

Original Research Article

Photoprotective Effect of a *Polyopes affinis* (Harvey) Kawaguchi and Wang (*Halymeniaceae*)-Derived Ethanol Extract on Human Keratinocytes

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Abstract

Purpose: To investigate the photoprotective effect of the ethanol extract of the red marine alga, *Polyopes affinis* (PAE) against ultraviolet B (UVB) radiation on cultured human keratinocytes.

Methods: The 2',7'-dichlorodihydrofluorescein diacetate method was used to detect intracellular reactive oxygen species (ROS) generated by H₂O₂ treatment or UVB radiation. Cell viability was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Superoxide anion or hydroxyl radical was detected using an electron spin resonance spectrometer after reaction with the nitron spin trap. Lipid peroxidation was assayed by determining the level of 8-isoprostane. Protein carbonyl formation was determined using a protein carbonyl ELISA kit. The degree of oxidative DNA damage was determined using an alkaline comet assay. Apoptosis was assessed by apoptotic bodies and DNA fragmentation.

Results: PAE significantly scavenged the free radical 1,1-diphenyl-2-picrylhydrazyl, as well as hydrogen peroxide- and UVB-induced intracellular ROS. Furthermore, PAE showed 23 % scavenging effect of the superoxide anion and 33 % of the hydroxyl radical. PAE also absorbed UVB rays in the 280 – 320 nm range. PAE significantly decreased cellular damage resulting from UVB-induced oxidative stress to lipids, proteins, and DNA. Furthermore, PAE-treated keratinocytes showed significant reduction in UVB-induced apoptosis, as exemplified by fewer apoptotic bodies and reduced DNA fragmentation.

Conclusion: These results suggest that PAE protects keratinocytes against UVB-induced oxidative stress by absorbing UVB rays and scavenging ROS, thereby reducing injury to cellular constituents.

Keywords: Human keratinocytes, *Polyopes affinis*, Reactive oxygen species, Red algae, Ultraviolet B, Apoptosis, DNA fragmentation

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INTRODUCTION

Ultraviolet B (UVB; 280–320 nm) exposure leads to the production of reactive oxygen species (ROS) in skin cells, both in vivo and in vitro. UVB-induced intracellular ROS, together with the

resultant oxidative stress, plays an important role in many inflammatory skin disorders, the development of skin cancer, and skin aging [1]. Many studies have demonstrated that the formation of ROS followed by lipid peroxidation, protein modification, and/or induction of DNA

lesions is a predominant mechanism of UV radiation-induced photodamage [2]. The superoxide anion, hydrogen peroxide, hydroxyl radical, singlet oxygen, and peroxy radical are among the deleterious ROS induced by UV/UVB radiation [3,4].

Polyopes affinis (Harvey) Kawaguchi & Wang, called also *Carpopeltis affinis*, is an edible red seaweed. In Korea, *P. affinis* is widely cultivated on Jeju Island [5]. Previous reports showed that a methanolic extract of *Carpopeltis affinis*, exhibited high scavenging activity against the superoxide anion, hydroxyl radical, and H₂O₂ [6]. Moreover, recent investigations demonstrated that *P. affinis* alleviated airway inflammation in a murine model of allergic asthma [7] and inhibited a rat mast cell-dependent atopic allergic reaction [8]. However, the photo-protective effects of *P. affinis* against UV radiation have not yet been reported. Therefore, this study investigated the actions of an ethanol extract derived from *P. affinis* (PAE) against UVB-induced cellular damage in human HaCaT keratinocytes.

EXPERIMENTAL

Extraction of *P. affinis*

The *P. affinis* was collected from Gimnyeong, (Jeju, Republic of Korea) in July 16, 2008. The alga was identified and authenticated by Dr Dong Sam Kim, Jeju Biodiversity Research Institute (Jeju, Republic of Korea). The voucher specimen (A08-0000870) has been deposited at the herbarium of Jeju Biodiversity Institute. Material obtained from a desiccated specimen was extracted with 80 % ethanol at room temperature for 24 h. The extract was then evaporated under vacuum.

Reagents

The 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, N-acetyl cysteine (NAC), 5,5-dimethyl-1-pyrroline-N-oxide (DMPO), 2',7'-dichlorodihydrofluorescein diacetate (DCF-DA), and Hoechst 33342 dye were purchased from Sigma Chemical Company (St. Louis, MO, USA). All other chemicals and reagents were of analytical grade.

Cell culture

Human keratinocytes (HaCaT cells) were obtained from the Amore Pacific Company (Gyeonggi-do, Republic of Korea) and maintained at 37 °C in an incubator with a humidified atmosphere of 5 % CO₂. The cells

were cultured in Dulbecco's modified Eagle's medium containing 10 % heat-inactivated fetal calf serum, streptomycin (100 µg/ml), and penicillin (100 U/ml).

Detection of the DPPH radical

PAE at a concentration of 50, 100, or 200 µg/ml and 2 mM NAC were added to a solution of DPPH (0.1 mM) in methanol. The mixture was shaken vigorously and then allowed to stand at room temperature for 3 h. The amount of unreacted DPPH was measured at 520 nm using a spectrophotometer.

Detection of intracellular ROS

The DCF-DA method was used to detect intracellular ROS generated by H₂O₂ treatment or UVB radiation. To detect ROS in H₂O₂-treated HaCaT keratinocytes, the cells were seeded into each well of a 96-well plate at a density of 1.5 × 10⁵ cells/well. Sixteen hours later, they were treated with PAE at a concentration of 50, 100, or 200 µg/ml. After a 30 min incubation at 37 °C, H₂O₂ (1 mM) was added to the wells, and the plates were again incubated for 30 min at 37 °C. At this time, DCF-DA solution (25 µM) was added to the wells. Ten minutes later, the fluorescence of 2',7'-dichlorofluorescein (DCF) was detected and quantified using a PerkinElmer LS-5B spectrofluorometer (PerkinElmer, Waltham, MA, USA). To detect ROS in UVB-irradiated cells, keratinocytes were treated with PAE as described above and exposed to UVB radiation 1 h later at a dose of 30 mJ/cm². The UVB source was a CL-1000M UV Crosslinker (UVP, Upland, CA, USA), which was used to deliver an energy spectrum corresponding to wavelengths of 280 – 320 nm. The irradiated cells were incubated for an additional 24 h at 37 °C, at which time DCF-DA solution (25 µM) was added. The fluorescence was detected as described above.

Cell viability assay

The effect of PAE on the viability of HaCaT cells was assessed as follows. Cells were seeded into each well of a 96-well plate at a density of 1 × 10⁵ cells/well. They were treated 16 h later with PAE (12.5, 25, 50, 100 or 200 µg/ml). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) stock solution (50 µl, 2 mg/ml) was added to each well to yield a total reaction volume of 200 µl. Four hours later, the plate was centrifuged at 800 × g for 5 min, and the supernatants were aspirated. The formazan

crystals in each well were dissolved in dimethylsulfoxide (DMSO, 150 μ l), and the absorbance at 540 nm was read on a scanning multi-well spectrophotometer.

Detection of the superoxide anion

The superoxide anion produced via the xanthine/xanthine oxidase system was reacted with the nitron spin trap, DMPO. The DMPO/ \cdot OOH adducts were detected using a JES-FA electron spin resonance (ESR) spectrometer (JEOL, Tokyo, Japan) as described previously [9]. Briefly, ESR signalling was recorded 5 min after 20 μ l of xanthine oxidase (0.25 U/ml) was mixed with 20 μ l each of xanthine (5 mM), DMPO (1.5 M), and PAE (100 μ g/ml). The ESR spectrometer parameters were set as follows: magnetic field of 336 mT, power of 1.00 mW, frequency of 9.4380 GHz, modulation amplitude of 0.2 mT, gain of 500, scan time of 0.5 min, scan width of 10 mT, time constant of 0.03 sec, and temperature of 25 $^{\circ}$ C.

Detection of the hydroxyl radical

The hydroxyl radical generated by the Fenton reaction ($\text{H}_2\text{O}_2 + \text{FeSO}_4$) was reacted with DMPO. The resultant DMPO/ \cdot OH adducts were detected using a JES-FA ESR spectrometer as described previously [10]. The ESR spectrum was recorded 2.5 min after a phosphate buffer solution (pH 7.4) was mixed with 0.2 ml each of DMPO (0.3 M), FeSO_4 (10 mM), H_2O_2 (10 mM), and PAE (100 μ g/ml). The ESR spectrometer parameters were set as follows: magnetic field of 336 mT, power of 1.00 mW, frequency of 9.4380 GHz, modulation amplitude of 0.2 mT, gain of 200, scan time of 0.5 min, scan width of 10 mT, time constant of 0.03 sec, and temperature of 25 $^{\circ}$ C.

UV/visible light absorption analysis

The UVB absorption spectra of PAE diluted in DMSO at a ratio 1:500 (v/v) was determined via UV scanning at 200 – 500 nm using a Biochrom Libra S22 UV/visible light spectrophotometer (Biochrom Ltd, Cambridge, UK).

Lipid peroxidation assay

Lipid peroxidation in HaCaT cells was assayed by determining the level of 8-isoprostane secreted into the culture medium with a commercial enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI, USA), according to the manufacturer's instructions.

Protein carbonyl formation

The extent of protein carbonyl formation in HaCaT cells was determined using an OxiselectTM protein carbonyl ELISA kit (Cell Biolabs, San Diego, CA, USA), according to the manufacturer's instructions.

Single cell gel electrophoresis (comet assay)

The degree of oxidative DNA damage in HaCaT cells was determined in an alkaline comet assay. A suspension of HaCaT keratinocytes was mixed with 0.5 % low-melting agarose (LMA, 75 μ l) at 39 $^{\circ}$ C. The mixture was then spread on a fully frosted microscopic slide pre-coated with 1 % normal melting agarose (200 μ l). After solidification of the agarose, the slide was covered with another 75 μ l of 0.5 % LMA and immersed in a lysis solution (2.5 M NaCl, 100 mM Na-EDTA, 10 mM Tris, 1 % Triton X-100, and 10% DMSO, pH 10) for 1 h at 4 $^{\circ}$ C. The slides were subsequently placed in a gel electrophoresis apparatus containing 300 mM NaOH and 10 mM Na-EDTA (pH 13) for 40 min to allow for DNA unwinding and the expression of alkali-labile damage. An electrical field was then applied (300 mA, 25 V) for 20 min at 4 $^{\circ}$ C to draw the negatively charged DNA toward the anode. The slides were washed three times for 5 min each time at 4 $^{\circ}$ C in a neutralizing buffer (0.4 M Tris, pH 7.5), stained with propidium iodide (75 μ l, 20 μ g/ml), and observed under a fluorescence microscope equipped with an image analyser (Kinetic Imaging, Komet 5.5, UK). The percentage of total fluorescence in the comet tails and the tail lengths for 50 cells per slide were recorded.

Nuclear staining with Hoechst 33342

HaCaT cells were treated with PAE at a concentration of 100 μ g/ml and exposed to UVB radiation 1 h later. After an additional 24 h incubation at 37 $^{\circ}$ C, the DNA-specific fluorescent dye Hoechst 33342 was added to each well, and the cells were incubated for 10 min at 37 $^{\circ}$ C. The stained cells were visualised under a fluorescence microscope equipped with a CoolSNAP-Pro colour digital camera (Media Cybernetics, Rockville, MD, USA). The degree of nuclear condensation was evaluated, and the apoptotic cells were quantified.

DNA fragmentation assay

Cellular DNA fragmentation was assessed by analysing the extent of cytoplasmic histone-associated DNA fragmentation using a kit (Roche

Diagnosics, Portland, OR, USA), according to the manufacturer's instructions.

Statistical analysis

All values are expressed as the mean \pm the standard error. The results were subjected to analysis of variance (ANOVA) followed by Tukey's test to analyse differences between means. In each case, $p < 0.05$ was considered statistically significant.

RESULTS

Scavenging capacity of PAE against free radicals

PAE scavenged the DPPH radical in a concentration-dependent manner, with 4 % of the radicals scavenged at a concentration of 50 $\mu\text{g/ml}$, 12 % at 100 $\mu\text{g/ml}$, and 14 % at 200 $\mu\text{g/ml}$. The well-known ROS scavenger, NAC (2 mM), was used as a positive control and scavenged 89 % of the radicals (Fig 1A, black bars). The scavenging capacity of PAE against H_2O_2 -induced intracellular ROS in HaCaT cells was also concentration-dependent, with 9 % of the ROS scavenged at 50 $\mu\text{g/ml}$, 11 % at 100 $\mu\text{g/ml}$, and 16 % at 200 $\mu\text{g/ml}$, compared with 54 % for NAC (Fig 1A light grey bars). PAE showed similar scavenging effects against UVB-induced intracellular ROS, with 14 % of the ROS scavenged at 50 $\mu\text{g/ml}$, 17 % at 100 $\mu\text{g/ml}$, and 13 % at 200 $\mu\text{g/ml}$, compared with 27 % for NAC (Fig 1A, dark grey bars).

The dose of UVB radiation yielding 50 – 60 % cell viability under the conditions of this study was 30 mJ/cm^2 . Therefore, this dose was chosen as the optimal dose for investigating the protective effects of PAE against UVB radiation. PAE was not cytotoxic towards HaCaT cells when added to the culture medium at 12.5, 25, 50, or 100 $\mu\text{g/ml}$. However, some cytotoxicity was observed at 200 $\mu\text{g/ml}$ (Fig 1B). Thus, 100 $\mu\text{g/ml}$ was chosen as the optimal concentration of PAE for further investigation.

The scavenging effects of PAE against the superoxide anion and the hydroxyl radical were next investigated by ESR spectrometry. The superoxide anion signal in the xanthine/xanthine oxidase system was 2504, but this signal was reduced by PAE treatment to 1938 (Fig 1C). PAE also caused a reduction in the hydroxyl radical signal produced by the Fenton reaction, from 3627 to 2418 (Fig 1D). The signals for the control

and PAE alone were 310 and 272, respectively, in the xanthine/xanthine oxidase system and 33 and 43, respectively, in the Fenton reaction.

Effect of PAE on UVB absorption

The effect of PAE on the absorption of UVB rays was determined using a UV/visible light spectrophotometer. PAE showed a high absorptive capacity for UV radiation in the range of UVB wavelengths (280 – 320 nm) (Fig 2).

Effect of PAE on UVB-induced oxidative lipid, protein, and DNA damage

The ability of PAE to inhibit membrane lipid peroxidation, protein carbonylation, and DNA damage in UVB-irradiated HaCaT cells was investigated 24 h after the cells were exposed to UVB rays. Lipid peroxidation was monitored by measuring the amount of 8-isoprostane secreted into the culture medium. As shown in Fig 3A, 8-isoprostane levels were increased in the medium of UVB-irradiated cells relative to untreated control cells and PAE-treated cells. However, pretreatment with PAE significantly prevented the increase. The concentration of 8-isoprostane in the medium of UVB-irradiated and PAE-pretreated, UVB-irradiated cells was 301 and 228 pg/ml , respectively.

Damaged protein accumulates in photodamaged skin, and protein carbonylation is a biomarker of oxidative stress-induced protein damage [11]. The protein carbonyl content in HaCaT cells exposed to UVB (13 nmol/mg) was significantly elevated relative to untreated control and PAE-pretreated cells; however, this value decreased to 11 nmol/mg in cells pretreated with PAE prior to UVB exposure (Fig 3B).

Finally, UVB-induced damage to cellular DNA was detected in an alkaline comet assay. Exposure of cells to UVB light increases the number of DNA breaks and, thus, the intensity of fluorescence due to propidium iodide-stained DNA in the tails of the comet-like structures formed in the assay. The level of fluorescence in the comet tails of the cells exposed to UVB was 37 % (Fig 3C), which is a significant increase compared with untreated control and PAE-treated cells. The corresponding value was 31 % in PAE-pretreated, UVB-irradiated cells, which is a significant decrease compared with UVB-irradiated cells.

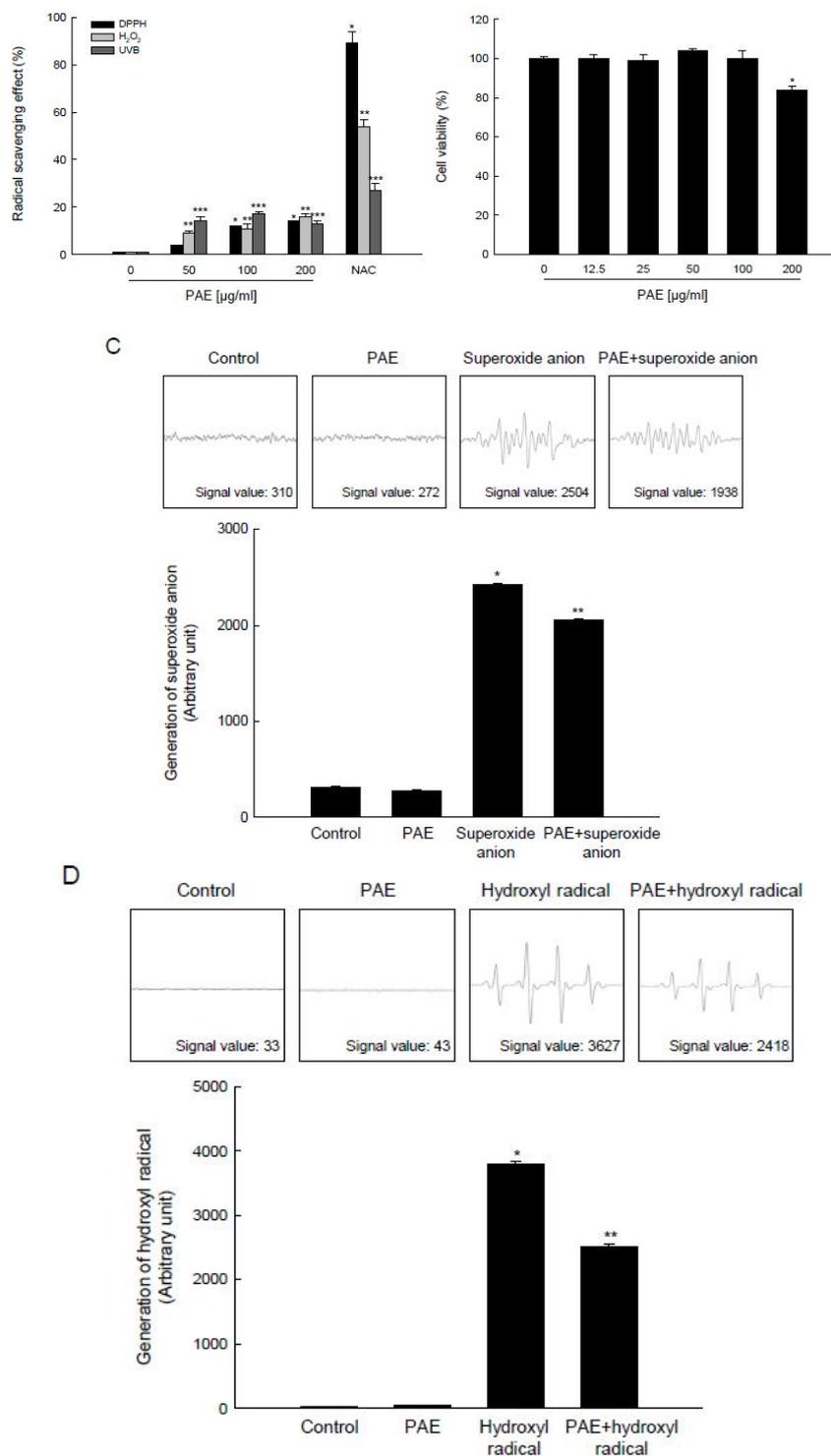


Fig 1: Effect of PAE on free radicals and cell viability. (A) The DPPH radical was detected spectrophotometrically at 520 nm, and intracellular ROS generated by H₂O₂ or UVB were detected spectrofluorometrically after DCF-DA staining. NAC served as the positive control. *, **, *** indicate significantly different from DPPH, H₂O₂-induced ROS, and UVB-induced ROS, respectively ($p < 0.05$). (B) Cells were seeded, and PAE was added at a final concentration of 12.5, 25, 50, 100, or 200 µg/ml. After 24 h, cell viability was determined using the MTT assay. *Significantly different from untreated control ($p < 0.05$). (C) The superoxide anion generated by the xanthine/xanthine oxidase system was reacted with DMPO, and the resultant DMPO/OOH adducts were detected using ESR spectrometry and quantified. Representative peak data are shown. *Significantly different from control ($p < 0.05$) and **significantly different from superoxide anion ($p < 0.05$). (D) The hydroxyl radical generated by the Fenton reaction was reacted with DMPO, and the resultant DMPO/OH adducts were detected by ESR spectrometry and quantified. Representative peak data are shown. *Significantly different from control ($p < 0.05$) and **significantly different from hydroxyl radical ($p < 0.05$)

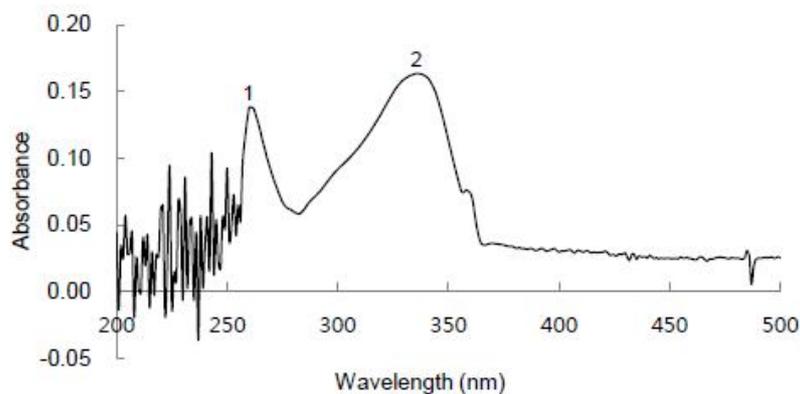


Fig 2: Effect of PAE on UVB absorption. The UV/visible light spectroscopic measurement was conducted in the spectral range from 200 to 500 nm. Peaks 1 and 2 indicate maximal absorbance at 261 and 336 nm, respectively

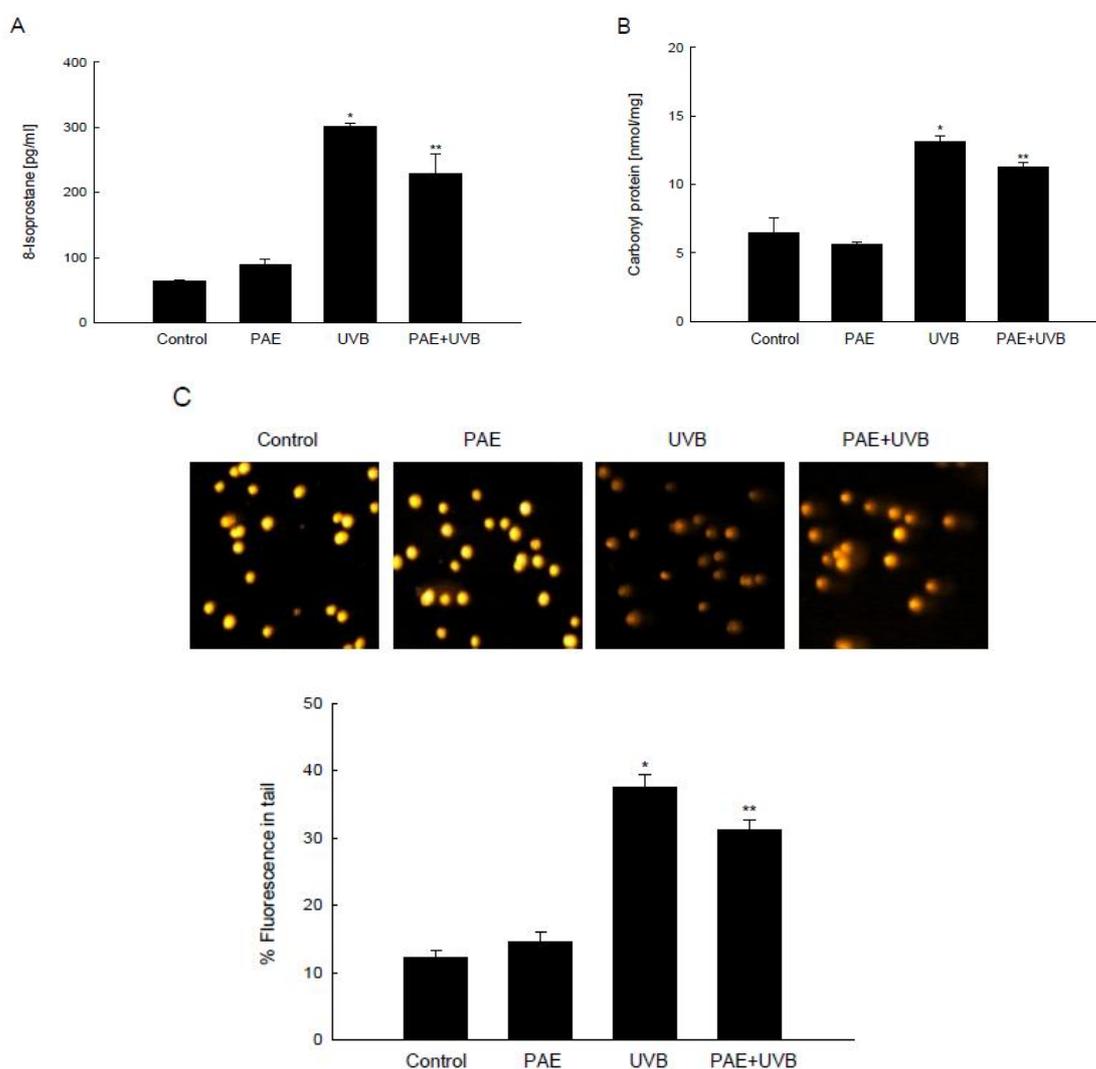


Fig 3: Protective effects of PAE in HaCaT cells subjected to oxidative stress induced by UVB radiation. Cells were treated with PAE for 1 h and then exposed to UVB radiation. (A) Following a 24 h incubation, lipid peroxidation was assayed by measuring the levels of 8-isoprostane secreted into the medium. (B) Protein oxidation was assayed by measuring protein carbonyl formation. (C) DNA damage was assayed using an alkaline comet assay. Representative images and the percentage of fluorescence resulting from propidium iodide-stained DNA in the comet tails are shown. *Significantly different from control ($p < 0.05$) and **significantly different from UVB-irradiated cells ($p < 0.05$)

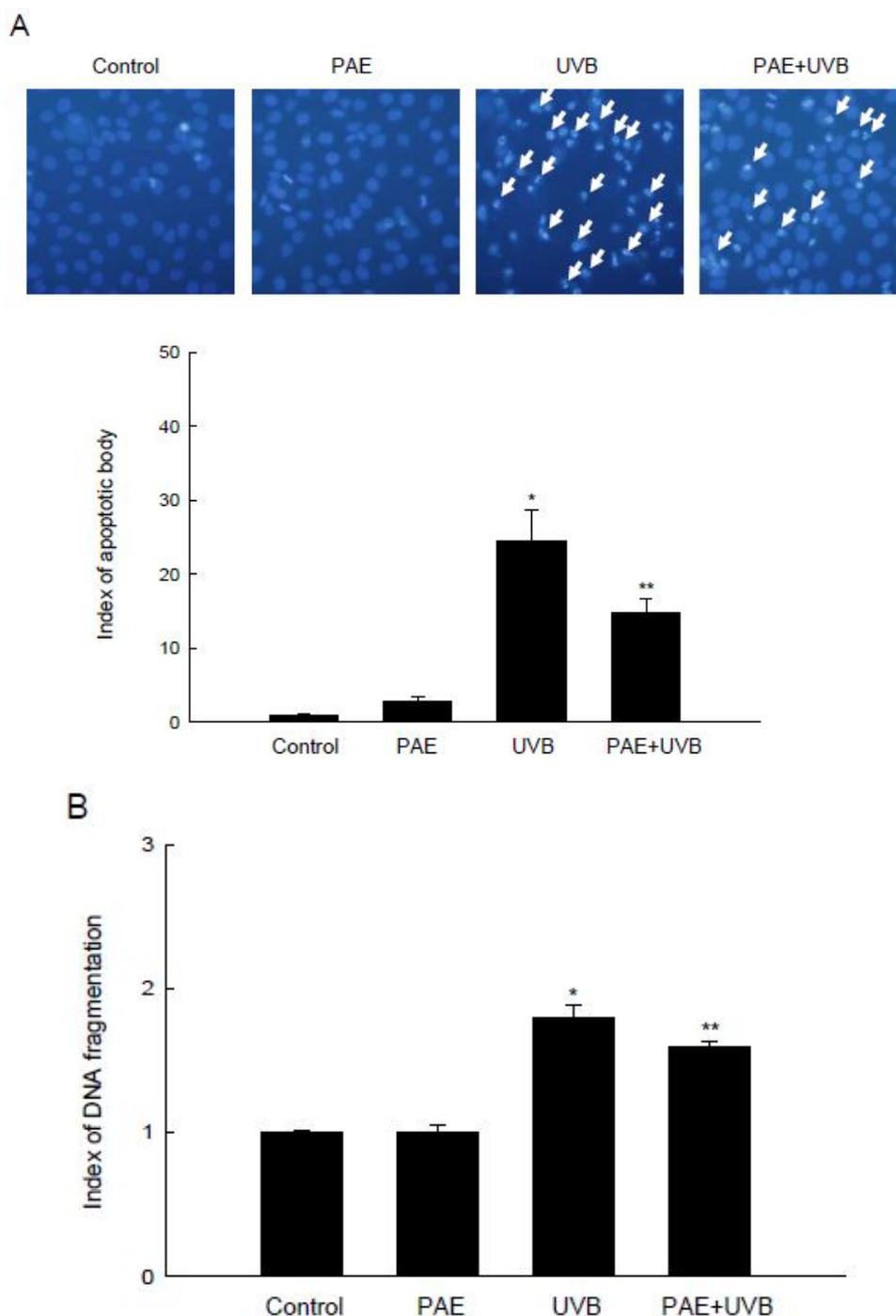


Fig 4: Effect of PAE on UVB-induced apoptosis. Cells were treated with PAE at a concentration of 100 $\mu\text{g/ml}$ and exposed to UVB radiation 1 h later. (A) Apoptotic bodies (arrows) were observed in UVB-irradiated cells stained with Hoechst 33342 dye and quantified by fluorescence microscopy. The number of apoptotic bodies was visibly reduced in UVB-irradiated cells that were pre-treated with PAE. *Significantly different from control ($p < 0.05$) and **significantly different from UVB-irradiated cells ($p < 0.05$). (B) DNA fragmentation was quantified via cytoplasmic histone-associated DNA fragmentation using a kit. *Significantly different from control ($p < 0.05$) and **significantly different from UVB-irradiated cells ($p < 0.05$)

Protective effect of PAE against UVB-induced apoptosis

Previous studies showed that UVB radiation induces apoptosis in human keratinocytes [12],

as evidenced by the presence of apoptotic bodies and DNA fragmentation. In the current study, the nuclei of untreated control and PAE-treated HaCaT cells were intact, in contrast to the significant nuclear fragmentation observed in

UVB-irradiated cells (Fig 4A). However, nuclear fragmentation was dramatically reduced when UVB-irradiated cells were pretreated with PAE, with a corresponding reduction in the apoptotic index from 24 to 15 (Fig 4A). Similarly, the cytoplasmic histone-associated DNA fragmentation index decreased from 1.8 in UVB-irradiated cells to 1.6 in cells that were pretreated with PAE prior to UVB irradiation (Fig 4B).

DISCUSSION

The chemical composition of red algae renders them useful to the food industry, as well as to various areas of medical and scientific research. Previous studies have shown that red algae exert antioxidant, anti-acetylcholine esterase, and anti-inflammatory actions [13]. Moreover, Heo *et al* [6] demonstrated that methanolic and aqueous extracts of red algae (e.g., *C. affinis*) collected on Jeju Island contain antioxidant compounds that strongly scavenge ROS, including the hydroxyl radical, the superoxide anion, H₂O₂, and DPPH.

This study is the first to show that an ethanol extract derived from the edible red algae *P. affinis* can inhibit UVB radiation-induced damage in human keratinocytes, likely due in part to its antioxidant properties. Notably, Lee *et al* [7] reported that *P. affinis* contains a number of polyphenolic compounds. Other investigators have similarly documented that brown and red algae possess an abundance of polyphenols, such as phlorotannins [14]. Phlorotannins possess antioxidant properties, in addition to anti-allergic, anti-bacterial, and anti-tumour properties [15]. Of importance to the current study, phlorotannins are also capable of absorbing UV radiation, thereby protecting cells against photodamage [16] and animals against UVB-induced skin carcinogenesis [17]. In addition, phlorotannins can inhibit melanogenesis and protect cells against photo-oxidative stress induced by UVB radiation [18]. Hence, phlorotannins are likely in part responsible for the photo-protective actions of PAE against UVB radiation.

Many studies report that UVB radiation induces ROS in epidermal keratinocytes [3]. Our study confirmed that PAE scavenged UVB-induced intracellular ROS, as well as H₂O₂-induced ROS, the superoxide anion, the hydroxyl radical, and the DPPH radical. We also demonstrated that PAE can absorb UVB photons; therefore PAE may reduce the number of UVB photons attacking cells.

UVB-generated ROS cause damage to lipid membranes, accumulation of modified protein carbonyls, and DNA strand breaks [19]. All of these processes may disrupt cellular function and contribute to apoptosis. We demonstrated the cytoprotective effects of PAE using cultured keratinocytes, which include the protection of cell membrane lipids from UVB-induced peroxidation damage and a reduction in the level of carbonylated proteins and DNA strand breaks. Furthermore, UVB radiation is a potent inducer of apoptosis, exerting its effects via ROS generation [20] in a highly complex process that involves several molecular pathways. PAE treatment suppressed UVB-induced apoptosis, as shown by a reduction in the number of apoptotic bodies, as well as DNA fragmentation.

CONCLUSION

PAE exhibits photo-protection against UVB-induced oxidative stress in human HaCaT keratinocytes by absorbing UVB rays, scavenging ROS, and attenuating injury to lipid, proteins, and DNA. Future studies will focus on identifying the active components of PAE with respect to UVB protection, and on elucidating the mechanisms of action of the active compounds.

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