ENHANCEMENT OF UV-B RADIATION – MEDIATE APOPTOSIS BY SKIN EPIDERMAL MUCUS OF PARROT FISH IN HaCaT HUMAN IMMORTALISED KERATINOCYTES

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ABSTRACTS

The UV-B radiation damaged cells responds to these alterations by either by inducing DNA repair activity or by inducing apoptotic death if the damage is too severe; failure of severely damaged cell to undergo cell death can lead to the formation of an initiator tumor cell. This investigation aimed to test whether extracts from the skin epidermal mucus (SEM) of parrot fish (*Scarus ghobban*), able to regulate the inflammatory process using the human keratinocyte HaCaT cell line. Cytotoxicity and apoptosis of SEM extracts were determined using MTT assay. Reults showed that HaCaT cells could survive when incubated in SEM at concentrations between 3µg and 10µg/mL. subsequentely, cell viability was compared between cultured HaCat cells exposed to seial dose of UV-B from 1 to 15 mJ/Cm^2 and containing 5 different concentrations of SEM(0,3,10, 30,100µ/mL). A increase in cell viability was observed following pretreatment with3 µg and 10µg/mL SEM. Propodium Iodide analysis showed that UV-B irradiated HaCaT cell at 15mJ/Cm^2 reduced the amount of live cells when compared to SEM pretreated in HaCaT cells in dose dependent manner. The present study demonstrated that SEM of parrot fish (Scarus ghobban) can attenuate inflammatory proteins triggered by UV-B. Hence, the of parrot fish SEM peptide, may provide new agents for skin anti inflammation, preventing damage due to UV-B.

Keywords: UV-B radiation, Parrot fish, Scarus ghobban, SEM, Cytotoxicity, Apoptosis, Propodium iodide

2. MATERIAL AND METHODS

2.1. Sample collection, preparation and partial 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 Preparation: 0.1002 g of sample was weighed and dissolved in 10 ml of methanol and diluted to 25 ml with methanol (Stored at -4º 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. The samples were extracted in 1.5 ml of 100% methanol and homogenized. Partial purification of fish mucus was carried out by Silica gel chromatography.

2.2. Cell Line: HaCaT –Human Immortalized Keratinocytes

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.

2.3. Experimental Design

Extraction of FME from the Parrot fish *Scarus ghobban*, the FME were divided into 6 groups, 30 minutes before irradiation, test doses (3,10,30 and 100 µg/ml) of FME were added. Preliminary cytotoxicity studies were carried out.

Group 1: Normal keratinocytes
Group 2: UV-B irradiated keratinocytes
Group 3: UV-B irradiated with FME (100 µg/ml)
Group 4: UV-B irradiated with FME (30 µg/ml)
Group 5: UV-B irradiated with FME (10 µg/ml)
Group 6: UV-B irradiated with FME (3 µg/ml)

2.4. 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/Cm2 dose was prescribed.

Sources of UVR are characterized in radiometric units. The terms dose (J/m2) and dose rate (W/m2) pertain to the energy and power, respectively, striking a unit surface area of an irradiated object (Jagger, 1985). UV-B radiation emitted by the narrowband TL20/W01 is 2.3 WATT. The tube emits radiation in the range of 280 nm-320 nm, peaked at 312 nm.

2.5. Treatment of the cells

Thirty minutes prior to irradiation two test-dose (3 µg/ml and 10 g/ml) of FME were added to the grouped normal Keratinocytes. Before exposure to UV light, the cell cultures were washed twice with PBS.

2.6. Assessment of Cytotoxicity by MTT (3-(4, 5 dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) Assay

The cells were trypsinized when they were at 80% confluence and seeded in a 96-well plate at the density of 15 x 103 cells / well. The cells were incubated in a CO2 incubator at 37°C and 5% CO2 under controlled humidified atmosphere overnight to allow them to attach to the plate. After overnight incubation, the cells were exposed to FME at different concentrations (0, 1, 3, 10, 30 and 100 µg/ml), respectively for 30 min and then exposed to UV-B irradiation for 15 mJ/Cm2 and further incubated for another 24 h before cytotoxicity assessment. Then 50 µl MTT (5 mg/ml stock) was added to the cells and further incubated for 3 h at 37°C. At the end of incubation period, the contents of the plate were discarded by simple decantation and the plates were dried overnight at room temperature. The purple-coloured formazon crystals formed were dissolved in 100 µl of dimethyl sulfoxide by shaking at 400 rpm for 15 min at room temperature in a thermo shaker. The intensity of the colour developed was absorbed at 570 nm in a multimode microplate reader. For cell viability assessment two doses (3 and 10 µg/ml) were employed based on the cell morphology and cell density observed after 48 h treatment for cytotoxicity assay.

2.7. Live / Dead Cell Analysis by Confocal Microscopic Imaging

The cells were trypsinized when they were at 80% confluence and seeded at the density of 50 x 103 cells on cover slips (22 x 22 mm) placed inside a 6-well culture plate. The cells were incubated in a CO2 incubator at 37°C and 5% CO2 under controlled humidified atmosphere overnight to allow them to attach to

the plate. After overnight incubation, the cells were exposed FME at two different concentrations (3 and 10 µg/ml), respectively for 48 h. Another set of cells were pre-treated with test items for 24 h and then exposed UV-B irradiation for 15m J/Cm2 and then the cells were incubated further for another 24 h. At the end of 48 h, the spent media was removed and the cells were washed thoroughly with 1X PBS (pH 7.4). The cells were then added with calcein (1:50 dilution from 4 mM stock) and incubated for 20 min in dark at room temperature. After 20 min, cell were washed with 1X PBS (pH 7.4), propidium iodide (1:50 dilution from 1 mg/ml stock) was added and then immediately washed with 1X PBS (pH 7.4). The cover slips with cells were then mounted over another microscopic cover slip (22 x 40 mm) and then viewed under the confocal microscope (Carl-Zeiss 510 Meta). The images of the cells were taken under the illumination of Neon and Argon lasers with excitation and emission wavelengths of 488 nm and 535 nm, respectively. The live cells had taken up calcein and fluorescence green, while the dead cells had taken up propidium iodide and fluorescence red.

3. RESULTS

3.1. Effects of FME on UV-B radiation induced cytotoxicity on HaCaT cells.

The effect of UV-B radiation on HaCaT cell viability by using MTT assay was recorded **Fig. 1A, 1B,** showed the cytotoxicity, which detected viable cells by assessing the capability of cells to reduce MTT to a formazan product by mitochondria. The cytotoxicity was increased in UV-B-irradiated cells at 15 mJ/Cm2. Cytotoxicity was restored significantly in SEM pre-treated HaCaT cells particularly in 3 µg/ml and 10 µg/ml. Therefore, these doses were chosen as the optimal doses for investigating the protective effects of SEM against UV-B radiation at 15 mJ/Cm2.

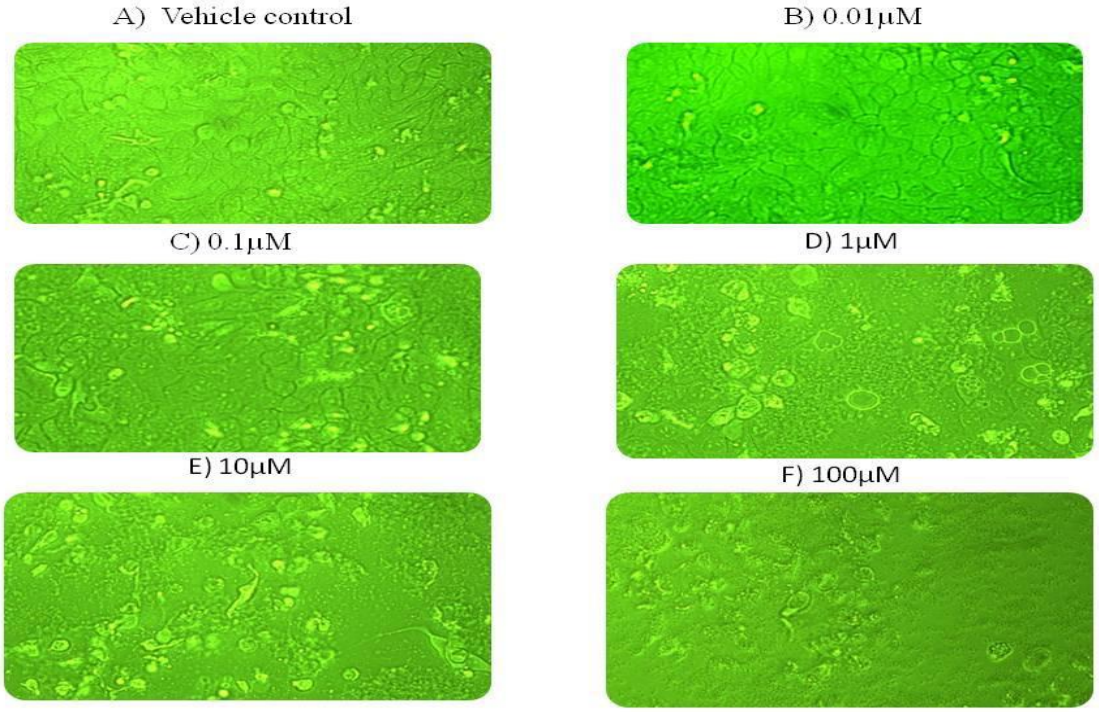
3.2. Effects of FME on UV-B radiation induced Live/dead cell analysis using PI staining

Propodium iodide stained the dead cells as fluorescence red whereas the live cells took calcein and observed as fluorescence green. The result showed that the UV-B-irradiated HaCaT cells at 15 mJ/Cm2 reduced the amount of live cells **Figure. 3B** when compared to control **Fig. 2A, 3A** and SEM pretreated restored the effect of UV-B radiation at 15 mJ/Cm2 in HaCaT cells in dose dependent manner **Figure. 3C**.

3.3. Effects of FME 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/Cm2 induced cell death in HaCaT cells through a typical apoptotic pathway, while this cell death was significantly inhibited by pre-treated SEM in a dose-dependent manner in HaCaT cells, when exposed to UV-B irradiation at 15 mJ/cm2 **Fig. 4A, B, C**.

MTT ASSAY



Doxorubicin-treated HaCaT Cells: Figure 1A: HaCaT cell line after the incubation for 24 hrs with the standard drug doxorubicin in 5 different concentrations (0.01 µM, 0.1 µM, 1 µM, 10 µM, 100 µM). Control-HaCaT cells.

SEM Treated HaCaT Cells

UV-B irradiation decreased the viability of HaCaT cells. Cell viability was not affected by SEM treatment, when it was treated with 100µg/mL and 30µg/mL reduced the cell viability, when compared to 10 µg/mL and 3 µg/mL. Data represents as mean ± S.D for all experiments done in triplicate.

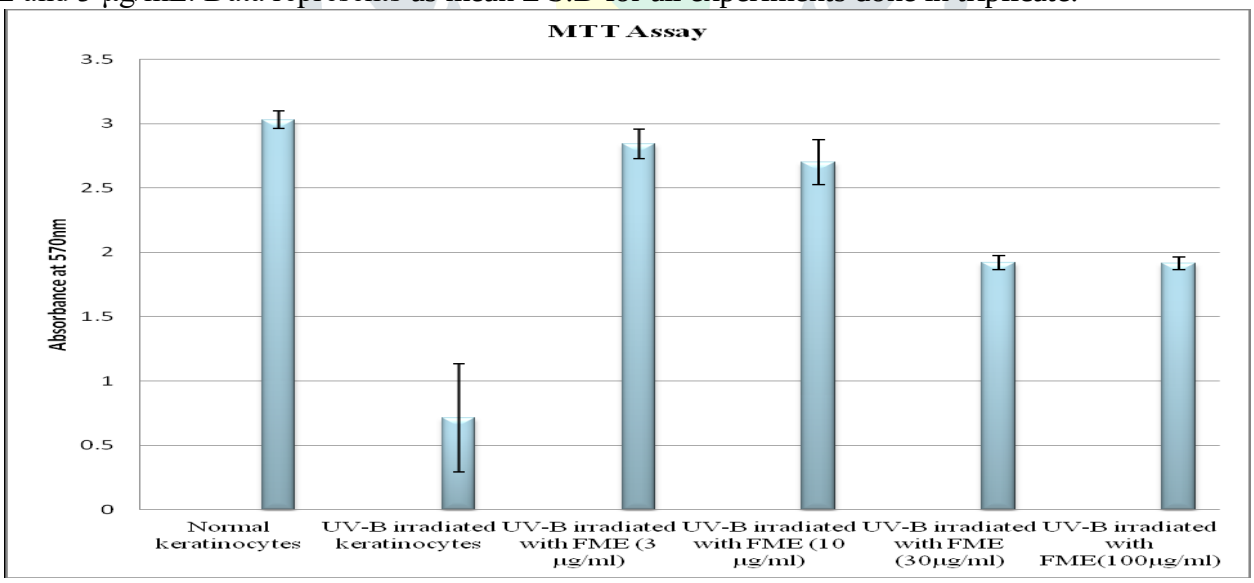
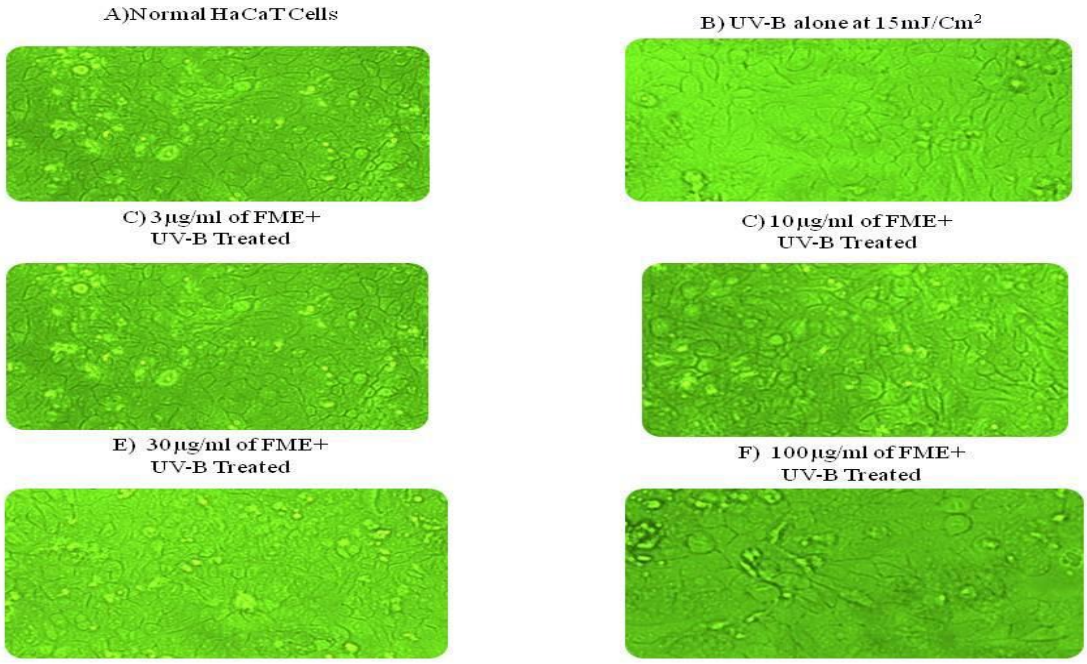


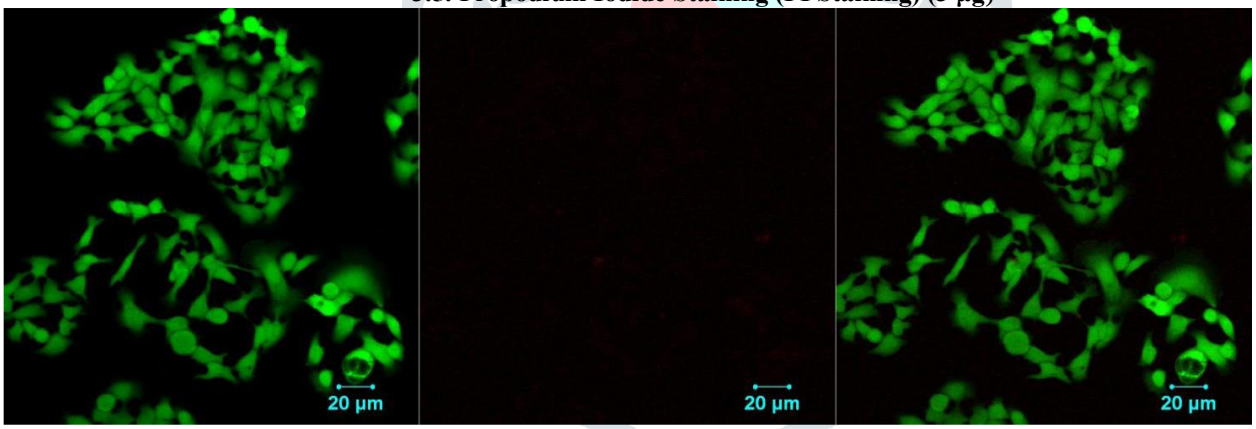
Figure 1B: Data represents the effect of SEM on HaCaT Cells.

Effect of SEM on UV-B radiation induced cytotoxicity

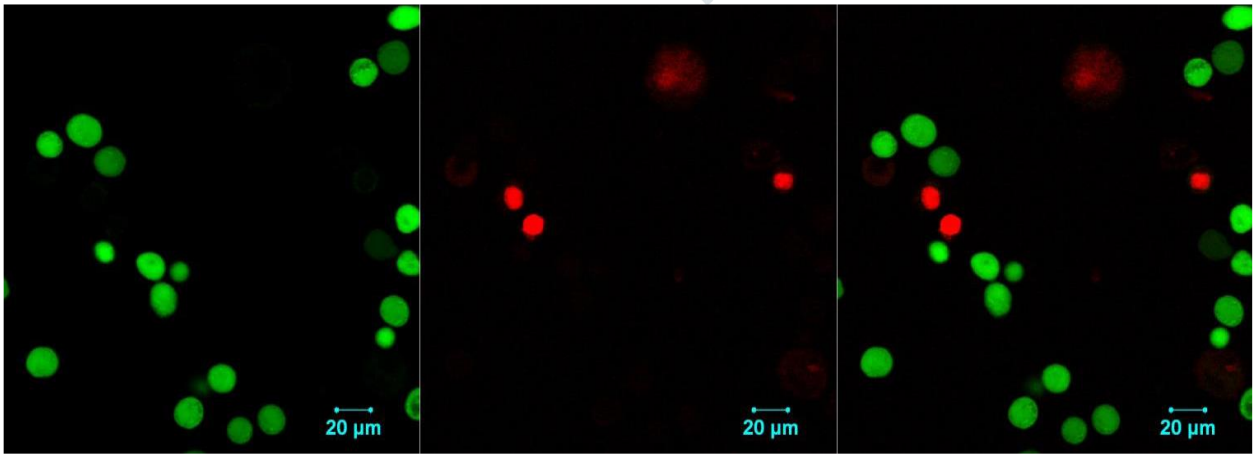


SEM Treated HaCaT Cells: Figure 2A. HaCaT cell line pre-treated for 30min with the Parrot fish *Scarus ghobban* mucus extract-SEM in 4 different concentrations (3 µg/ml, 10 µg/ml, 30 µg/ml, and 100 µg/ml) and then irradiated at 15mJ/Cm2.

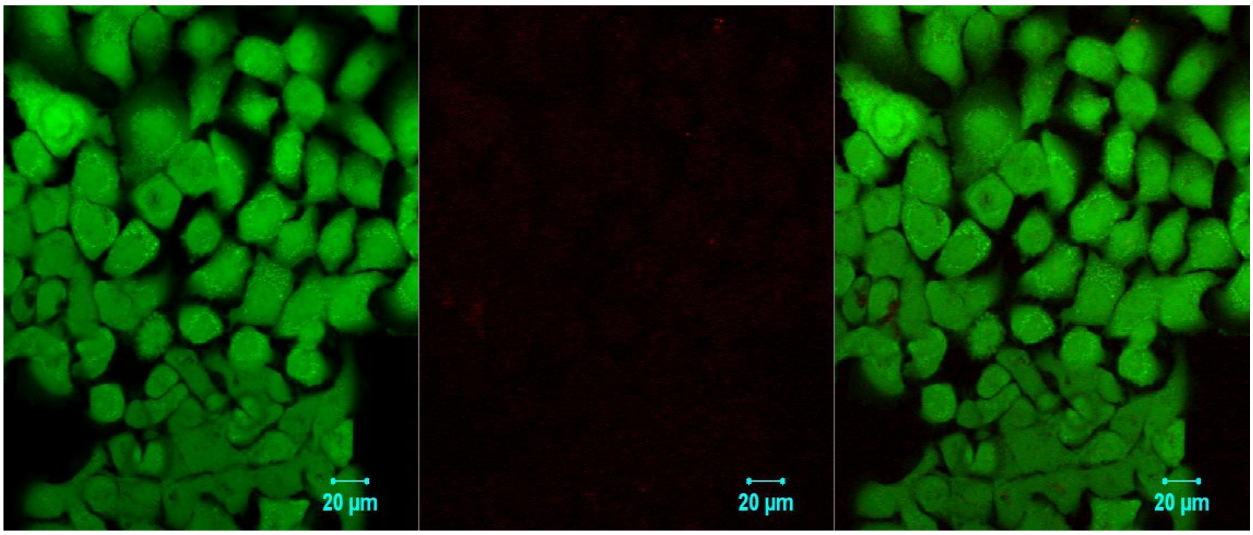
3.5. Propodium Iodide Staining (PI Staining) (3 µg)



CONTROL: Figure 3A. Live/dead cell analysis of the HaCaT cells without UV-B irradiation with 3 µg of SEM using PI staining.

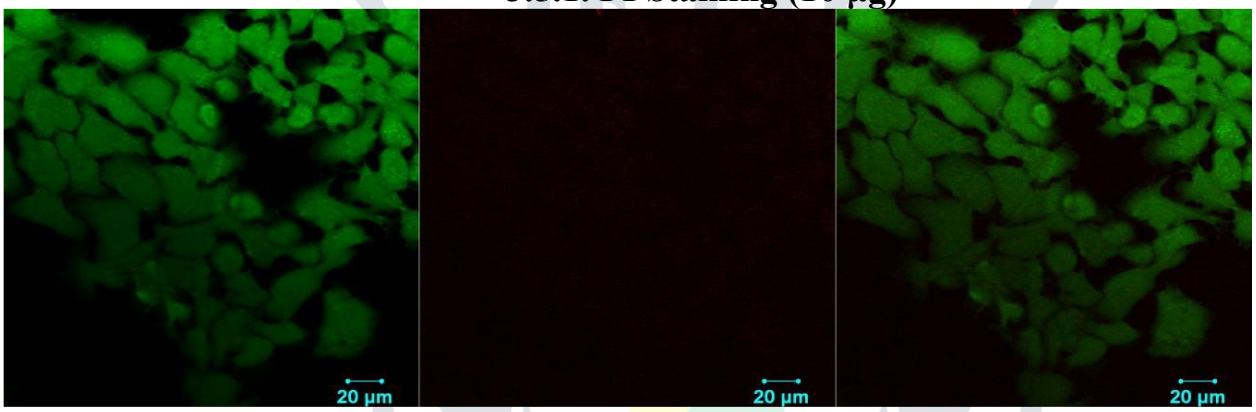


UV TREATED: **Figure 3B. HaCaT cells after UV-B irradiation for 15m J/Cm2 for live/dead cell analysis using PI staining.**

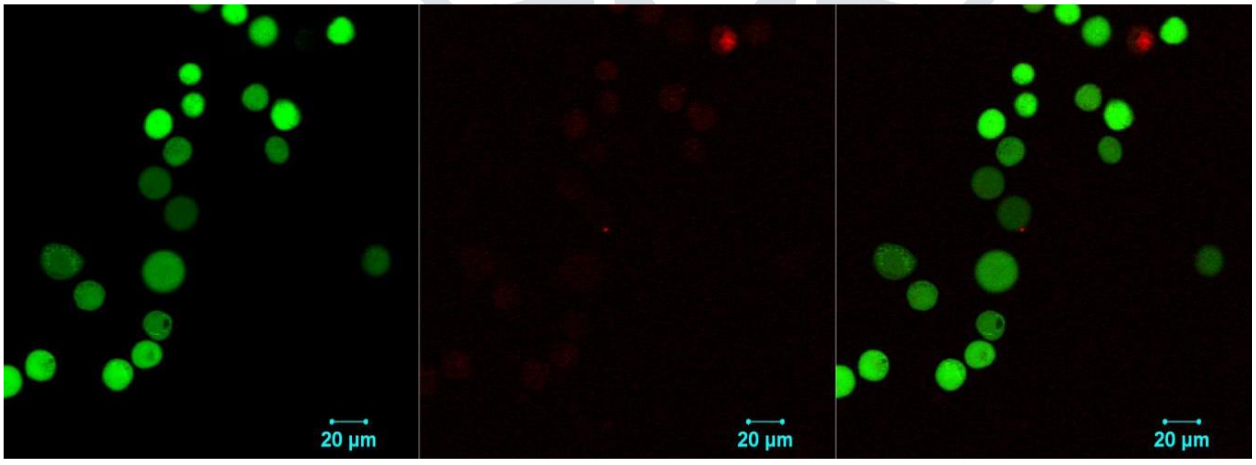


With SEM UV Treated: Figure 3C HaCaT cells after UV-B irradiation for 15m J/ Cm2 with 3 µg of SEM for live/dead cell analysis using

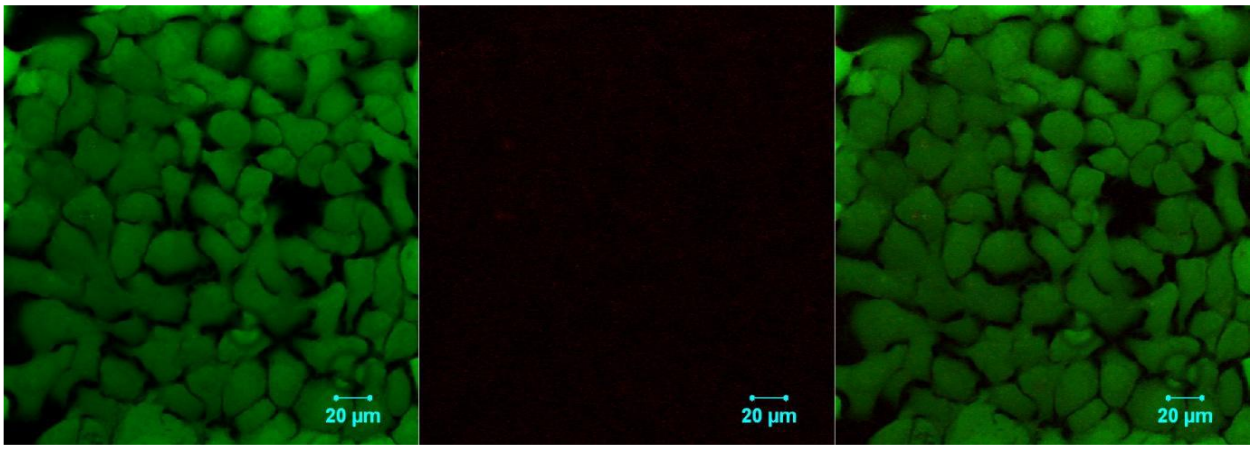
3.5.1. PI Staining (10 µg)



CONTROL: Figure 4A. Live/dead cell analysis of the Normal HaCaT cells without UV-B irradiation with 10 µg of SEM using PI staining.



UV TREATED: Figure 4B. HaCaT cells after UV-B irradiation for 15m J/ Cm2 for live/dead cell analysis using PI staining.



WITH SEM UV TREATED: Figure 4c) HaCaT cells after UV-B irradiation for 15m J/ Cm2 with 10 µg of SEM for live/dead cell analysis using PI staining.

Discussion

In the present study, we investigated the effects of SEM derived from parrot fish well known for secreting mucus, to ascertain whether they attenuate UV-based cell damage. We found that SEM extracts attenuated the effects of UV-B on HaCaT cells, as determined by both morphological and molecular analyses of inflammatory related proteins. Having established that SEM extract doses 10 µg/ml were not detrimental to HaCaT cell survival, we then tested cell survival in the presence of various concentrations of extracts with UV-B exposure. We demonstrated the capacity for SEM extracts to inhibit the process of and protein expression profiling supported this. In the absence of extract (control) HaCaT cells showed cytoplasmic condensation, cell shrinkage, membrane budding and accumulation of the condensation of chromatin after being exposed to UV-B. It was experimentally authenticated by Hoechst staining and PI staining.

The UV absorbent properties of animal mucus have been well studied in fish and corals. In corals, the algal symbionts help determine the mucus composition, including the release of MAAs which protect against UV (Shick et al., 1995). Other biomolecules that may act against photochemical damage to UV-B include cytosolic water-soluble reductants and membrane-bound lipid-soluble antioxidants. In tropical fish species, MAAs present within the epithelial mucus are critical for absorbing UV-A and UV-B, with strong absorbance peaks between 290 and 400 nm (Zamzow and Losey, 2002). Analysis of its mucus composition has primarily focused on the existence of water-soluble peptides that may be required for non-specific communication, showing that there are three major water-soluble peptides that are released in large amounts from secretory cells and diffuse into the surrounding seawater (Kuanbradit et al., 2012).

The antitumor/cytotoxic marine peptides vary considerably in size and structural complexity. They can be classified into four types: (1) linear depsipeptides; (2) cyclic depsipeptides; (3) linear peptides, and (4) marine protein hydrolysates. Many of the depsipeptides contain atypical amino acids with post-translational modifications such as carbamoylation or N- and O-methylation or amino acids not found in common proteins. They exert their cytotoxic action by blocking tubulin polymerization, arresting cell cycle and triggering apoptosis (Anderson et al., 1997, Ting et al., 1990, Bai et al., 1990) . Pardaxin is a 33-amino acid peptide which manifests both antimicrobial and antitumor activities. It induced apoptosis by endoplasmic reticulum targeting and c-FOS activation (Ting et al., 2014). There is a trend to focus on protein hydrolysates, formed by the enzymatic digestion of marine proteins that are used as antitumor agents. The digested hydrolysates or peptides from oyster extract (Wang et al., 2010) exhibited in vivo antitumor activity in BALB/c mice and tunicate extract(Jumeri and kim, 2011) , tuna dark muscle(Hsu et al., 2011) [59] and squid gelatin(Aliman et al., 2011) demonstrated in vitro cytotoxic effect on several human cancer cell lines. Pardaxin with a lytic action has potent activity against canine perianal gland adenomas and thus has potential veterinary application(Pan et al., 2015). Bullacta exarata peptides BEPT II and BEPT II-1 manifested potent apoptotic activity toward PC-3 cells (Mao et al., 2013). P2, a polypeptide fraction from the traditional Chinese medicine Arca subcrenata also exerted an antiproliferative action on HeLa and HT-29 cells without affecting normal hepatocytes. Antitumor effect in S-180 tumor-bearing mice was observed (Hu et al., 2012). The cyclic octapeptide reniochalistatin E from the marine sponge Reniochalina stalagmites demonstrated

cytotoxic activity toward HL-60, HepG2, HeLa, MGC-803, and RPMI-8226 cancer cell lines (Zhan et al., 2014).

In conclusion, the present study demonstrated the ability of biomolecules derived from skin epidermal extract of parrot fish to attenuate UV-B damage. Without extract, HaCaT cell viability was significantly reduced upon exposure to UV-B at 15 mJ/cm2. This was determined based on morphological changes, live-dead staining assay and analysis of changes in the abundance of inflammatory related proteins. It was experimentally authenticated by PI staining. Subsequent research will be carried out to determine the exact factors in the parrot fish extracts that are responsible for these properties.

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