

Protective effects of halophyte complex extract against UVB-induced damage in human keratinocytes and the skin of hairless mice

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Abstract. *Limonium tetragonum*, *Triglochin maritimum*, *Artemisia scoparia* and red ginseng have been used as folk remedies for treating a variety of diseases. In the current study, the protective effects of halophyte and red ginseng against ultraviolet (UV)-induced skin damage were investigated. Halophyte red ginseng complex extract (HRCE) was prepared and its effects on UV-B irradiated human keratinocytes and mouse skin were studied through ELISA, Western blotting immunofluorescence and histological staining. HRCE inhibited peroxide-induced damage in human keratinocytes. HRCE also inhibited UVB-induced collagen and elastin degradation in human keratinocytes and mouse skin. In addition, HRCE inhibited mast cell infiltration in the skin of mice irradiated with UVB light. This effect was likely due to HRCE inhibiting the activation of MAPK and NF- κ B. By protecting the skin from UVB-induced skin damage, HRCE has the potential to be used in the treatment and prevention of UV-induced skin damage and photoaging.

Introduction

Skin is the largest organ in the body. It is the first barrier that protects the body from external agents. The skin consists of a stratum corneum composed of keratinized epithelial cells, an epidermis composed of keratinocytes, and a dermis containing fibrous collagen and elastin (1). Collagen is a major matrix protein produced by fibroblasts. It is particularly rich in the skin (dermis). Collagen contributes to mechanical

firmness of the skin. It helps cell adhesion and induction of cell division and differentiation (2). Skin aging and damage are caused by UV light, genetic factors, oxidative stress, and environmental exposure. Skin aging is divided into photoaging caused by UV exposure and endogenous aging caused by physiological factors. However, UV exposure is the most common cause of skin damage and aging (3,4). UV spectrum is divided into UV-A (320-400 nm), UV-B (290-320 nm), and UV-C (290-100 nm). In particular, UV-B causes oxidative stress such as reactive oxygen species (ROS) on the skin. It can result in transient and persistent DNA damage with increased expression of aging factors such as matrix metalloproteinase (MMPs) (5,6). Increased expression of MMPs can degrade collagen in the dermis and reduce its production. Inhibited expression of MMPs is an important factor in regulating collagen metabolism and promoting collagen production (7,8). MMP-1 is called collagenase. It mainly decomposes type 1 collagen in the dermis. Expression of MMPs is initiated by the activation of the MAPK signaling pathway by ROS (9). UVB stimulation can activate phosphorylation of ERK1/2, JNK, and p38 kinase. Activated MAP kinase can promote phosphorylation of p65 and p50 proteins as important subunits of the NF- κ B transcription factor, thereby increasing the amount of transcription factors translocated into the nucleus. Activated and translocated into the nucleus, NF- κ B can promote the transcription of proteins such as MMP-1, resulting in collagen degradation (10,11).

Limonium tetragonum is a biennial plant of the Plumbaginaceae family. This plant contains active ingredients such as myricetin, myricetin glycosides, tannins, and caprolactam. It has been used in folk medicine to treat uterine bleeding, oligomenorrhea, and dysgalactia (12). *Triglochin maritimum* is a perennial plant that has been reported to have antioxidant and anti-inflammatory effects (13). *Artemisia scoparia* is a perennial plant in the Asteraceae family found mainly in India and Pakistan. Its main chemical components have been reported to be flavonoids, coumarin, ketone, and chromogen (14). *A. scoparia* has been used as a folk remedy for its antipyretic, anticholesterol, antiseptic,

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antibacterial, diuretic, and vasodilator properties (15). Red ginseng is a herbal medicine that has been used for a long time in oriental medicine. Its main chemical component is ginsenoside. It has been reported to be effective in alleviating diseases related to oxidative stress (16). The purpose of this study was to investigate inhibitory effects of *L. tetragonum*, *T. maritimum*, *A. scoparia*, and red ginseng complex against UVB-induced photoaging and the mechanism of action involved in such effects.

Materials and methods

Materials. Dulbecco's modified Eagle medium (DMEM) and fetal bovine serum were purchased from Gibco; Thermo Fisher Scientific, Inc. Penicillin/streptomycin antibiotics came from Invitrogen; Thermo Fisher Scientific, Inc. EZ-Cytox reagent and EZ-western Lumi Pico Alpha were obtained from DoGenBio. Protease inhibitors, tert-butyl hydroperoxide (tBHP), L-ascorbic acid, and o-toluidine blue were purchased from Sigma-Aldrich. Radio-immunoprecipitation assay buffer (RIPA buffer) was purchased from Thermo Fisher Scientific, Inc. ELISA Kit for Collagen Type I was purchased from Cloud-Clone Corp.. Collagen, elastin, MMP-1, MMP-9, JNK, p-JNK, ERK, p-ERK, p38, p-p38, NF- κ B, P-NF- κ B, and HRP conjugated secondary antibody (Santa Cruz Biotechnology, Inc.) and actin antibody (Biosciences) were also used in this study.

Plant material and extract. *Limonium tetragonum* used in the experiment was collected from Sinsido. *Artemisia scoparia* was collected from Sohwangasagu. *Triglochin maritimum* *Linnaeus* was collected from Simwon-myeon. Plants were identified by Dr Ahn Jin-Gap. Red ginseng root was purchased from Jinandang Farming Association Corporation. Halophyte red ginseng complex extract (HRCE) was produced from raw materials of complex *Limonium tetragonum* 2: *Artemisia scoparia* 1: *Triglochin maritimum* 1: Red ginseng 2, and ethanol 50% with the extraction ratio of 20:1. HRCE was filtered using a 0.45- μ m filter, concentrated with a rotary pressure reducer, dried with a freeze dryer, and stored at -20°C .

Cell culture. Human keratinocyte (HaCaT) cell line was purchased from CLS Cell Lines Service GmbH. These cells were cultured and maintained in DMEM media supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin in a 5% CO_2 incubator at 37°C .

Animal and experimental design. Male hairless mice at five weeks of age were purchased from Orient Bio Inc. These mice were housed in an air-conditioned room with temperature of $22\pm 2^{\circ}\text{C}$, humidity of 50-60%, and a 12/12 h day/night cycle. These mice were given a commercial-standard laboratory diet and water at will. All procedures were performed in compliance with guiding principles for animal care and use committee of Jeonju University Institutional Animal Care and Used Committee guidelines (approved no. JJU-IACUC-2018-5). Animals were adapted to the laboratory environment for one week prior to the experimentation. The number of mice in each experimental group was five. HRCE (200 mg/kg), HRCE

(100 mg/kg) + L-ascorbic acid (AA) (25 mg/kg), and L-ascorbic acid (AA) (50 mg/kg) were dissolved in saline and oral administered at one week before UVB irradiation and continued until the termination of the experiment. The UVB-irradiated control group was administered with saline. Irradiated groups received saline, HRCE, or AA. Dorsal skin area of mice was exposed to UVB radiation from LF-215.M lamp (emission peak at 312 nm; Uvitec). Using an electronic controller, UVB dosage at a fixed distance from lamps to the dorsal skin surface of mice was regulated to be $300\text{ mJ}/\text{cm}^2$. The exposure time was 3 min thrice a week for two weeks. At the end of the experiment, the mice were euthanized through cervical dislocation. The dorsal dermis was collected and stored at -80°C for western blotting and fixed in 4% paraformaldehyde for histological analysis.

Cell viability. Cell viability assay was measured using EZ-Cytox reagent. HaCaT cells (1×10^5 cells/ml) were seeded into 96-well plates and incubated for 24 h. These cells were then pretreated with 100 $\mu\text{g}/\text{ml}$ of HRCE or 50 $\mu\text{g}/\text{ml}$ of L-ascorbic acid (AA) for 1 h and subsequently stimulated with 400 μM of tBHP for 16 h. After 16 h, 10 μl of EZ-Cytox reagent was added to each well. Cells were then incubated for 4 h. The absorbance of each well was then measured at 450 nm with a microplate reader (Tecan). The concentration of tBHP treatment was determined in previous experiments. HaCaT cells were treated with various concentrations of tBHP for 16 h, and the survival rate was $\sim 60\%$ compared to the negative control at 400 μM concentration, which was determined to be a suitable concentration for the experiment.

ELISA assay. Culture supernatants of HaCaT cells treated with or without HRCE or AA for 48 h after UVB radiation were used to measure concentrations of Type I collagen. The protocol used was in accordance with the outlined protocol of the manufacturer of the Kit without modification.

Immunofluorescence staining. In cell culture slide chambers, HaCaT cells were pretreated with or without HRCE or AA for 24 h and then stimulated with or without 20 mJ/cm² of UVB irradiation. These cells were fixed and permeabilized by 100% ice-cold methanol for 10 min at -20°C . Cells on slides were blocked with 1% BSA for 1 h at room temperature and incubated with collagen antibodies overnight at 4°C . These cells were then washed with PBS and further incubated with goat anti-mouse IgG, (H + L) Alexa Fluor™ plus 488 conjugated secondary antibodies for 1 h. Slides were washed with PBS and mounted with DAP mounting medium. Visualization was under a Zeiss fluorescence Microscope (Zeiss Co.).

Protein extraction. HaCaT cells (2×10^5 cells/ml) were pretreated with HRCE or AA for 1 h and then treated with UVB irradiation (20 mJ/cm²). These cells were incubated for 15 min or 1 h, washed with ice-cold PBS, and centrifuged at 2,000 rpm at 4°C for 2 min. The supernatant was discarded and cell pellets were suspended in 0.1 ml of ice-cold RIPA buffer. Tubes were vortexed and incubated on ice for 15 min with gentle shaking. After incubation, tubes were centrifuged at 12,000 rpm for 15 min at 4°C to pellet cell debris. The

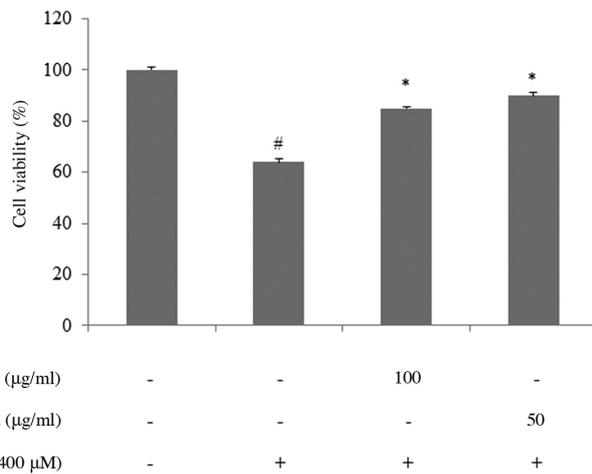


Figure 1. Effect of HRCE on cell viability in tBHP-stimulated HaCaT cells. HaCaT cells were pretreated with HRCE or L-ascorbic acid (AA) and subsequently stimulated with tBHP. The cells were then incubated with EZ-Cytox reagent, after which cell viability was assessed. Data are presented as the mean \pm SD. [#]P<0.05 vs. untreated cells; ^{*}P<0.05 vs. tBHP treated cells. HRCE, halophyte red ginseng complex extract; tBHP, tert-butyl hydroperoxide; AA, L-ascorbic acid.

supernatant (protein lysates) was transferred into new tubes and stored at -80°C for subsequent use.

For protein extraction from tissue samples, at the end of the UVB irradiation in animal experiment described above, the dorsal dermis was removed and 0.2 g of the tissue from each mouse was grounded in liquid nitrogen and placed in 0.2 ml of RIPA buffer. Tissues were then incubated on ice for 5 min before centrifugation at 12,000 rpm for 15 min at 4°C . The supernatant was transferred into new tubes and stored at -80°C for subsequent use.

Western blot analysis. Proteins (45 μg) were separated on 7.5% SDS-PAGE gels and transferred onto PVDF membranes. After blocking with 5% BSA prepared with 1% Tween-20 in 20 mmol/l TBS (pH 7.4), membranes were then incubated overnight with specific primary antibodies to be assessed. These membranes were washed and incubated with appropriate horseradish peroxidase-conjugated secondary antibody. Protein expression was detected and visualized using a chemiluminescence detection system. The density of each band in an immunoblot was analyzed using ImageJ gel analysis software developed by the National Institutes of Health.

Histopathological examination. Dorsal skin tissues were fixed in 10% neutral buffered formalin, washed with 1X PBS five times, dehydrated in graduated ethanol, cleared in xylene, embedded in paraffin, and sectioned at 5 μm in thickness. Sections were then stained with H&E for measuring epidermal thickness, trichrome for collagen fiber analysis, and toluidine blue for mast cell quantification. All staining procedures were done using their respective protocols with little or no modifications. Histopathological changes were examined under a light microscope (Leica). Transepidermal water loss (TEWL) was expressed as an average value after five repeated measurements using a gpskin barrier (Amicogen).

Statistical analysis. Data are presented as mean \pm SD. Statistically significant differences among groups were determined by one-way analysis of variance (ANOVA) followed by Tukey's test. Statistically significant difference was considered at $P<0.05$.

Results and Discussion

HRCE recovers tBHP-induced cell damage. Exposure of UVB can lead to over-production of ROS on the skin, resulting in oxidative stress. Increased intracellular ROS can cause skin diseases, including photoaging, inflammation, and carcinogenesis (17). Therefore, protecting the skin against oxidative stress might be a strategy to prevent UVB-related skin damage. The cytoprotective effect of HRCE in tBHP-stimulated HaCaT cells was investigated. As shown in Fig. 1, stimulated cells without HRCE treatment showed significantly lower survival rates than unstimulated cells. However, the survival rate was significantly higher when cells were treated with 100 $\mu\text{g/ml}$ of HRCE or 50 $\mu\text{g/ml}$ of AA. This implies that HRCE and AA can protect skin against damaging effects of peroxide.

HRCE protects collagen and elastin from cell damage caused by UVB. In the skin, collagen and elastin are important for maintaining elasticity, strength, and structure. A decrease in collagen and elastin expression has been observed in photoaged skin (18,19). Therefore, the effect of HRCE on the expression of collagen and elastin in cell damage induced by UVB was investigated. As shown in Fig. 2A, the amount of collagen released into the culture medium of UVB-stimulated cells without HRCE treatment was significantly reduced compared to that in the unstimulated experimental group. Results of immunofluorescence staining (Fig. 2B) also confirmed that collagen density was decreased in cells stimulated by UVB. However, HRCE significantly increased collagen synthesis, which was reduced by UVB. Intracellular collagen and elastin protein expression were examined by western blot analysis (Fig. 2C). Results showed that HRCE recovered UVB-induced collagen and elastin degradation. AA can act as a photoprotective agent. It can stimulate collagen synthesis, protect against damage caused by UVB radiation, and relieve inflammation in the skin (20). In this study, AA also prevented collagen loss. Moreover, HRCE showed similar or slightly less inhibitory effects on collagen and elastin degradation than AA. In general, the reduction of collagen and elastin in the skin is driven by the expression of matrix metalloproteinases (MMP) (21). For this reason, the expression of MMPs was also investigated in this study.

HRCE suppress the expression of MMPs. Exposure to UV can increase the expression of matrix metalloproteinases (MMP) in human skin. MMPs can degrade extracellular matrix (ECM) such as collagen, fibronectin, and elastin and cause photoaging (21,22). The effect of HRCE on protein expression of MMPs called collagenase was investigated. As shown in Fig. 3, HRCE suppressed the expression of MMP-1 and MMP-9 that was increased by UVB. In particular, inhibition of HRCE on MMP-9 expression was superior to that of AA, the reference compound. According

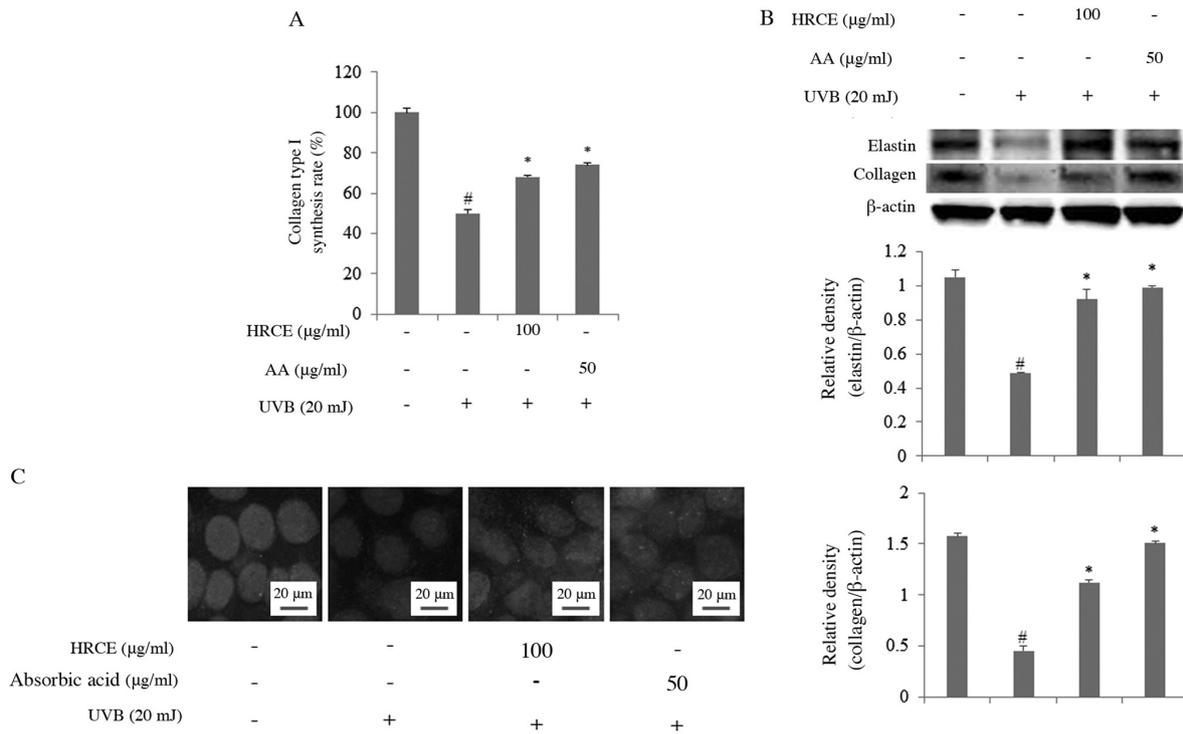


Figure 2. Effects of HRCE on UVB-induced collagen degradation in HaCaT cells. HaCaT cells were pretreated with HRCE or AA and then irradiated with UVB rays. (A) The release of collagen into the culture medium was investigated using an ELISA kit. The expression levels of (B) collagen and elastin were assessed by performing western blotting using total protein extract. Collagen density in the cell structure was examined by (C) immunofluorescence staining. Data are presented as the mean \pm SD. [#] $P < 0.05$ vs. untreated cells; ^{*} $P < 0.05$ vs. UVB treated cells. HRCE, halophyte red ginseng complex extract; UVB, ultraviolet B; AA, L-ascorbic acid.

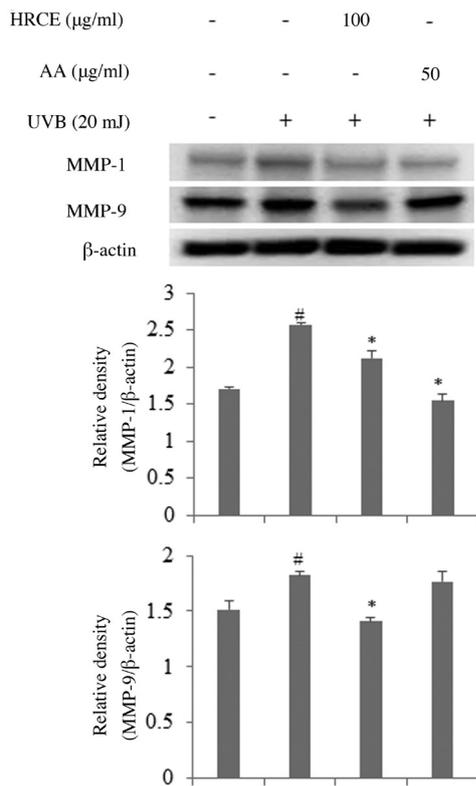


Figure 3. Effects of HRCE on MMP-1 and MMP-9 expression in UVB-irradiated HaCaT cells. HaCaT cells were pretreated with HRCE or AA and then irradiated by UVB rays. Expression levels of MMP-1 and MMP-9 were investigated via western blot analysis using total protein extract. Data are presented as the mean \pm SD. [#] $P < 0.05$ vs. untreated cells; ^{*} $P < 0.05$ vs. UVB treated cells. HRCE, halophyte red ginseng complex extract; UVB, ultraviolet B; AA, L-ascorbic acid.

to previous studies, MMP-1 degrades Collagen types I and III while MMP-9 degrades Collagen type IV and elastin (22,23). Therefore, HRCE has collagen and elastin protective effect by suppressing the expression of MMP-1 and MMP-9.

HRCE suppresses phosphorylation of Mitogen-activated protein kinase (MAPK) proteins. Next, pathways stimulated by irradiation with UVB were investigated. Fig. 4A shows the degree of phosphorylation of MAPKs in cells exposed to UVB. Results showed that HRCE had no inhibitory effect on phosphorylation of c-Jun N-terminal kinases (JNK) or p38 mitogen-activated protein kinases (p38). However, the phosphorylation inhibitory effect of extracellular signal-regulated kinases (ERK) was significantly evident. As HRCE inhibited the phosphorylation of ERK, the activation of NF- κ B transcription factor by HRCE was then investigated. As a result (Fig. 4B), it was confirmed that the phosphorylation of NF- κ B transcription factor of HaCaT cells exposed to UVB was significantly inhibited by HRCE. According to previous studies, when the skin is exposed to UV, ROS overproduction occurs, causing MAPK (ERK, JNK, and p38) phosphorylation and NF- κ B activation, which then promotes the expression of MMPs genes and proteins, causing collagen degradation and inflammation (24,25). Thus, the cell protective effect from UVB of HRCE might be due to the inhibition of ERK and NF- κ B signaling pathway.

HRCE prevents UVB-induced skin damage and morphological changes. Because HRCE was confirmed to have preventative

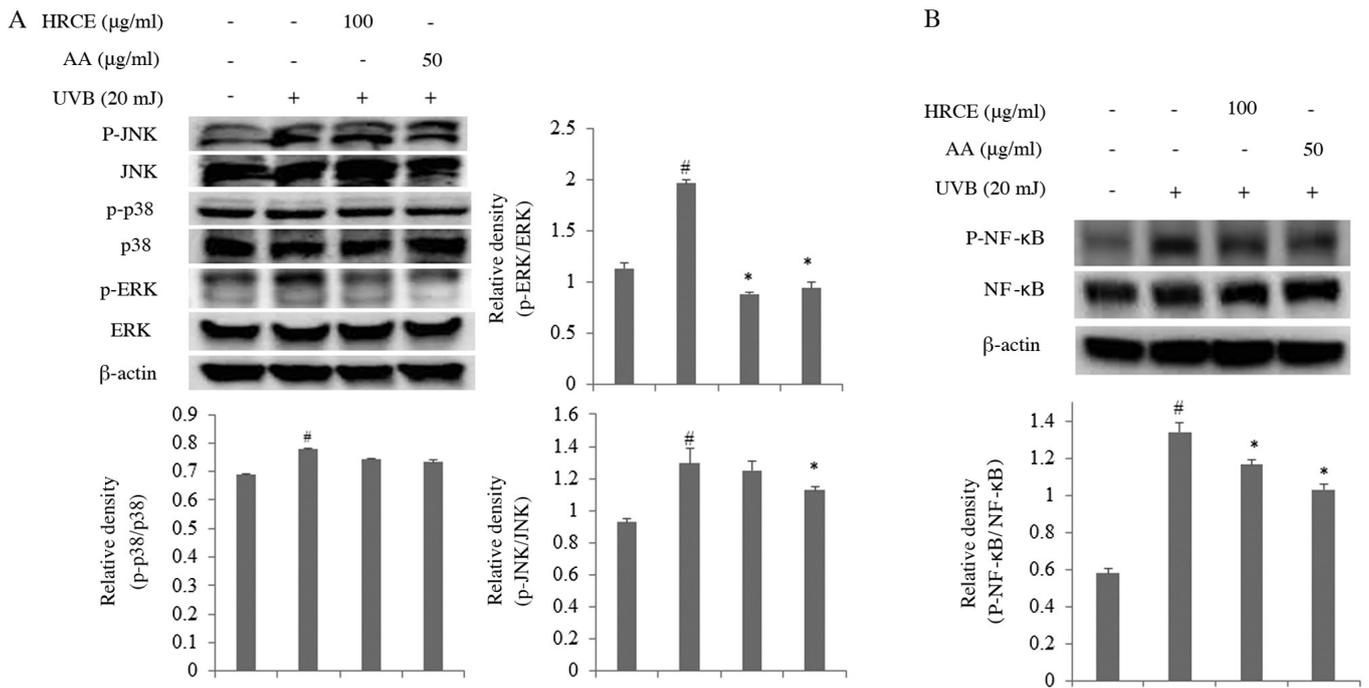


Figure 4. Effects of HRCE on the activation of (A) MAPK and (B) NF-κB. HaCaT cells were pretreated with HRCE or AA and irradiated by UVB rays. Expression levels of MAPK and NF-κB were investigated via western blot analysis using total protein extract. Data are presented as the mean ± SD. #P<0.05 vs. untreated cells; *P<0.05 vs. UVB treated cells. HRCE, halophyte red ginseng complex extract; UVB, ultraviolet B; AA, L-ascorbic acid; p, phosphorylated.

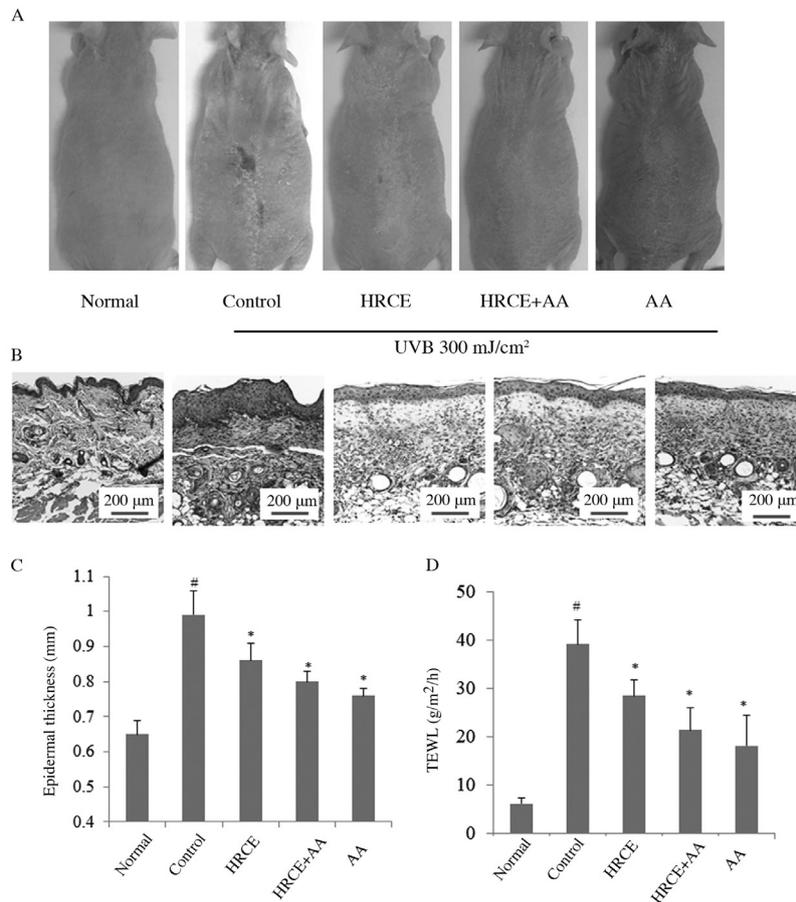


Figure 5. Effects of HRCE on UVB-induced edema and TEWL of mouse skin. Mice were irradiated with UVB three times a week for a total of 2 weeks. HRCE and AA were orally administered for 2 weeks, except for the control group. (A) The morphological changes of mice in different groups are presented. (B) Representative hematoxylin & eosin staining demonstrated epidermal thickness. (C) Epidermal thickness was subsequently measured for each group. (D) TEWL was measured. Data are presented as the mean ± SD. #P<0.05 vs. normal group; *P<0.05 vs. control group. HRCE, halophyte red ginseng complex extract; UVB, ultraviolet B; TEWL, transepidermal water loss; AA, L-ascorbic acid.

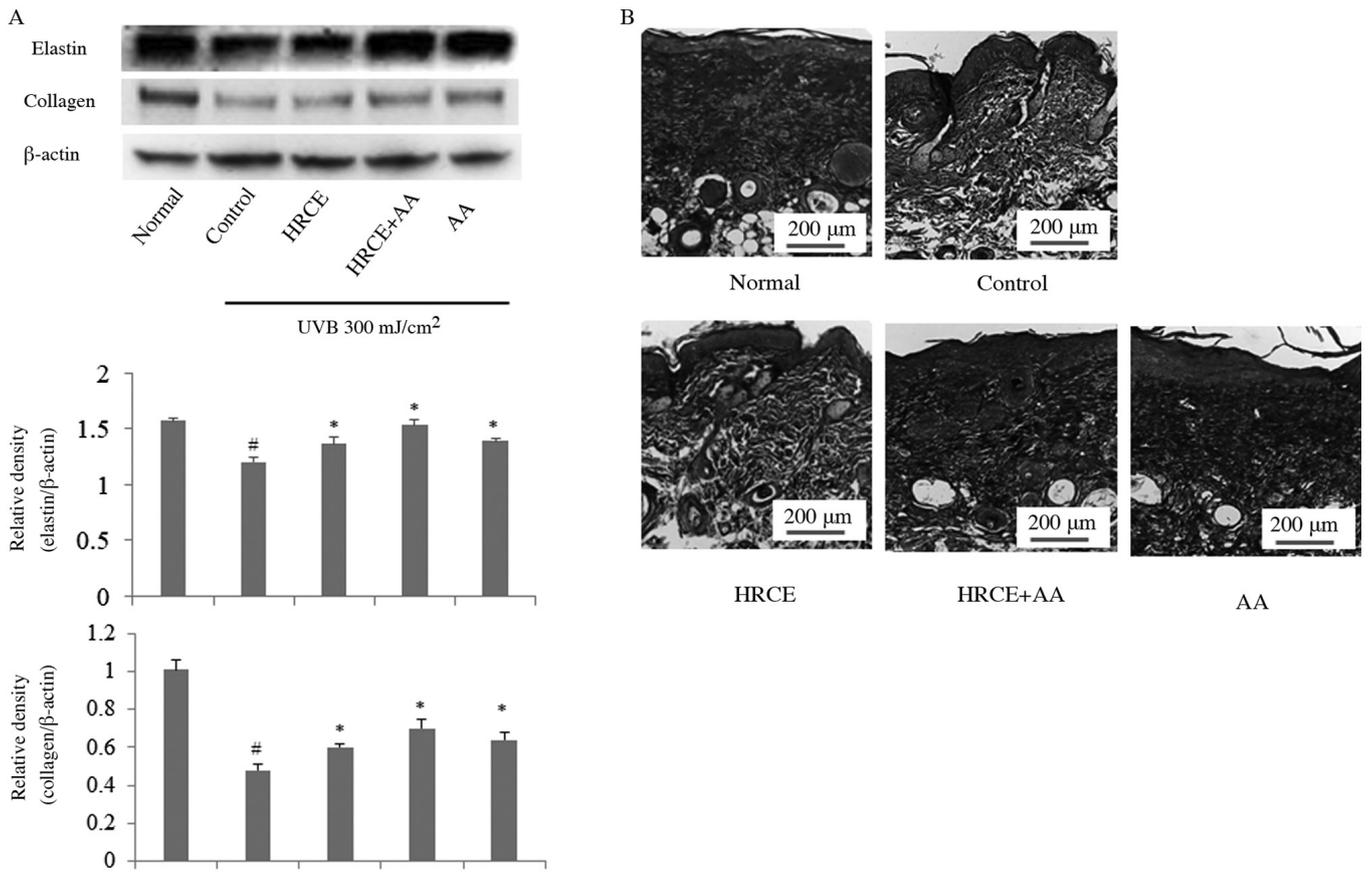


Figure 6. Effects of HRCE on UVB-induced collagen degradation in mouse skin. Mice were irradiated with UVB three times a week for a total of 2 weeks. HRCE and AA were orally administered for 2 weeks, except for the control group. (A) Expression levels of collagen and elastin are investigated by western blot analysis using skin protein extract. (B) Representative trichrome stained tissue of five mice for collagen fiber visualization is presented. Data are presented as the mean \pm SD. [#]P<0.05 vs. normal group; ^{*}P<0.05 vs. control group. HRCE, halophyte red ginseng complex extract; UVB, ultraviolet B; AA, L-ascorbic acid.

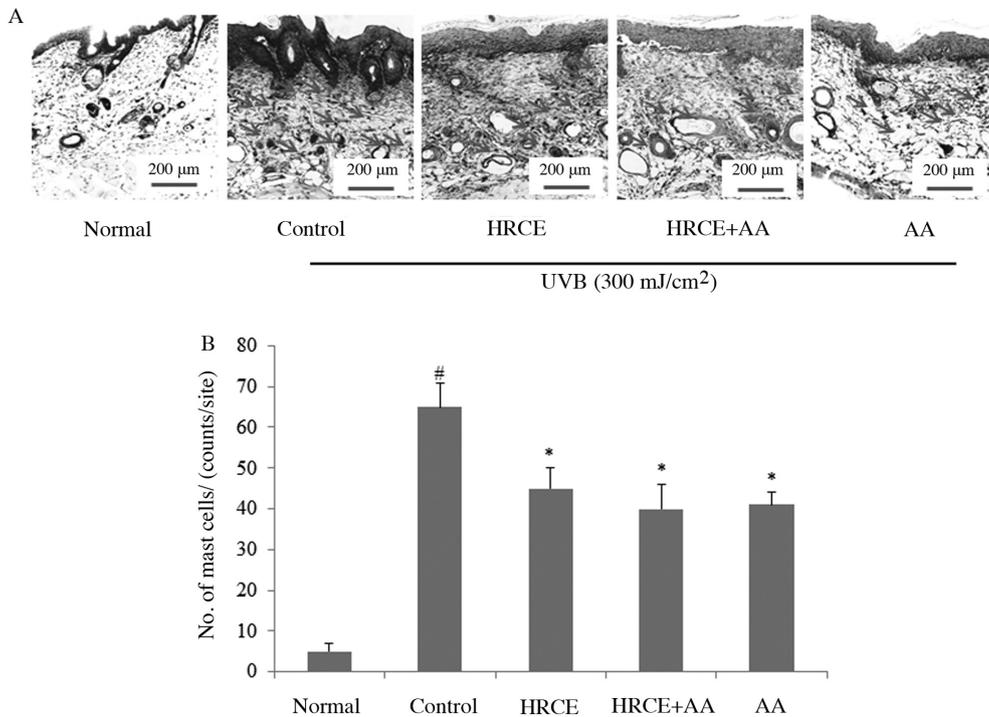


Figure 7. Effects of HRCE on the UVB-induced infiltration of mast cells in mouse skin. Mice were irradiated with UVB. HRCE and AA were orally administered for 2 weeks, except for the control group. (A) Representative images of toluidine blue staining indicating the mast cells from five mice. (B) The number of mast cells from five murine tissue sections was counted under a microscope in a blinded manner. Data are presented as the mean \pm SD. [#]P<0.05 vs. normal group; ^{*}P<0.05 vs. control group. HRCE, halophyte red ginseng complex extract; UVB, ultraviolet B; AA, L-ascorbic acid.

effect against photoaging and skin damage at the cell level, the efficacy of HRCE was then evaluated in *in vivo* studies. Improvement of clinical signs and symptoms of UV-induced skin damage by HRCE was evaluated by measuring epidermal thickness and transepidermal water loss (TEWL). Results revealed that repeated irradiation with UVB (300 mJ/cm²) caused skin edema and dryness (Fig. 5A). As shown in Fig. 5B and C, skin thickness values of UVB irradiated mice were increased. However, when mice were treated with HRCE or AA, skin thickness was reduced. TEWL values of mice in the HRCE group were significantly reduced (Fig. 5D). TEWL is a measure of the function of the stratum corneum. A healthy stratum corneum layer can prevent foreign substances from penetrating the skin. It can also prevent moisture loss (26). The reduction of TEWL with HRCE administration suggests that HRCE can maintain the function of the stratum corneum after UV damage.

HRCE prevents UVB-induced collagen and elastin degradation. Collagen can maintain skin's strength and elasticity along with keratin formation (27). Elastin is a major protein in the extracellular matrix that helps the skin to return to its original shape when the skin is subjected to physical pressure (28). Collagen and elastin are main targets in studies about UV-induced skin damage. Western blot and trichrome staining were performed to investigate the inhibitory effect of HRCE on collagen degradation. According to Fig. 6A, collagen and elastin of mice decomposed by UVB irradiation were recovered by administration of HRCE. Trichrome staining showed that collagen density increased when mice were administered with HRCE (Fig. 6B). Through animal experiments, the improvement effect of HRCE on UV-induced collagen and elastin degradation was consistent with results from cell experiments.

HRCE prevents infiltration of mast cells to the skin. It is known that infiltration of mast cells occurs in skin after UVB exposure (29). Toluidine blue staining showed that mast cells infiltrated the skin after UVB irradiation (Fig. 7A and B). However, the infiltration of mast cells was significantly reduced in skin of mice of the HRCE-administered group and the AA-administered group. Previous studies have reported that mast cells increased by UV in the skin might increase the risk of developing basal cell carcinoma (30). These effects are thought to be due to direct suppression of mast cell invasion or the reduction of oxidative stress by HRCE. These results suggest that HRCE can be used not only to protect collagen and elastin degradation by UVB, but also to prevent skin inflammation and basal cell carcinoma. Therefore, it is considered that there is a need for research on the effects of HRCE on inflammatory cytokines and inflammatory reactions caused by UV rays.

In conclusions, to the best of our knowledge, this is the first study to reveal that HRCE can prevent degradation of collagen and elastin caused by UV exposure using HaCat cells and hairless mice. It is thought that HRCE can inhibit the expression of MMPs by blocking the activation of MAPK and NFκB signaling pathways at the cellular level. In addition, the collagen protective effect of HRCE in cell experiments was consistent with results from animal experiments. Therefore,

HRCE is a potential health functional material that can prevent skin damage caused by UV rays such as skin aging, wrinkles, blemishes, and freckles. It has possible application in the food and cosmetics industry.

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Availability of data and materials

The datasets used and/or analyzed data during the study are available from the corresponding author on reasonable request.

Authors' contributions

JYS and SIJ designed the research. JHP, JYS, DNC, HJK and YTL performed the experiments. JYS, BOC and SIJ analyzed the data. SIJ and JYS wrote the manuscript draft. BOC, SIJ and JYS reviewed and edited the final manuscript. SIJ managed the research project. JYS and SIJ confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Mice were handled and experiments were carried out based on Jeonju University Institutional Animal Care and Use Committee guidelines with permission to carry out the experiment obtained from Jeonju University (approval no. JJU-IAC UC-2018-5).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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