

Protective Effects of *Gracilaria lemaneiformis* Extract against Ultraviolet B-Induced Damage in HaCaT Cells

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ABSTRACT

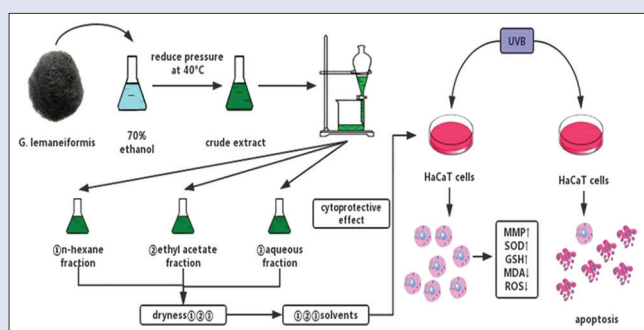
Background: *Gracilaria lemaneiformis* is an edible red marine macroalga that contains various active components including phycoerythrin, polysaccharides, and phenolics. In our previous work, crude ethanolic extracts of *G. lemaneiformis* exhibited potential antioxidative and anti-photoaging activities. Therefore, in this study, we aimed to further investigate the antioxidative and anti-photoaging activities of different fractions of *G. lemaneiformis* (n-hexane, ethyl acetate, and aqueous fractions [HF, EAF, and AQF, respectively]) using ultraviolet B (UVB)-induced HaCaT cells. **Materials and Methods:** Solvents with different polarity were used to fractionate crude ethanolic extract of *G. lemaneiformis*. The physicochemical properties of HF, EAF, and AQF were detected by gas chromatography–mass spectrometry, Fourier transform-infrared spectroscopy, and high-performance liquid chromatography–mass spectrometry. The antioxidant and antiapoptotic effects of the fractions were evaluated by estimating the levels of reactive oxygen species, antioxidant enzymes, and mitochondrial membrane potential (MMP) in UVB-exposed HaCaT cells. **Results:** According to our results, fatty acids, chlorophyll-a, and soluble polysaccharides were, respectively, present in the HF, EAF, and AQF. Furthermore, both EAF and AQF decreased the UVB-induced apoptosis by decreasing MMP. These results also suggest that EAF and AQF provide cytoprotective effects by activating the antioxidant enzymes. The soluble polysaccharides of AQF and chlorophyll-a of EAF were positively correlated with antioxidant activity. In addition, AQF exhibited stronger antioxidant and anti-photodamage properties than that of other fractions in UVB-radiated HaCaT cells. **Conclusion:** The results of this study indicated that water-soluble polysaccharides from *G. lemaneiformis* may be suitable to use as a natural anti-photodamage agent.

Key words: Antiapoptotic, *Gracilaria lemaneiformis*, HaCaT, oxidative stress, ultraviolet B

SUMMARY

• *Gracilaria lemaneiformis* is a red marine macroalga that is widely distributed in various Chinese coasts. Previous studies have reported that polysaccharides from *G. lemaneiformis* show various bioactive functions, such as antioxidant, anticancer, antiviral, anti-inflammatory, and immunomodulation effects. In this study, the different anti-photodamage constituents from *G. lemaneiformis* were investigated by separating different polar solvents. The results show that pretreatment with ethyl acetate fraction and aqueous fraction (AQF) dramatically increased the expression levels of superoxide dismutase and glutathione and

decreased the production of malondialdehyde in ultraviolet B-induced HaCaT cells. AQF, which contained soluble polysaccharides, exhibited the highest antioxidant and antiapoptotic activities among all fractions of *G. lemaneiformis*. Thus, our findings demonstrated that water-soluble polysaccharides from *G. lemaneiformis* could be used as a natural anti-photodamage agent.



Abbreviations used: UVB: Ultraviolet B; GC-MS: Gas chromatography–mass spectrometer; HPLC-MS: High-performance liquid chromatography–mass spectrometry; ROS: Reactive oxygen species; MMP: Mitochondrial membrane potential; HF: n-hexane fraction; EAF: Ethyl acetate fraction; AQF: Aqueous fraction; CF: Crude fraction; HPLC: High-performance liquid chromatography; FTIR: Fourier transform-infrared spectroscopy; DMEM: Dulbecco's modified Eagle's medium; PBS: Phosphate-buffered saline; DCFH-DA: Dichlorodihydrofluorescein-diacetate; GSH: glutathione; MDA: Malondialdehyde; SOD: Superoxide dismutase; ANOVA: Analysis of variance; PPB a: Pheophorbide a; HO-PPB a: Hydroxyl-pheophorbide a.

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INTRODUCTION

Ultraviolet B (UVB) radiation (280–320 nm) is a major component of UV light, which mainly originates from solar radiation.^[1] Previous studies have reported that excessive UVB irradiation might be detrimental to the skin causing inflammation,^[2,3] photoaging,^[4] and photocarcinogenesis.^[5,6] Most changes on the skin, such as pigmentation, roughness, and wrinkling, are commonly thought to negatively affect human appearance and also cause serious psychological issues.^[4,7,8] Many studies have suggested that dermal photoaging is linked to excessive accumulation of reactive oxygen species (ROS) in UVB-irradiated cells. The overproduction of ROS damages cellular structures.

Furthermore, ROS has been shown to promote UVB-induced apoptosis of keratinocytes.^[9–11] In addition, antioxidants have been reported to

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attenuate ROS-induced damage to keratinocytes.^[12-14] Therefore, there is a need for the development of more effective antioxidant agents to mitigate dermal photoaging.

Recent studies have suggested that the antioxidant properties of marine algae can attenuate UVB-induced apoptosis of keratinocytes. For example, eckstonolol extracted from *Ecklonia cava* protects against UVB-induced ROS accumulation in human keratinocytes,^[15] and the red alga *Bonnemaisonia hamifera* has UVB-absorbing properties and scavenges UVB-induced ROS.^[16] In addition, diphlorethohydroxycarmalol, a phlorotannin, derived from *Ishige okamurae* protects keratinocytes from UVB-induced DNA damage by regulating the nucleotide excision repair.^[17]

Gracilaria lemaneiformis is a red marine macroalga that is widely distributed in various Chinese coasts. Previous studies have reported that polysaccharides from *G. lemaneiformis* might exhibit various bioactive functions, such as antioxidant, anticancer, antiviral, anti-inflammatory, and immunomodulation.^[18-21] The liposoluble constituents of *G. lemaneiformis* were reported to exhibit the tyrosine phosphatase 1B inhibitory activity, which is used to treat type 2 diabetes mellitus and obesity.^[22] In our previous study, we showed that crude ethanolic extracts of *G. lemaneiformis* inhibited oxidative stress and prevented skin photoaging. In addition, a nonpolar fraction from ethanolic extracts with poor antioxidant activity showed similar results. However, there is no sufficient evidence regarding the cytoprotective and antioxidative effects underlying the differences in polarity in response to UVB irradiation. Many studies have also suggested that differences in polarity of the constituents might induce variations in biological effects.^[23]

Therefore, in this study, we investigated the antioxidant and cytoprotective effects of different fractions obtained from ethanolic extract of *G. lemaneiformis* using HaCaT human keratinocytes as the model.

MATERIALS AND METHODS

Plant material

In this study, *G. lemaneiformis* was obtained from Naozhou Island of Zhanjiang city, South China, in June 2016. Samples were washed and dried at 30°C ± 2°C. The dried seaweeds were stored at 4°C. The specimen was identified by Prof. Enyi Xie (Guangdong Ocean University).

Extraction

The extraction was conducted based on the solvent reflux extraction method that was carried as previously described.^[24] Briefly, *G. lemaneiformis* powders were placed in the flask with ethanol; the optimized extraction conditions are as follows: 70% ethanol; solvent/material ratio, 50:1; extraction thrice; and 30 min for each time. The extract was filtered and then concentrated under reduced pressure at 40°C to obtain the crude fraction (CF). CF was dissolved in distilled water and filtered. The filtrate was successively partitioned with n-hexane and ethyl acetate as the solvents yielding their respective fractions (n-hexane fraction [HF] and ethyl acetate fraction [EAF]). HF and EAF were concentrated under reduced pressure using a rotary evaporator. The aqueous fraction (AQF) was also concentrated. CF and solvent fractions (HF, EAF, and AQF) were all weighed to calculate the yield.

Analysis and identification

HF was analyzed by gas chromatography–mass spectrometry (GC-MS) (QP2010; Shimadzu). CF, EAF, and AQF were qualitatively analyzed by Fourier transform-infrared spectroscopy (FTIR) (FTS175C-UMA500; Bio-Rad). They were purified with silica gel column chromatography,

and the components were identified using high-performance liquid chromatography (HPLC)-MS (6430B; Agilent).

Cell culture

HaCaT cell line was purchased from Huons Co. Ltd. (Gyeonggi-do, China) and was cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (FBS) and 1% antibiotics (penicillin-streptomycin) at 37°C and 5% CO₂ in a humidified incubator.

Cell viability assay

HaCaT cells (5×10^3) were inoculated on 96-well culture plates and treated with various concentrations of CF, HF, EAF, and AQF for 24 h. Control cells were not irradiated by UVB light and were not pretreated with different fractions. Before UVB treatment, adherent cells were washed twice with 15- μ L phosphate-buffered saline (PBS). Then, a 100- μ L FBS-free medium was added. In this study, cells were exposed to UVB radiation (80 mJ/cm²). Cell viability was analyzed using MTT assay (Beyotime, China) on a microplate reader (Epoch; BioTek, USA) and according to the manufacturer's protocol.

Flow cytometry-based analysis of apoptosis

After 24 h of treatment with different fractions following UVB exposure, the cells were harvested and the percentage of apoptotic cells was analyzed using a fluorescein isothiocyanate (FITC)-labeled Annexin V apoptosis detection kit (Beyotime). The cells were stained with Annexin V-FITC according to the manufacturer's instructions. Apoptotic cells were analyzed using a FACSCalibur flow cytometer (Beckman Coulter, USA) with excitation at 488 nm. Data were analyzed using CellQuest software. Cells in early stages of apoptosis were positive for Annexin V.

Intracellular reactive oxygen species

The level of intracellular ROS was determined as previously described using a dichlorodihydrofluorescein-diacetate (DCFH-DA) probe (Beyotime).^[25] In this experiment, cells were pretreated with different fractions for 24 h and then exposed to UVB radiation (80 mJ/cm²). Next, the cells were harvested, washed twice with PBS, and labeled with 10- μ M DCFH-DA at 37°C for 20 min. The intracellular ROS levels were detected by flow cytometry (Beckman Coulter, USA), with excitation at 488 nm.

Mitochondrial membrane potential ($\Delta\psi_m$) analysis

Mitochondrial membrane potential (MMP) was detected using a lipophilic cationic fluorescent dye, named JC-1, which changes from green to red with increasing levels of MMP in the mitochondria. HaCaT cells were cultivated with different fractions for 24 h and exposed to UVB radiation (80 mJ/cm²). Subsequently, JC-1 was added to each well, and the cells were incubated for an additional 30 min at 37°C. Next, the cells were washed with PBS. The stained cells were analyzed with an Olympus IX73 fluorescence microscope.

Antioxidant enzyme activities

Cells were pretreated with different fractions for 24 h. After exposure of the cells to UVB radiation, they were put in cold lysis buffer. The lysates were separated by centrifugation at $10,500 \times g$ for 20 min at 4°C. Supernatants were used to detect the levels of glutathione (GSH), malondialdehyde (MDA), and superoxide dismutase (SOD). GSH activity was detected by conducting the dithio-bis-nitrobenzoic acid direct method. MDA was estimated by detecting the thiobarbituric acid reactive substances, and SOD activity was determined by the hydroxylamine method. The analyses were performed based on the reagent kit (Nanjing Jiancheng Bioengineering Institute, China).

Statistical analysis

All data are presented as mean \pm standard deviation. Significance of the differences between multiple groups was tested using one-way analysis of variance by SPSS 18.0 software (SPSS, Inc., Chicago, USA). Differences were regarded as significant when $P < 0.05$.

RESULTS

HF was analyzed by GC-MS.^[26] Its components were mostly saturated and unsaturated fatty acids, aldehydes, and sterols. The top four most abundant compounds were n-hexadecanoic acid (38.5%), oleic acid (25.4%), arachidonic acid (12.8%), and cholesterol (4.9%). The lower amounts of oleic acid and arachidonic acid suggest that there were only a small fraction of unsaturated fatty acids present in HF, although the unsaturated ethylenic bonds (C=C) in the chemical structure gave them reductive properties.

EAF was obtained as an oily substance with deep green color including most of hydrophobic pigments. According to the FTIR spectrum [Figure 1], EAF exhibited maximal absorbance at 2927 cm^{-1} and 2856 cm^{-1} linked with symmetric and antisymmetric C–H stretching vibrations of methylene (CH_2), the peaks in 1693 cm^{-1} and 1730 cm^{-1} were associated with the ester C = O and keto C = O band of the chlorophyll, respectively. While the bands around 1616 cm^{-1} were contributed to the C = C in chlorophyll and its derivatives, the bands in the region starting from 1490 cm^{-1} to 1440 cm^{-1} were the features of the chlorine and quinone ring bands,^[27,28] indicating that the structure was similar to chlorophyll-a. Some chlorophyll-a derivatives in the EAF were identified as pheophorbide-a (PPB-a, [M + H] m/z 593) and hydroxyl-PPB-a (HO-PPB a, [M + H] m/z 609) by HPLC-MS [Figure 2]. These results were consistent with previous reports in which the pigments of *G. lemaneiformis* mainly included phycoerythrobilin, chlorophyll-a, and their derivatives.^[29]

The AQF was examined by FTIR [Figure 1]. The strong absorption peak from 3500 cm^{-1} to 3200 cm^{-1} represented O–H stretching vibrations of polysaccharides. The absorption peak at 2926 cm^{-1} represented C–H stretching vibrations of the aliphatic compound. The absorption band at 1653 cm^{-1} was a band possessing C = O stretching vibrations.^[30] The absorption peaks at 1076 cm^{-1} , 931 cm^{-1} , and 892 cm^{-1} were presented the characterization of glycosidic bond from 3,6-anhydro-l-galactose and d-galactose.^[31] Some monosaccharides in AQF were identified

as glucose ([M + H] m/z 203) and galactose ([M + H] m/z 219) by HPLC-MS [Figure 2]. Thus, the major components in the AQF were soluble polysaccharides, which have been reported to exhibit antioxidant activity.^[32]

Effects of *Gracilaria lemaneiformis* fractions on ultraviolet B-induced HaCaT cell cytotoxicity

To assess the effects of different fractions of *G. lemaneiformis* on the proliferation of HaCaT cells after exposure to UVB, the cells were treated with different concentrations of these preparations for 24 h and exposed to UVB radiation (80 mJ/cm^2). The cytotoxicity was detected using MTT assay. The results showed that cell viability was reduced to 32.2% after UVB irradiation. However, pretreatment of the cells with different concentrations of CF, EAF, and AQF enhanced cell viability [Figure 3a and b].

Antiapoptotic effects of *Gracilaria lemaneiformis* fractions

The results of flow cytometric analysis showed that compared to the control cells (rate of apoptosis in nonirradiated cells [1.0%]), apoptosis was significantly elevated after irradiation (4.5%). The cells pretreated with CF, HF, EAF, and AQF before irradiation showed low rates of apoptosis [3.6%, 4.4%, 2.5%, and 2.2%, respectively; Figure 4]. The percentage of apoptotic cells was the lowest after pretreatment with AQF. These results indicated that solute polysaccharides efficiently reduced the rate of apoptosis in UVB-irradiated HaCaT cells.

Effects of *Gracilaria lemaneiformis* fractions on reactive oxygen species generation and ultraviolet B-induced damage to HaCaT cells

As can be seen from Figure 5, compared with nonirradiated cells, UVB exposure enhanced the production of ROS ($P < 0.05$). Pretreatment with CF, EAF, and AQF decreased the production of UVB-induced ROS. AQF showed the highest activity against ROS activation, and HF showed poor antioxidant activity.

Effects of *Gracilaria lemaneiformis* fractions on mitochondrial membrane potential

MMP plays a key role in ROS generation and mitochondrial apoptosis. As shown in Figure 6, aggregates of the JC-1 dye showed red/green

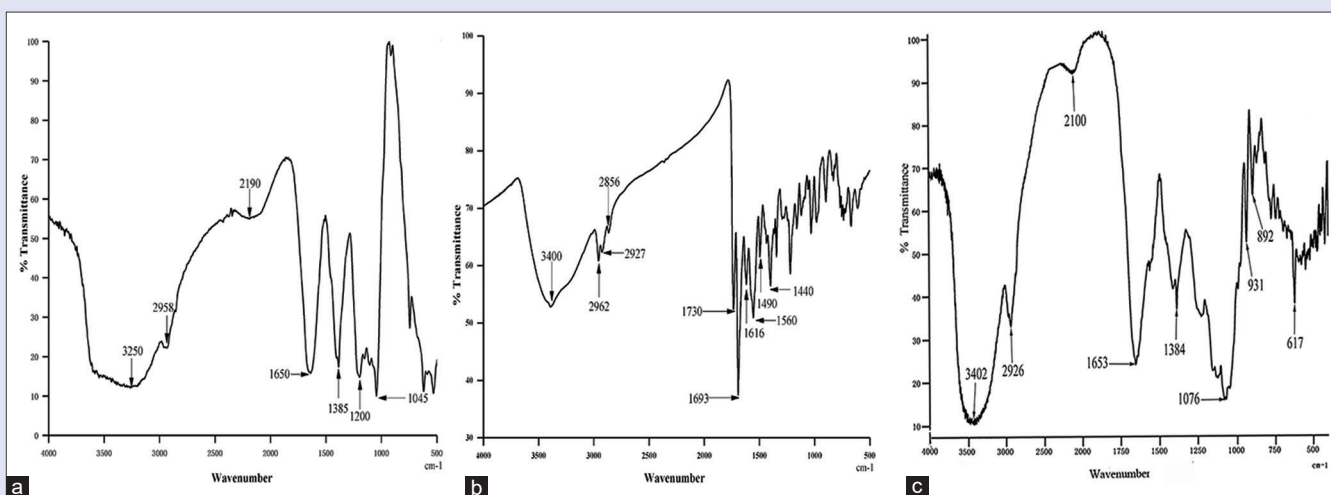


Figure 1: The CF, EAF, and AQF were examined with Fourier transform-infrared spectroscopy. (a): CF; (b): EAF; (c): AQF. CF: Crude fraction; EAF: Ethyl acetate fraction; AQF: Aqueous fraction

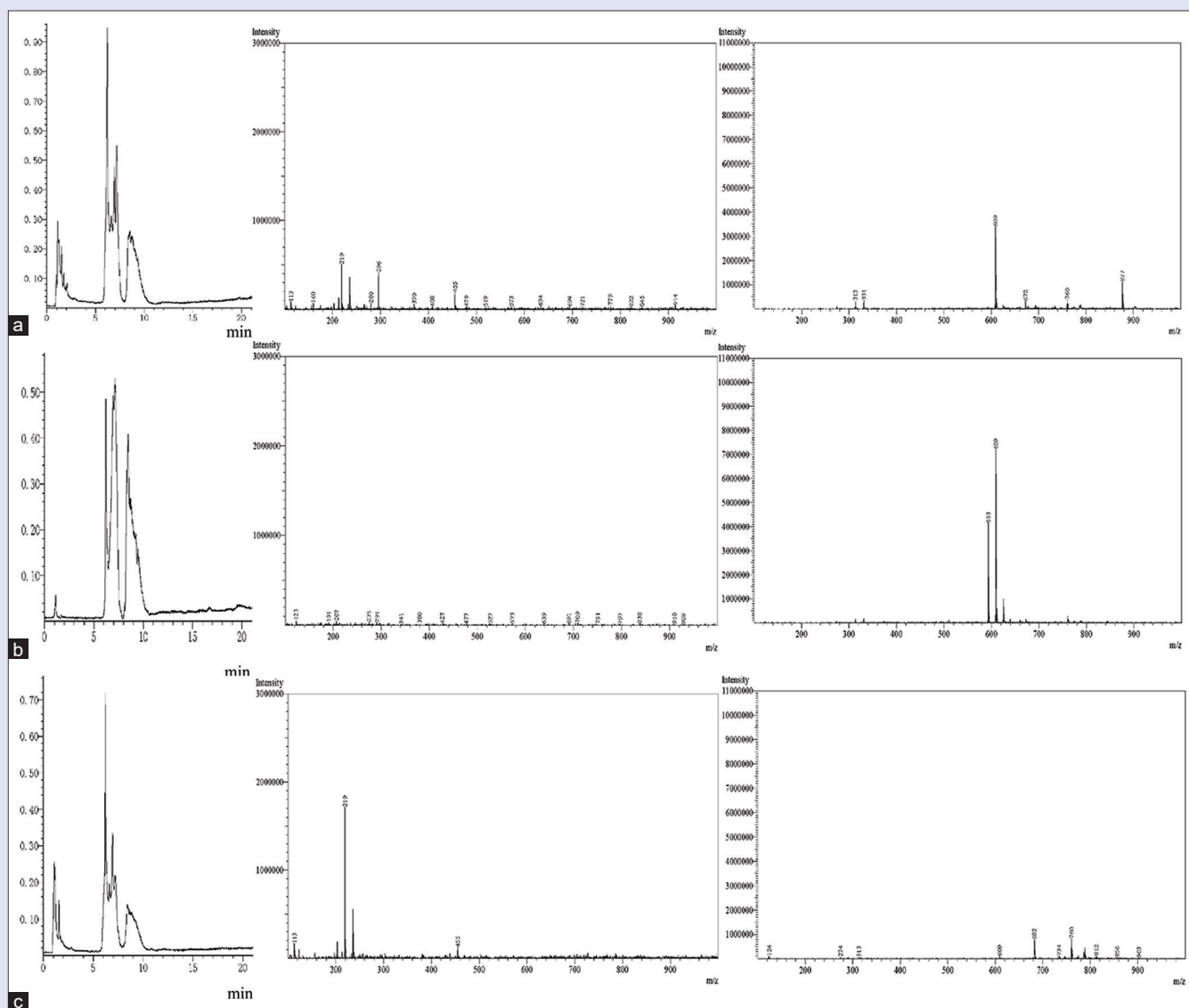


Figure 2: The CF, EAF, and AQF were analyzed by high-performance liquid chromatography–mass spectrometry. (a) CF; (b) EAF; (c) AQF. CF: Crude fraction; EAF: Ethyl acetate fraction; AQF: Aqueous fraction

fluorescence, and the monomer shows green fluorescence. After UVB exposure, the green fluorescence was dramatically increased in HaCaT cells, whereas pretreatment with CF, EAF, and AQF resulted in an increase in red/green fluorescence and a decrease in green fluorescence. These observations have demonstrated that the different fractions of *G. lemaneiformis* protect keratinocytes against UVB-radiated cytotoxicity by regulating the MMP.

Effects of *Gracilaria lemaneiformis* fractions on the activity of antioxidant enzymes

The contents of MDA, GSH, and SOD were evaluated as indicators of oxidative stress. As shown in Figure 7, the level of intracellular MDA was dramatically increased after UVB irradiation compared with that in the negative control group ($P < 0.05$). Pretreatment with different fractions of *G. lemaneiformis* decreased intracellular MDA levels in HaCaT cells by UVB irradiation. The level of MDA in the AQF pretreatment group was dramatically lower than that in other fractions ($P < 0.05$). Moreover, intracellular levels of GSH and SOD were significantly higher following

pretreatment with AQF than those following treatment with other *G. lemaneiformis* fractions ($P < 0.05$). These results demonstrated that fractions with antioxidant activities might enhance protection against UVB-induced cell damage and that solute polysaccharides were one of the primary components showing antioxidant activity in *G. lemaneiformis*.

DISCUSSION

Photodamage from exposure of UV radiation is a leading cause of skin aging. Various treatments for photoaging skin have focused on the use of natural products from plants or seaweeds, which provide equal efficacy with fewer side effects compared with chemical agents.^[33,34] Previous studies have reported that marine algae contain phenolic compounds, namely phycoerythrin and polysaccharides with antioxidant activity, which show beneficial effects against UV-induced skin damage.^[35,36] Moreover, *G. lemaneiformis* polysaccharides have been widely reported as antioxidant-rich components,^[37,38] however, the other phytochemical constituents have not been fully elucidated. In this study, screening of the chemical composition showed that fatty acids,

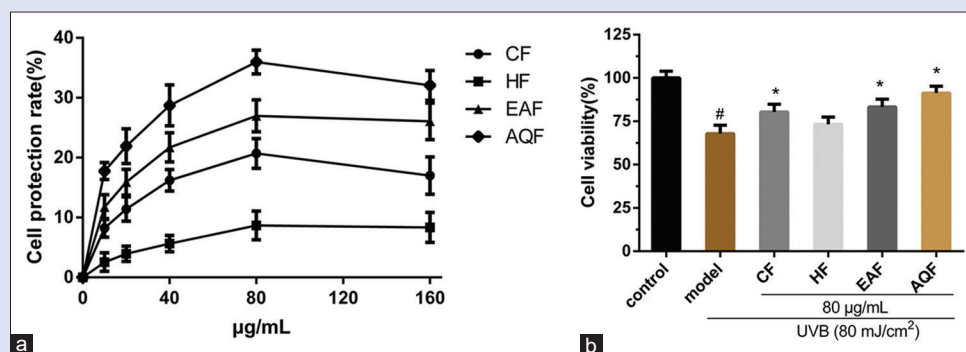


Figure 3: (a) Extracts of *Gracilaria lemaneiformis* inhibited UVB-induced cell injury. The cells were cultured for 24 h in Dulbecco's modified Eagle's medium and then in fresh medium in the presence of 10, 20, 40, 80, or 160 µg/mL extracts. After incubation for 24 h, the cells were exposed to UVB radiation (80 mJ/cm²) and MTT assay was performed. (b) Effects of *Gracilaria lemaneiformis* fractions on UVB-irradiated cytotoxicity in HaCaT cells. Data were compared with control or UVB model groups by one-way analysis of variance; #Indicates test versus control cells $P < 0.05$; *Indicates test versus UVB model group with $P < 0.05$. UVB: Ultraviolet B

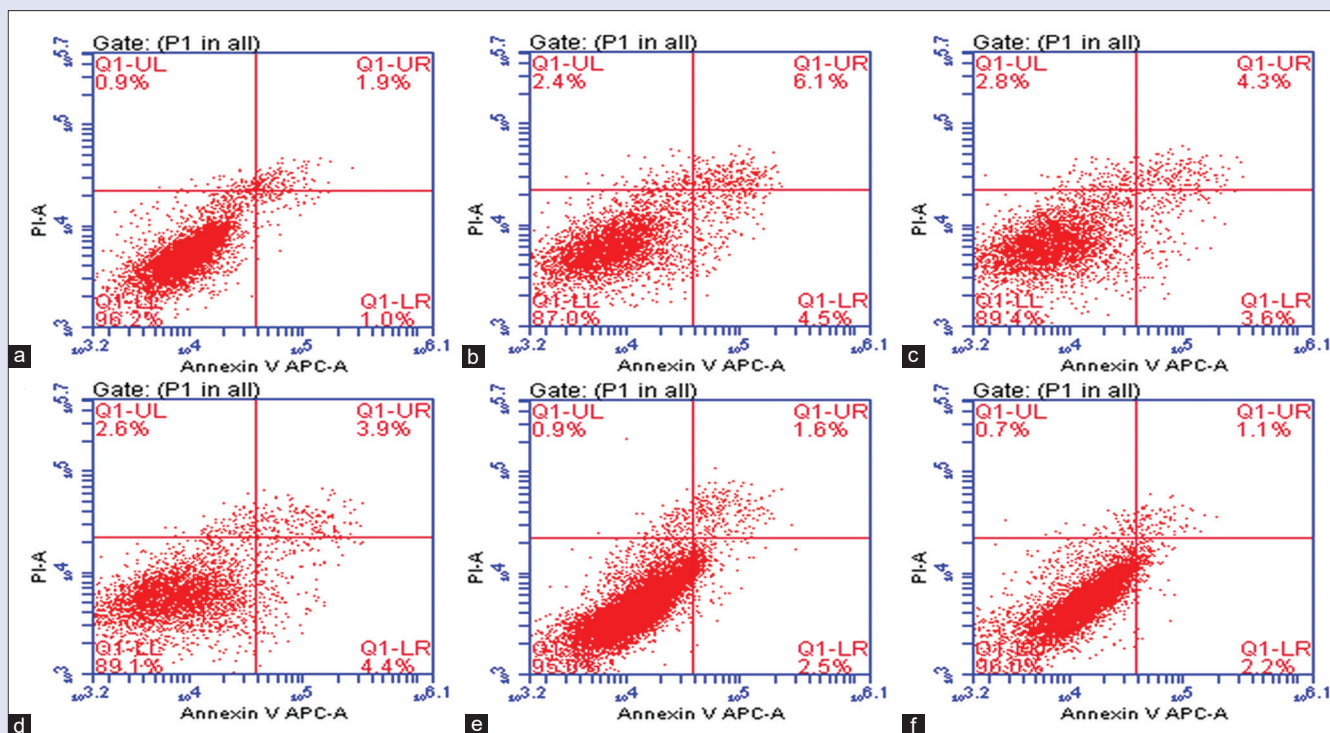


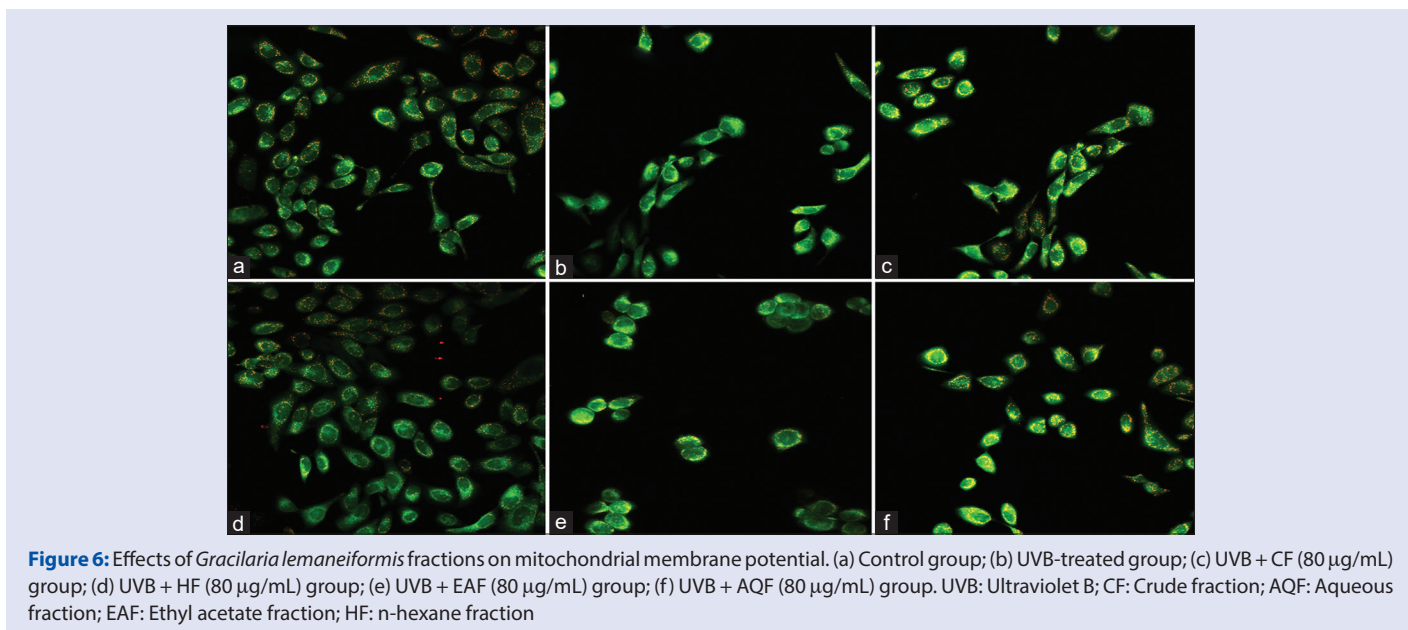
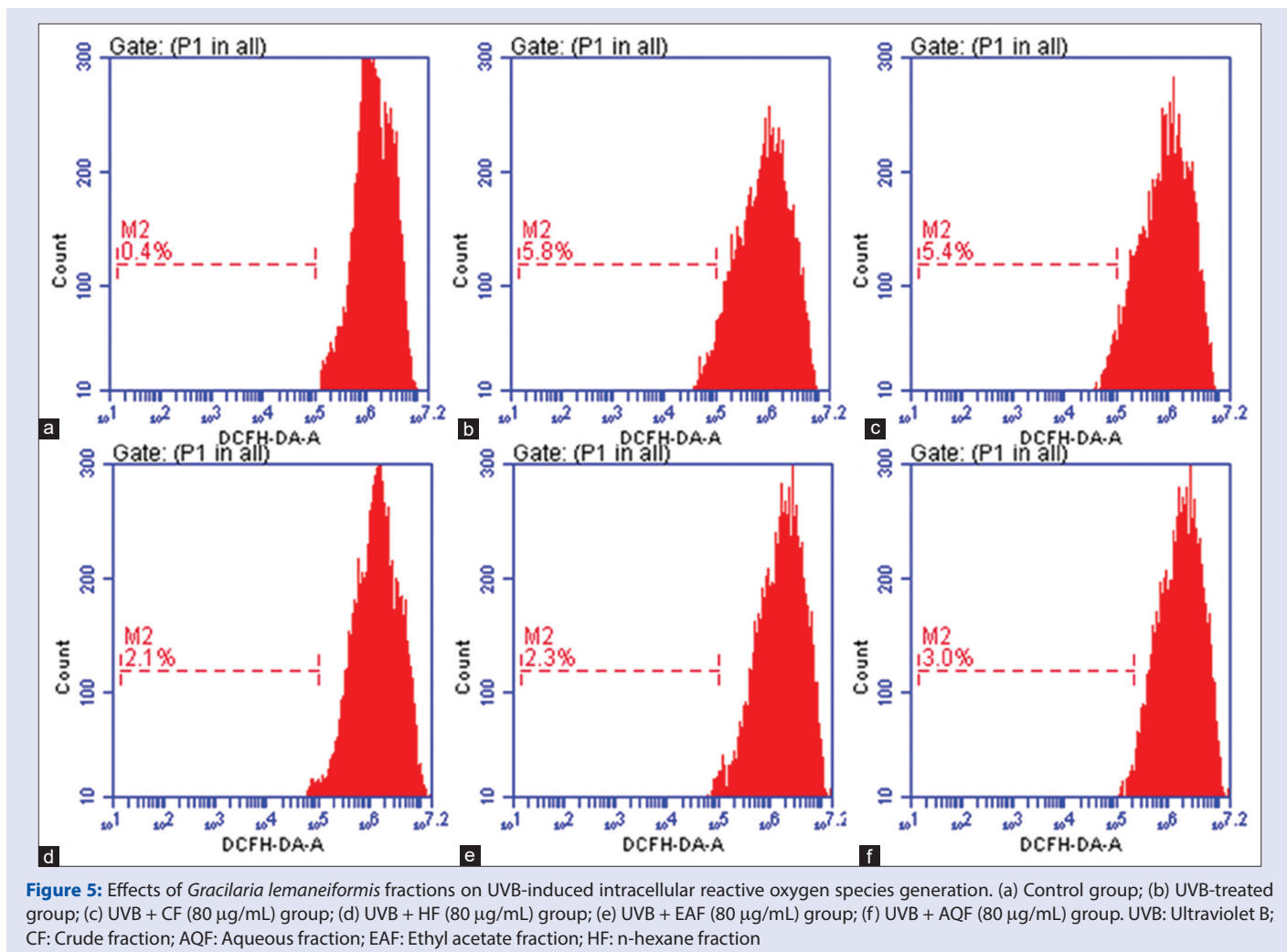
Figure 4: Inhibitory effects of *Gracilaria lemaneiformis* fractions on UVB-radiated apoptosis in the HaCaT cells. (a) Control group; (b) UVB-treated group; (c) UVB + CF (80 µg/mL) group; (d) UVB + HF (80 µg/mL) group; (e) UVB + EAF (80 µg/mL) group; (f) UVB + AQF (80 µg/mL) group. UVB: Ultraviolet B; CF: Crude fraction; AQF: Aqueous fraction; EAF: Ethyl acetate fraction; HF: n-hexane fraction

aldehydes, phytoalcohol, chlorophyll-a derivatives, and water-soluble polysaccharides were the primary components of *G. lemaneiformis*.

Consistent with the results of phytochemical screening, we found that HF primarily contained fatty acids, including n-hexadecanoic acid, oleic acid, arachidonic acid, and cholesterol; EAF contained chlorophyll-a derivatives, including PPB-a, HO-PPB-a, PPT-a, and HO-PPT-a; and AQF primarily contained soluble polysaccharides. The results of *in vitro* cytoprotective assay showed that cell viability was significantly increased in a concentration-dependent manner following pretreatment with EAF and AQF for 24 h and subsequent exposure to UVB radiation. Notably, AQF showed the highest cytoprotective effects against UVB irradiation among all fractions. Similarly, previous studies have demonstrated

that some antioxidants from seaweeds have beneficial effects against skin photodamage and that soluble polysaccharides contribute to cytoprotection against UVB-induced cell damage.^[39,40] However, HF, which contained fatty acids, did not exhibit any cytoprotective effects. This might be because the proportion of saturated fatty acids, which show poor antioxidant activity, was too high in HF.

ROS is mainly produced in the mitochondria and is degraded by endogenous antioxidants, including SOD and GSH peroxidases. UV irradiation is associated with excessive formation of ROS, leading to cellular senescence and mitochondrial dysfunction, an important feature of apoptosis.^[41] Previous studies have also indicated that ROS formation is directly related to the loss of MMP.^[42] Furthermore,



another study reported that hyperpolarization of MMP enhances ROS generation and damages organelles, including mitochondria,

lipid membranes, and proteins.^[43,44] Hence, in this study, we analyzed the relationship between MMP and ROS generation and found that

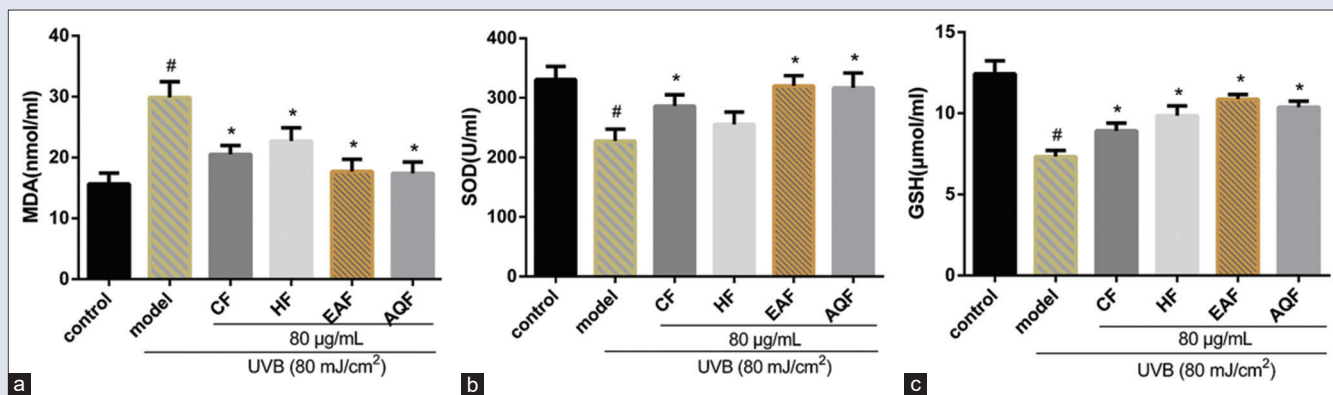


Figure 7: Effects of *Gracilaria lemaneiformis* extracts on the MDA, SOD, and GSH levels in HaCaT cells exposed to UVB. Cells were incubated with 80 µg/mL extracts for 24 h and irradiated by 80 mJ/cm² UVB. The activities of MDA (a), SOD (b), and GSH (c) were then measured using a reagent kit. Versus the control and $P < 0.05$ indicated by[#], versus UVB model group and $P < 0.05$ indicated by*. UVB: Ultraviolet B; MDA: Malondialdehyde; SOD: Superoxide dismutase; GSH: Glutathione

Table 1: Fractions and chemical components from *Gracilaria lemaneiformis*

Fraction	Yield (mg/g dried seaweed)	Chemical components
CF	12.2	Phenols, terpenoids, steroids, glycosides
HF	6.0	Fatty acids including n-hexadecanoic acid, oleic acid, arachidonic acid, and cholesterol
EAF	1.2	Chlorophyll-a derivatives including PPB a, HO-PPB a, PPT a, HO-PPT a
AQF	3.9	Soluble polysaccharides

Fractions and chemical components. CF: Crude fraction; HF: n-hexane fraction; EAF: Ethyl acetate fraction; AQF: Aqueous fraction; HO-PPB a: Hydroxyl-pheophorbide a; HO-PPT a: Hydroxyl-Pheophytin a

pretreatment with EAF or AQF increases MMP while inhibiting intracellular ROS generation. Growing evidence has indicated that activation of cellular antioxidant defense mechanisms, such as enzymes (SOD and CAT) and non-enzymatic molecules (GSH), can decrease ROS generation by converting ROS into non-toxic products and maintain the cellular redox balance.^[45] In the study, we found that EAF and AQF treatment significantly increased the expression levels of SOD and GSH and decreased the levels of MDA. Therefore, our findings suggested that pretreatment with soluble polysaccharides from *G. lemaneiformis* alleviated UVB-induced photodamage to cells by blocking ROS production, preventing mitochondrial dysfunction, enhancing the expression levels of endogenous antioxidants, and by blocking mitochondrion-mediated apoptosis.

CONCLUSION

In this study, different extracts of *G. lemaneiformis* were investigated for their cytoprotective and antioxidative effects. Our analysis of the chemical constituents revealed that aldehydes, phytoalcohol, chlorophyll-a derivatives, and water-soluble polysaccharides were the primary components of *G. lemaneiformis*. These constituents were responsible for the cytoprotective activity against UVB-induced HaCaT cell damage. AQF, which contained soluble polysaccharides, exhibited the highest antioxidant and antiapoptotic activities among all fractions from *G. lemaneiformis*. Thus, our findings demonstrate that water-soluble polysaccharides from *G. lemaneiformis* might be used as a natural anti-photodamage agent.

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Conflicts of interest

There are no conflicts of interest.

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