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Preservation effects of Scarin on human Keratinocytes (HaCaT) against UV-B

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Abstracts

Increased exposure to Ultraviolet-B (UV-B) radiation is the major risk for various skin injuries. Numerous studies have shown that bioproducts could demonstrate photopreventive efficacy against UV-B damage. Herein, we aimed to investigate the preventive effects of Scarin (Scarus ghobban) on preservation of human keratinocytes, and scavenging intracellular reactive oxygen species (ROS) against UV-B. This study demonstrated feasibility of scarin on cosmeceutical application via ultraviolet B induced damage protective effects such as intracellular ROS and decrease of apoptotic cell death. To investigate protein secondary structural characterization used Fourier transform infrared spectroscopy; examination of scavenging intracellular ROS by Fluorescent probe and apoptotic cell death by fluorescent microscope. Secondary structure of skin epidermal mucoprotein (SEM) was alpha-helical, beta-turn and random coils with tyrosine as aminoacid side chains and henceforth so named as Scarin (Scarus ghobban). Scarin pre-trated significantly decreased the effects of UV-B irradiate at 15mJ/cm^2 in HaCaT cells I dose dependent manner. The florescent microscopical observation revealed that the cell death was significantly reduced by pre-treated scarin in a dose dependent manner in HaCaT cells when exposed to UV-B irradiated at 15mJ/cm^2. Through these results we verified that the scarin has effects on scavenging intracellular ROS and protection of human keratinocytes against UV-B.

Key Words: *Intracellular ROS, Keratinocytes, Scarin, Scarus ghobban, SEM, Ulraviolet-B radiation.*

Introduction

Solar ultraviolet (UV) radiation induces many types of skin damage, including sunburn, photoaging, corneal injury, and inflammation; extreme UV exposure can even compromise the immune system (Matsumura and Ananthaswamy, 2004; Liu *et al*., 2012). UV radiation consists of three kinds of energy: UVA (~320-540 nm), UVB (~280-320 nm), and UVC (~100-320 nm) light. While UVA and UVB light both provoke skin damage, the latter is stronger than the former and accordingly, is associated with graver biological insults. UVB radiation is mostly absorbed in the epidermis, where it mainly affects epidermal cells, especially keratinocytes (Zeng *et al*., 2014). However, ~10-30% of UVB energy can reach the upper dermis, and hence, UVB light is also hazardous to fibroblasts and the extracellular matrix (Everett *et al*., 1966; Rosette and Karin, 1996). Additionally, UVB light interacts with

intracellular chromophores and photosensitizers to severe oxidative stress in skin cells, together with transient as well as permanent genetic damage. At a more fundamental level, these oxidative reactions activate cytoplasmic signal transduction pathways associated with cell growth, differentiation, and senescence, and ultimately, connective tissue degradation (Helenius *et al*., 1999). UVB radiation triggers two predominant types of cytotoxic damage to the skin that interfere with normal cellular functions, and finally culminate in photodamage, photoaging, and photocarcinogenesis (Sander *et al*., 2004**).** First, UVB light directly damages DNA via the formation of thymine-thymine cyclobutane dimers (Budden and Bowden, 2013). Second, UVB light indirectly damages DNA, lipids and proteins via generation of unwarranted levels of reactive oxygen species (ROS) (Wäster and Ollinger, 2009; Lee *et al*., 2013). Although the skin has efficient antioxidant defense mechanisms against a variety of endogenous and exogenous insults (Lyu and Park, 2012), an overabundance of UVB-induced ROS disrupts this defensive capacity and precipitates irrevocable oxidative injury (Scandalios, 2002).

There is increasing demand for antioxidants in the pharmaceutical and health food industries as well as the food processing and preservation industries. Therefore, some artificial antioxidants including butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), and tertiary butylhydroquinone (TBHQ) show stronger antioxidant activities and have been widely applied in food preservation for retarding lipid oxidation (Guo et al., 2015, Agrawal et al., 2016). However, these synthetic antioxidants might cause liver damage and carcinogenesis (Agrawal et al., 2016). Therefore, there has been a major interest in searching for new, natural, and efficient antioxidants from various sources as alternatives to synthetic antioxidants. Bioactive peptides consist of 2 to 20 amino acid residues and are inactive in the amino acid sequence of their parent proteins, and they can be released by in vitro enzymatic hydrolysis without destroying their nutritional value (Sila and Bowgatef,2016). These low molecular weight (MW) peptides are considered to have easy absorption, high activity, and contain no hazardous immunoreactions (Agrawal et al., 2016, Makinen et al., 2016). Recently, seafood-derived peptides with antioxidant properties have been prepared and identified from different aquatic organisms, such as the dark muscle of tuna (Saidi et al., 2014, Chi et al., 2015), skin of Alaskan Pollock (Guo et al., 2013), skin and head of bluefin leatherjacket (Chi et al., 2015a, Chi et al., 2015b), viscera and carcass of Nile tilapia (Silva et al., 2014), gonad of jellyfish (Zhang et al., 2018), monkfish muscle (Chi et al., 2014), skin of Nile tilapia (Hu et al., 2017), and pectoral fin of salmon (Ahn et al., 2014). This research indicates that seafood-derived protein hydrolysates and peptides have strong antioxidant activity and could serve as functional ingredients in food systems to protect food quality by reducing oxidative stress. In addition, bioactive protein hydrolysates and/or peptides can be applied as ingredients of functional foods due to their low cost, safety, and high nutritional and physiological value (Sila et al., 2016).

Several mechanisms have been proposed to elucidate their properties, including metal ion-chelating ability, radical-scavenging activity, and aldehyde adduction (Zhou and Decker, 1999). The antioxidative peptides are histidine and hydrophobic residues rich (Chen et al., 1995). The high hydrophobicity favors their distribution at the water-lipid interface and enhances the radical-scavenging activity at the lipid phase (Ranathung et al., 2006). The histidine-containing peptides may account for the hydrogen-donating ability, lipid peroxyl radical-trapping ability, and metal-chelating ability from the imidazole group (Chan and Decker, 1994). Many studies derived from fish peptides

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focusing the properties of radical scavenging, lipid peroxide radical-trapping ability, metal chelating, scavenging intracellular reactive oxygen species, enhanced expression catalase, glutathione and superoxide dismutase (Chi et al., 2015,Wang et al., 2014, Himava et al., 2004). However, ther was no research focusing on the peptides derived from skin epidermal mucoprotein (SEM) extracts of parrot fish. Hence, we aimed to investigate the protective effect of scarin (*Scarus ghobban*) on preservation of human keratinocytes and intracellular reactive oxygen species (ROS) against UV-B. *Scarus ghobban* belongs to Actinopterigii, perciformes, scaridae, scarus found in shallow lagoons,seagrass beds and reef habitats. Scarin extracted from the skin epidermal mucus *of scarus ghobban* is a type of protein which is natural alpha-helical peptide,beta-turn and random coil with tyrosine aminoacid side chains.

2.0. Material and methods

2.1. Sample Collection, Extraction and Purification

Live specimens of the fish *Scarus ghobban* were collected from Nagapattinam as by-catch. Epithelial mucus was sampled by scraping a dull scalpel blade along the dorsal flank of live fishes, anterior to posterior. Mucus of the fish was collected from the dorsal region of the skin using blunt edged scalpel. Mucus was not collected from Ventral side of the fish to avoid urine and intestinal excreta (Chong *et al.,* 2005). The fish was placed on a flat non slippery surface with its head and eyes covered by palms to reduce the photophobic response (fear of light). Using a dull blade, mucus was gently scraped off the entire dorsal flank of fish as described by Zamzow (2004). Mucus sample was taken from the anterior section by moving from the head towards the anus using a spatula and stored in the sterile Amber bottle and stored in ice, to avoid bacterial contamination and proteins degradation during the transportation Sample Preparation: 0.1002 g of sample was weighed and dissolved in 10 ml of methanol and diluted to 25 ml with methanol (Stored at -4° C). Preparation of 0.2 % Acetic acid: 0.2 ml of Acetic acid mixed with 100 ml of distilled water, 75 ml of 0.2 % Acetic acid mixed with 25 ml of methanol and Filtered through and 0.45 μm nylon vacuum filter and sonicated. Each sample was then mixed using a sonicator in an ice bath (Unisonics) for 20 min and left to leach for 24 h at room temperature. The extracts were then centrifuged for 5 min at 18 000 × *g* and the supernatant was used for laboratory spectral UV analysis. Samples were extracted in 1.5 ml of 100% methanol and homogenized. Partial purification of Parrot fish *Scarus ghobban* fish mucus**:** Partial purification of fish mucus was carried out by Silica gel chromatography.

2.2. Fourier Transform Infrared Spectroscopy (FTIR):

FTIR characterization of scarin was performed with a Perkin Elmer-Spectrum RX1 instrument. FME were prepared in the forms of pellet. Then the sample was mixed with KBr (Potassium Bromide) to make a 13 mm diameter pellet. The spectra of the scarin sample were obtained with a frequency range of 4000-400 cm^{-1} resolution. Fourier-transform infrared spectra were recorded on a Perkin-Elmer spectrometer equipped with a TGS detector. A Perkin-Elmer model 4000 data station was used for acquisition, storage and analysis. Samples were placed in a thermostatically controlled Beckmann FH-01CFT microcell fitted with CaF2 windows. The sample compartment was continuously purged with dry air to eliminate absorption by water vapour in thespectral region. Spectral conditions were analysed following procedure explained by Haris *et al*., (1975) and Perkins *et al*., (1988) and the

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conditions were as follows: number of scans, 200; spectral resolution, 4 cm-1; sample thickness of 50 μm using a Teflon spacer; sample temperature, 20°C.

2.3. Cell Line: Culture of Human Keratinocyte (HaCaT) Cell Line

HaCaT cells were grown in DME/HAMS F-12 medium containing 10% FBS, 10,000 IU/ml penicillin and 10,000 μg/ml streptomycin in a 25 Cm2 culture flask in a CO2 incubator at 37°C and 5% CO2 under controlled humidified atmosphere. Once the cells reached ~90% confluency, they were trypsinized using trypsin (0.05%) EDTA (0.54 mM) solution washed thoroughly with media and subcultured into a 75 Cm2 culture flask for expansion. This process was repeated twice till the cells attained a consistent growth phase. Once after the cells attained consistent growth phase, they were trypsinized at 80% confluency and then utilized for the assay.

2.4. Experimental Design and procedure: Extraction of scarin from the Parrot fish *Scarus ghobban*, the scarin were divided into 6 groups, 30 minutes before irradiation, test doses (3,10,30 and 100 μg/ml) of scarin were added. Preliminary cytotoxicity studies were carried out. Group 1: Normal keratinocytes, Group 2: UV-B irradiated keratinocytes, Group 3: UV-B irradiated with scarin (100 μg/ml), Group 4: UV-B irradiated with scarin (30 μg/ml), Group 5: UV-B irradiated with FME (10 μg/ml), and Group 6: UV-B irradiated with scarin (3 μg/ml). Irradiation procedure: HaCaT cells were cultured in 6 well plates. Media had been removed and washed with phosphate based saline (PBS). For irradiation purpose, a broadband UV-B irradiation was applied using TL 20 W/20 fluorescent tubes served as a UV-B source in the range of 280-320 nm, peaked at 312 nm. 15 mJ/Cm^2 doses were prescribed.Sources of UVR are characterized in radiometric units. The terms dose (J/m^2) and dose rate (W/m^2) pertain to the energy and power, respectively, striking a unit surface area of an irradiated object (Jagger, 1985). UV-B radiation emitted by the narrow band TL20/W01 is 2.3 WATT. The tube emits radiation in the range of 280 nm-320 nm, peaked at 312 nm. Treatment of the cells: Thirty minutes prior to irradiation two test-doses (3 μg/ml and 10 g/ml) of scarin were added to the grouped normal Keratinocytes. Before exposure to UV light, the cell cultures were washed twice with PBS.

2.5. Intracellular ROS

Evaluation of protective effect of scarin on UV-B induced ROS generation: Intracellular ROS levels were measured by using a non-fluorescent probe, 2, 7-diacetyl dichlorofluorescein diacetate (DCFH-DA). DCFH-DA is transported across the cell membrane in acetylated form and deacetylated by esterase to generate the non fluorescent 2', 7'-dichlorofluorescein (DCFH). This compound is trapped inside of the cells. DCFH is converted to highly fluorescent DCF through the action of hydrogen peroxide generated by the presence of peroxidase (Hafer *et al*., 2008). Fluorescence measurements were made with excitation and emission filters set at 485±10 nm and 530±12.5 nm, respectively. The results were expressed as percentage fluorescence intensities. Further, the cells were subjected to fluorescence microscopic analysis using blue filter (460 nm). Reagents: 1. Phosphate Buffer Saline and 2. 2, 7-diacetyl Dichlorofluorescein diacetate (DCFH-DA). Procedure: The percentage of ROS levels was estimated in the control, UV-B irradiated, scarin treated HaCaT Cells. Briefly, an aliquot of the isolated cells were made up to a final volume of 2Ml in PBS (PH 7.4).

Then 1 mL of cell suspension was taken to which 10 µl DCFH-DA (10 µM) was added and incubated at 37° C for 30 minutes. Then scarin pretreated and UV-B irradiated HaCaT were incubated for 30 min in 6 well plates with 10 µM/mL of DCFH-DA in PBS. Finally, cells were washed thrice with PBS and the fluorescence intensity was recorded using Spectrofluorometer and the images were captured using fluorescence microscope (460 nm).

2.6. Hoechst 33258 Staining

Hoechst 33258 stains nuclei of both live and dead cells when examined by fluorescence microscope. Cells were scored apoptotic if their nuclei showed chromatin condensation and marginalization of nuclear heading or other apoptotic morphology. Often apoptotic nuclei fragmented into smaller structures.

Results

3.1. FTIR analysis of Scarin, *Scarus ghobban*

FTIR spectra of methanolic extracts of *S. ghobban* parrot fish epidermal mucoprtein showed functional groups such as carboxylic acids, aliphatic amines, aromatics, phenol and alkynes. Mostly aliphatic amines presence showed in the spectra showed in the **Figure.1.** Fourier transforms infrared spectroscopy (FTIR): Lyophilized Fish mucus sample was mixed with KBr (IR grade) and made into a pellet. The pellet was immediately put into the sample holder and FTIR spectra were recorded in the range of 400-4000 cm^{-1} for sample. Characterization of the scarin was carried with a Spectrum one FT-IR Spectrometer. Determination of secondary structure of protein/peptide: Secondary structure of skin epidermal mucoprotein was estimated using a FTIR spectrometer in the presence of 50 mM sodium phosphate buffer (NaPB) and IR peak observed in the range of 4000 to 400 cm^{-1} (**fig.1**. The contents of α-helix, ß-turn and random coils with Tyr as side chain were calculated according to Barth, 2007. The FTIR spectrum of mucoprotein for *S.ghobban* confirmed the presence of α-helix, ß-turn and random coil with tyr as amino acid side chains. In the presence of 50% trifluoroethanol, the peptide became structured, exhibiting a high level i.e. of α-helical folding structure. Then, the α-helical conformation of scarin was confirmed in the presence of structure promoting solvent.

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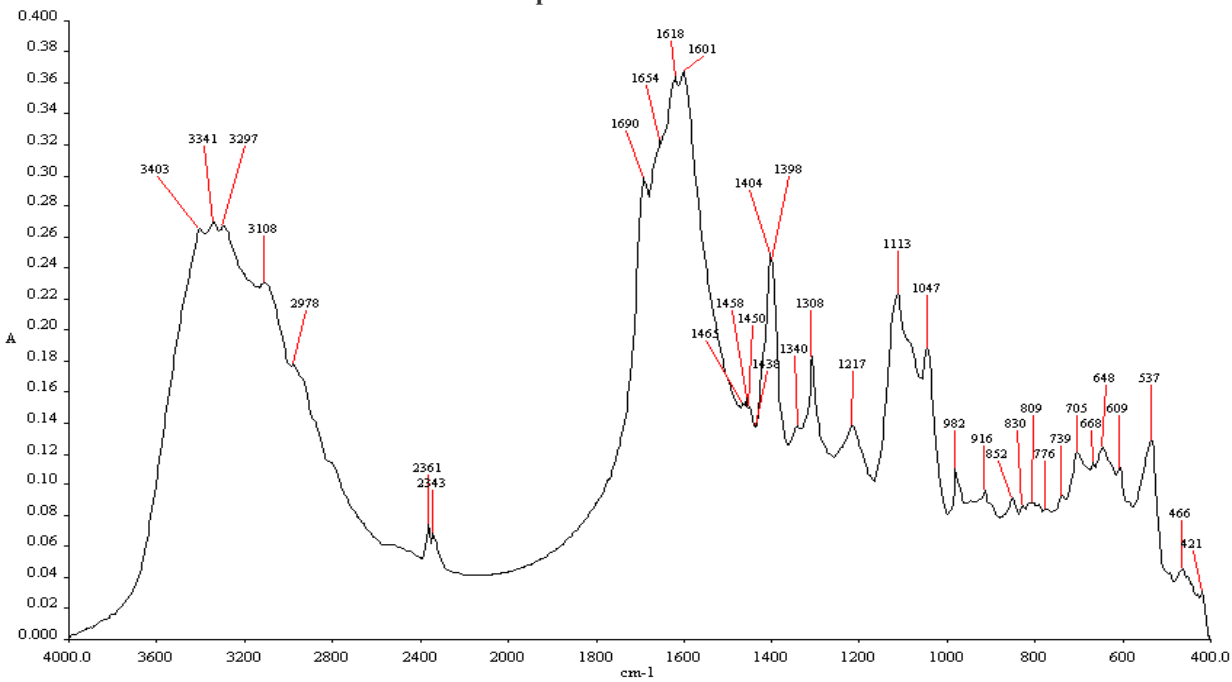

Fig. 1. FTIR spectra of scarin, *S. ghobban*

3.1. Effect of Scarin on UV-B radiation induced Intracellular ROS in HaCaT Cells.

The DCFH-DA method measures intracellular generation of hydrogen peroxide, a circuitous method for estimating ROS. In this study, HaCaT cells treated with scarin at different concentrations showed no significant difference in dose dependent manner (**Figure. 2**). UV-B-irradiated HaCaT cells at 15m J/Cm2 showed increase in the intracellular ROS concentration. Scarin pre-treated significantly decreased the effect of UV-B radiation at 15m J/Cm2 in HaCaT cells in dose dependent manner (**Figure. 3**).

Scarin pretreated HaCaT cells

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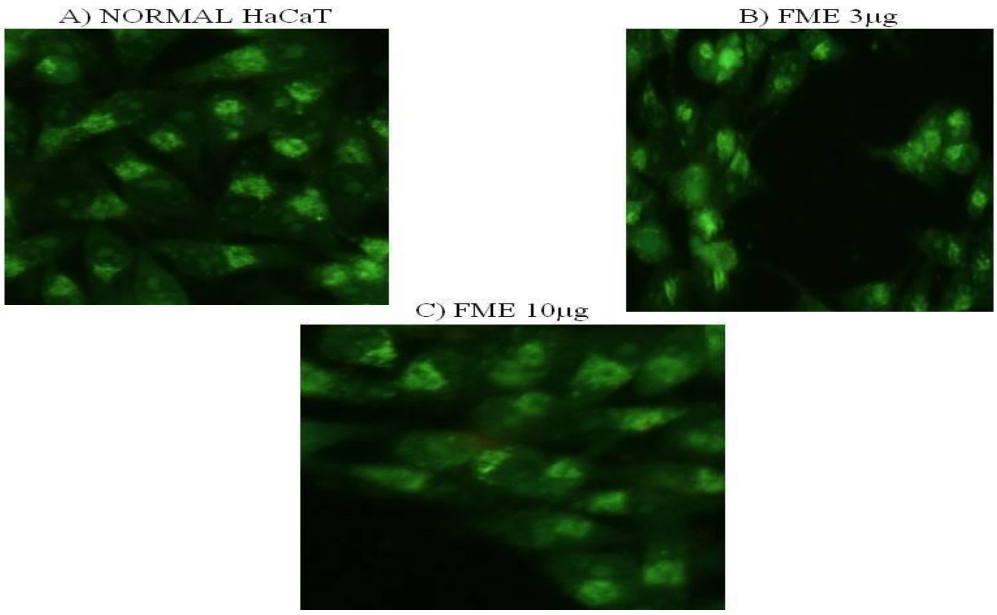

Fig. 2. A) Normal HaCaT Cells, B) HaCaT cells treated with 3 µg of scarin, C) Normal HaCaT cells treated with 10 µg of scarin

Measurement of ROS levels in UV-B irradiated and scarin pretreated UV-B irradiated HaCaT cells

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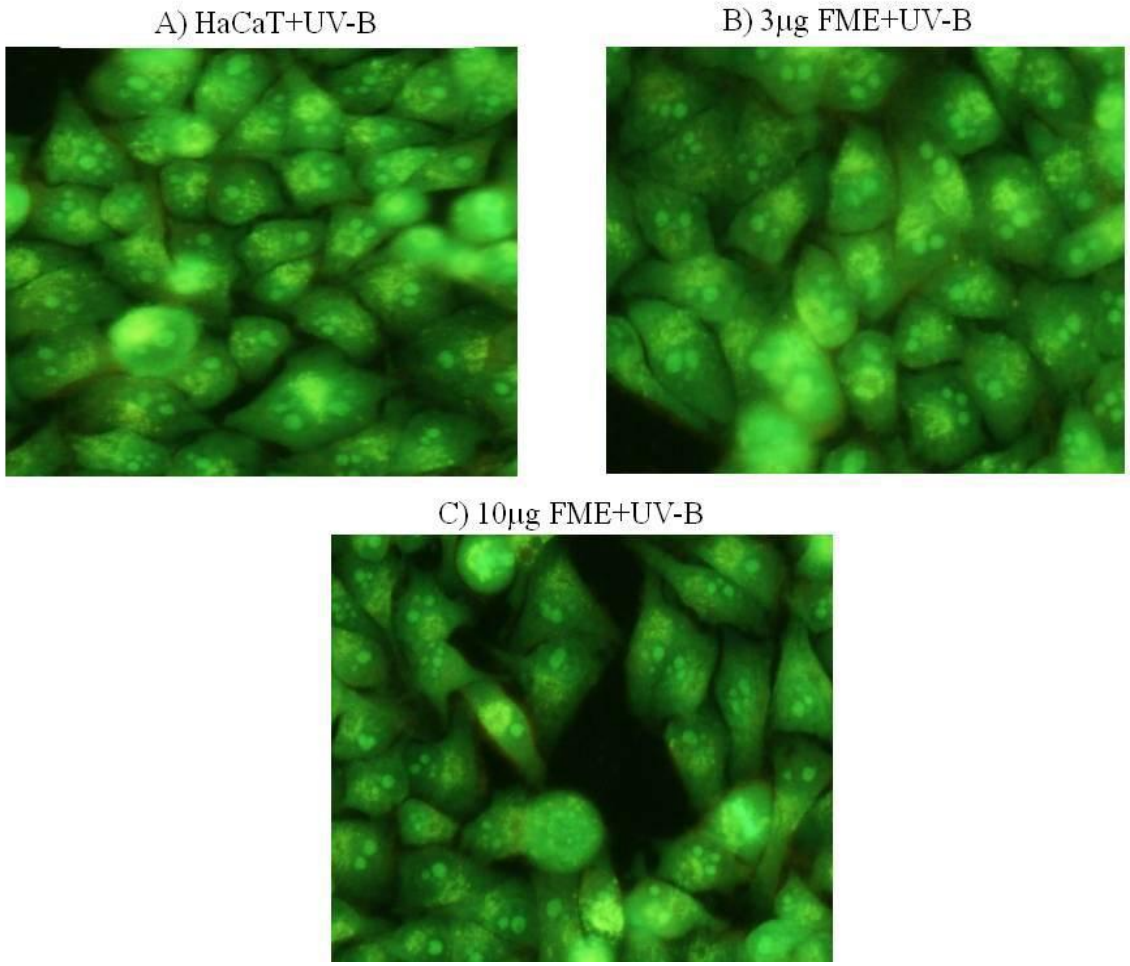

Fig. 3. A) HaCaT Cells irradiated with UV-B alone for 15mJ/Cm^2 B) HaCaT cells irradiated with UV-B for 15m J/Cm^2 with 3 µg of scarin, C) HaCaT cells irradiated with UV-B for 15m J/Cm^2 with 10 µg of scarin

3.2. Nuclear staining with Hoechst 33258

Hoechst 33258 staining was employed to image the cellular morphological changes in cells treated with Scarin: Effects of scarin on apoptosis of HaCaT cells induced by UV-B irradiation: Chromatin condensation is morphological marker of the apoptotic process. The nuclei with chromatin condensation were observed in the cells after UV-B irradiation. This observation revealed that UV-B irradiation at 15 mJ/Cm^2 induced cell death in HaCaT cells through a typical apoptotic pathway, while this cell death was significantly inhibited by pre-treated scarin in a dose-dependent manner in HaCaT cells, when exposed to UV-B irradiation at 15 mJ/cm^2 **Figure. 4, 5.**

Effect of scarin on Normal HaCaT cells

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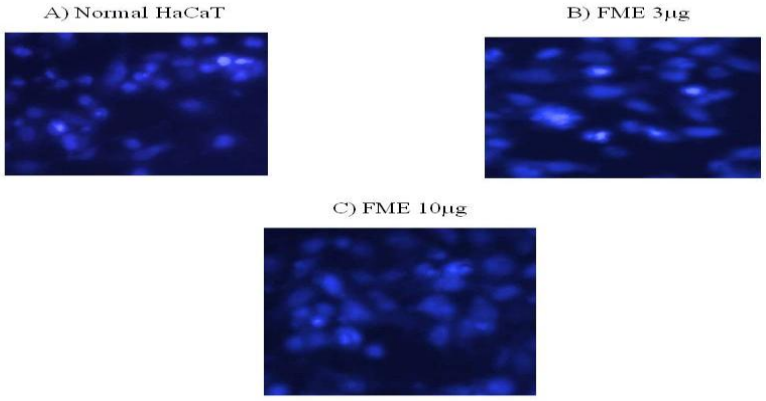

Fig. 4. A) HaCaT cells with no treatment used as control, B) HaCaT cells treated with 3 µg of scarin, C) HaCaT cells treated with 10 µg of scarin.

Effect of pre-treated scarin on HaCaT cells irradiated with UV-B for 15mJ/Cm^2.

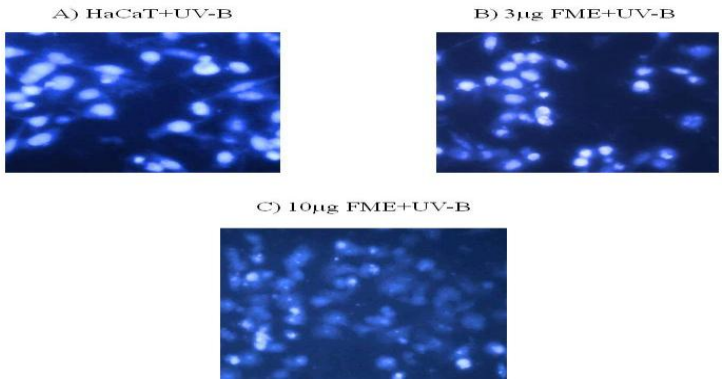

Fig. 5. A) HaCaT Cells irradiated with UV-B alone for 15 mJ/Cm^2, B) HaCaT cells irradiated with UV-B for 15 mJ/Cm^2 with 3 µg of scarin, C) HaCaT cells irradiated with UV-B for 15m J/Cm^2 with 10 µg of scarin.

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Discussion

In the current study, we investigated the antioxidant and cytoprotective effects of scarin against UVB-induced damage in keratinocytes, the predominant cell type found in the human epidermis. Our findings indicate that scarin is not cytotoxic at concentrations of ≤ 3-10 μM to keratinocytes (Fig. 2,3), and may therefore find utility as part of a therapeutic arsenal against UVB-provoked skin damage. Because of its special chemical structure features, the hydroxyl group is an indispensable functional group of many natural antioxidants, including peptide and other polyphenols . In general, the phenolic hydroxyl group donation of a hydrogen atom acts to effectively quench ROS (Choi *et al*., 2002; Heim *et al*., 2002; Valentão *et al*., 2003). Consistent with these reports, the present results revealed that scarin efficiently scavenged intracellular ROS in HaCaT keratinocytes (Fig. 2,3), and also directly quenched the superoxide anion and the hydroxyl radical in cell-free systems (Fig.1, 2). Therefore, the ROS scavenging ability of scarin may be due to the Tyr side chain groups in its structure.

UVB-induced apoptosis is mediated by a number of molecular processes, which target the mitochondria and activate the mitochondria-initiated cell death pathway (Ji *et al*., 2015). These processes include changes in the opening of the mitochondrial permeability transition pore, and release of cytochrome c from the mitochondrion into the cytoplasm. Consequently, mitochondria rapidly lose their transmembrane potential during apoptosis, generating excessive amounts of intracellular ROS (Ricci *et al*., 2003) and attenuating cell viability. In addition to substantially protecting human keratinocytes from UVB-provoked programmed cell death and mitochondrial dysfunction (Fig.3-4), scarin also rescued cell viability in UVB-exposed HaCaT keratinocytes (Fig.3-4). Abnormal production of ROS leads to oxidative damage to macromolecules, including DNA, lipids, and proteins (Pashikanti *et al*., 2011; Ray *et al*., 2012). Like apoptosis and mitochondrial dysfunction, macromolecular injury contributes to considerable disruption of normal cellular functions. Notably, the present study showed that scarin pretreatment also prevented DNA strand breakage, lipid peroxidation, and protein carbonyl formation in HaCaT keratinocytes following exposure to UVB light (Fig. 3-4)

In general, molecular size, hydrophobicity, amino acid composition, and sequence are believed to playan essential role in antioxidant activity of peptides (Harnedy, 2012). Acidic and basic amino acids play a critical role in metal ion chelating and HO^- scavenging activities of peptide, which is related to carboxyl and amino groups in their side chains (Pan et al., 2016). Similar results were also reported by Chang et al. who found that basic (arginine, Arg) and acidic (Glu and aspartic acid (Asp)) amino acid residues were critical for their antioxidant activities (Chang et al., 2013). Chen et al. reported that glycine (Gly) residue might contribute significantly to antioxidant activity since the single hydrogen atom in the side chain of Gly serves as protons, and thus neutralises active free radical species (Chen et al., 2012).

Hydrophobic amino acids including proline (Pro),Tyr and Met in peptide sequences are believed to play an important role in scavenging free radicals because their large hydrophobic group can help them to facilitate the contacts with hydrophobic radical species (Xing et al., 2016). Pyrrolidine ring of Pro could increase the flexibility of peptides and also be capable of quenching singlet oxygen due to its low ionization potential (Mirzaei et al., 2015). Chen, Muramoto, Yamauchi, & Nokihara proved that Pro played an important role in the antioxidative activity of Pro-His-His (Chen et al., 1996). Dávalos,

Miguel, Bartolomé, & López-Fandiño confirmed that tryptophan (Trp), Tyr, and Met showed the highest antioxidant activity among all the amino acids (Davalos et al.,2004). Therefore, Tyr residues might be the important contributor for the antioxidant activity of scarin.

In addition, antioxidant activities of peptides highly rely on their molecular size, and shorter size peptides are deemed to obtain stronger radical scavenging and lipid peroxidation inhibition activities than their parent native proteins and long-chain peptides (Matsui et al., 2018). In the test, scarin exhibited good antioxidant activities on intracellular reactive oxygen species and apoptosis inhibition assays, which suggested that alpha-helical,beta turn and random coil with Tyr side chains could interact more effectively and easily with free radicals and inhibit the propagation cycles of radical scavenging. Therefore, more detailed study should be designed for clarifying the relationship between the activities and structures of the isolated peptides. Nevertheless, the biological efficacy of scarin *in vivo* and the underlying mechanisms of scarin action require further exploration.

Conclusion

In conclusion, the results of this study demonstrate that scarin suppresses the deleterious effects of UVB irradiation in human keratinocytes, including excessive intracellular ROS generation, oxidative damage to DNA, lipids, and proteins, and mitochondrial dysfunction. Moreover, scarin improves cell viability and inhibits apoptosis in UVB-exposed human keratinocytes. These data support the hypothesis that scarin might be utilized as a novel antioxidant agent to treat ROS-related skin disorders.

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