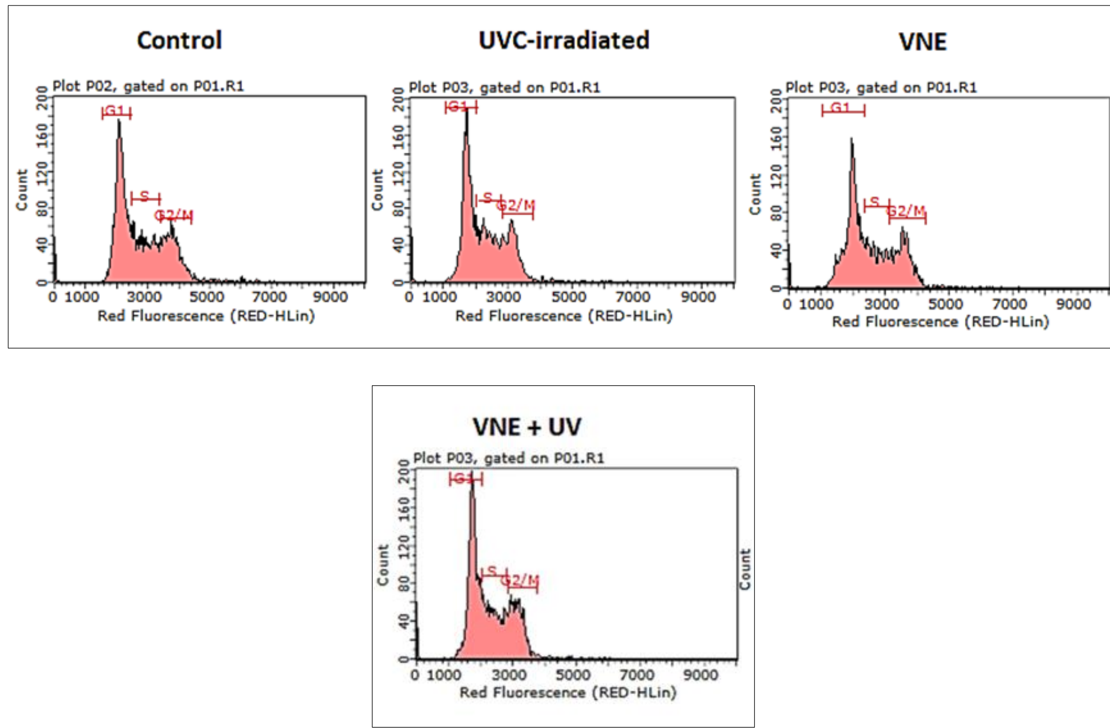
B

A) Representative figures showing the inhibition of ROS production by the VNE extract in UVC irradiated HCE cells. B) Quantification of the intracellular ROS produced by HCE cells. VNE extract (12.5 µg/mL) after UVC irradiation (254 nm and 200 J/m2) was added to HCE cells and ROS levels were monitored by DCFDA method using flow cytometry. Data represented as % DCFDA stained cell count vs treatment.UV- Ultraviolet C radiation, VNE – *Vitex negundo* ethanol extract

Figure 3 Inhibition of ROS production by *Vitex negundo* ethanol extracts

3.4. Assessment of the Cell cycle distribution in UVC and VNE treated HCE cells

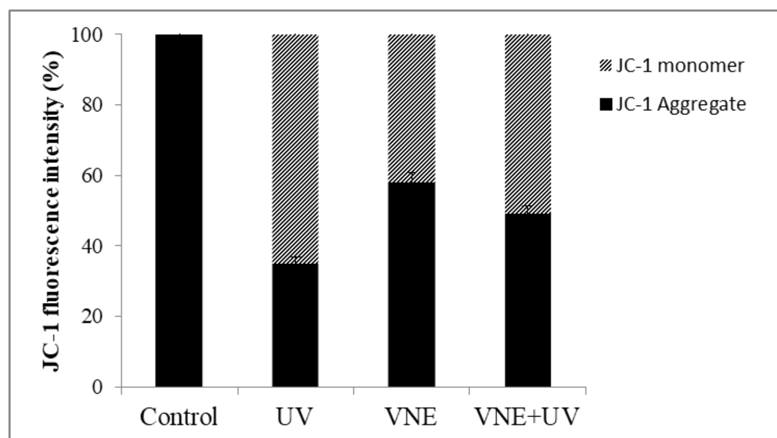
To further determine whether UVC-irradiation and VNE extract has any effect on the cell cycle distribution, it was detected by flow cytometry method.UVC irradiation caused an increase in the number of cells in the G1 phase, which indicates cell cycle arrest in this phase which also correlates that cell apoptosis has occurred. Furthermore, VNE extract reversed the UVC-induced increase in the G1 population in HCE cells (Fig. 4).



Cells were treated with VNE extract at a concentration 12.5 μg/mL after UVC irradiation of 254 nm wavelength and 200 J/m^2 energy density and then analysed by flow cytometry. UVC induced cell cycle arrest at G1 phase, which was reversed by VNE extracts. UVC- Ultraviolet C radiation, VNE – *Vitex negundo* ethanol extract.

Figure 4 Effect of UVC and *Vitex negundo* ethanol extract on the cell cycle phase distribution of human corneal epithelial cells

3.5. Assessment of Mitochondrial membrane potential (ΔΨm) in HCE cells



Flow cytometric analysis of the effect of UVC irradiation and VNE extract treatment on mitochondrial membrane potential by JC-1 staining. Dot plot shows spectral shift from red to green upon treatment. H_2O_2 (500 μM) treated cells served as positive control. JC-red represents cells with intact membrane potential and JC-green represents cells with collapsed membrane potential. Bar diagram presents the distribution of cell populations as the mean ± SD, n=3.

Figure 5 Mitochondrial membrane potential (ΔΨm) assessment in UVC irradiated and VNE extract treated HCE cells

In healthy cells with high ΔΨm, this dye reagent, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1), enter into mitochondria and accumulates complexes known as J-aggregates with red fluorescence. However, this dye cannot pass into the mitochondria of apoptotic or unhealthy cells where the cells will be with low or collapsed mitochondrial membrane potential and JC-1 remains in the cytoplasm as monomeric form, which gives green fluorescence. Apoptosis is mainly regulated by mitochondrial-mediated signaling; hence, we further checked whether VNE extract protects HCE cells from UVC-induced apoptosis by mitochondrial signaling regulation. The results showed that VNE extract treated HCE cells exhibited high intensity of red fluorescence which are termed as J-aggregates and weak intensity of green fluorescence when compared to UVC irradiated HCE cells termed as JC-1 monomers alone, which indicates that VNE extracts prevent the loss of mitochondrial membrane potential in UVC-irradiated HCE cells (Fig.5)

4. Discussion

Large number of medicinal plants and their purified constituents are reported to possess various beneficial therapeutic properties. The aim of the present study was to investigate the cytoprotective activity of the selected medicinal plants under UVC-irradiated stress. The present study showed VNE extract showed significant cytoprotective activity on HCE cells exposed to UVC radiation. The beneficial action being significantly increased number of live cells. UV radiation is considered to be a major environmental stimulus for the formation of ROS in the eye [4]. Oxidative stress due to ROS generation is the one of the sole cause of pathogenesis of a number of eye diseases such as photokeratitis, pterygia, cataract, glaucoma and macular degeneration [5, 6].ROS production at cellular level leads to lipid peroxidation, protein modification and DNA damage. So we checked the ability of the VNE extract to inhibit the ROS produced by UVC-irradiation in HCE cells. The result of our experiment demonstrated that UVC irradiation significantly enhanced the production of intracellular ROS leading to oxidative damage, and the VNE extract was effective in reducing the UVC-induced ROS in HCE cells. ROS production is attributed to various process of cellular injury which increases membrane permeability due to the peroxidation of membrane phospholipids leading to apoptosis [21].

The frequency of cell division is crucial for normal growth, development and maintenance of normal physiological conditions. The G1/S checkpoint is utmost essential in the cell cycle for normal cell division and the cell cycle regulator Cyclin/ CDK complex plays a key role in G1/S checkpoint [22]. In the present study, UVC treated HCE cells showed increased G1 phase. This might be due to the induction of p21 by p53 dependent transactivation. This results in signaling modulation and activation of certain cellular pathways leading to G1 phase arrest. While the post treatment with VNE extract in UVC-irradiated cells indicated a noticeable reduction in G1 phase cells, which visibly showed the progression of cells from the G1 phase to the next phase of the cell cycle. Previous studies report that during DNA damage, p53 gene is phosphorylated and plays a vital role in regulating checkpoints during the G1 and G2 phases of the cell cycle. These checkpoints prevent the cells from undergoing DNA replication or mitosis with impaired genomes [23]. In addition, the UVC-treated group showed a significant decrease in S phase. This may be due to the induction of p53 transactivation mediated by DNA-PKcs followed by depletion of Cyclin E and CDK2 [22]. But, treatment with VNE extracts permitted the cells to enter S phase by suppressing the abnormal G1/S phase arrest in UVC-irradiated conditions. The key factors involved in the pathogenesis of ocular surface diseases are increased ROS production which leads to oxidative stress [24, 25]. The basic energy required for cellular events and functions is supplied by the important cytoplasmic cell organelles such as mitochondria [26]. To detect whether UVC-induced oxidative stress in HCE cells altered the mitochondrial function, JC-1 staining probe was used to evaluate the changes in the mitochondrial membrane potential (ΔΨm).Several studies have revealed a strong association between inadequate antioxidant ability and mitochondrial dysfunction which acts as triggering event in apoptotic mitochondrial pathways in various cells [27]. Our results showed that UVC irradiation caused a substantial change in ΔΨm in HCE cells which might be due to ROS accumulation when compared to VNE extract treated and control cells. The association between ROS production and mitochondrial dysfunction may be the result of cellular response of HCE cells to increased oxidative stress after UVC irradiation [28]. UV radiation induced mitochondrial dysfunction will initiate apoptosis in HCE cells [29, 30].

The study provided a basis for the role of UVC radiation on cellular systems and emphasizing the importance of developing new natural cytoprotectants from plant sources. The search for protective agents, should thus aim for compounds with higher prophylactic and therapeutic potentials.

5. Conclusion

Therefore it can be concluded that medicinal plants might be considered as promising cytoprotective agents for protecting the corneal epithelium during occupational exposure especially farmers, outdoor labourers and fishermen at sea and soldiers at high altitude and outdoor workers involved in welding arcs, tanning booths where UV rays are

more intense. However, more intensive efforts through organized research can make a noteworthy difference to human health.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

There are no conflicts of interest between the authors.

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