Scopoletin downregulates MMP-1 expression in human fibroblasts via inhibition of p38 phosphorylation

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Abstract. Irradiation of keratinocytes by ultraviolet B induces cytokine production, which in turn activates fibroblasts to produce cytokines and increase matrix metallopeptidase (MMP)-1 protein expression. The present study investigated the effect and potential mechanisms of scopoletin on the regulation of MMP-1 expression in fibroblasts. Scopoletin was isolated from *Artemisia capillaris* crude extract. Treatment of fibroblasts with scopoletin resulted in a decrease in the protein expression of MMP-1 following stimulation with human keratinocyte (HaCaT) conditioned medium. To further explore the mechanism underlying this effect, the expression levels of proteins in the mitogen-activated protein kinase (MAPK) and nuclear factor-κB (NF-κB) signaling pathways were evaluated via western blot analysis. The mRNA expression levels of interleukin (IL)-1α and tumor necrosis factor (TNF) α were evaluated via reverse transcription-quantitative polymerase chain reaction. The effect of scopoletin on cell viability was assessed with the MTT assay. The results demonstrated that scopoletin treatment markedly decreased MMP-1, IL-1α and TNFα mRNA expression in fibroblasts stimulated with HaCaT conditioned medium (40 mJ/cm^2), without any apparent cell cytotoxicity, and in a dose-dependent manner. In addition, western blot analysis demonstrated that scopoletin reduced the phosphorylation of p38 MAPK in fibroblasts. In summary, the present study demonstrated that scopoletin inhibited MMP-1 and proinflammatory cytokine expression by inhibiting p38 MAPK phosphorylation. These findings suggest that scopoletin may have potential as a therapeutic agent to prevent and treat photoaging of the skin.

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Introduction

Ultraviolet rays can be classified into three types according to wavelength: Ultraviolet A (UVA; 320-380 nm), ultraviolet B (UVB; 280-320 nm), and ultraviolet C (UVC; 100-280 nm) (1). UVC is mostly absorbed by the ozone layer, and UVA and UVB reach the surface of the Earth. Although a small amount of UVB reaches the surface of the Earth, UVB is 500-800 times more harmful than UVA (2). Nowadays, UVB rays reaching the earth's surface are the predominant risk factor causing skin photoaging and disease, such as immune suppression and cancerization (3).

UVB destroys keratinocyte cells on the outer layer of the skin (epidermis) without penetrating the skin (4). Damaged keratinocyte cells secrete proinflammatory cytokines, including interleukin (IL)-1α, IL-1β, IL-6, IL-8, and tumor necrosis factor (TNF) α (5-9). There is evidence that UVB-irradiated keratinocytes induce TNFα and TNFα-dependent pathway. In specific, IL-1α induces a synergistic induction of TNFα in keratinocytes and fibroblasts (10,11). These types of proinflammatory cytokines activate the mitogen-activated protein kinase (MAPK) and nuclear factor-κB (NF-κB) pathways in fibroblasts on the dermis (12), a deeper layer of skin (13). The activation of MAPK cascades, such as extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 kinase phosphorylation, which in turn regulate activator protein-1 (AP-1), increases matrix metallopeptidase (MMP)-1 production (14,15). The activation of p38 MAPK leads to the induction of multiple proteins that are key to the inflammatory process, including a further induction of cytokine secretion. p38 MAPK signaling has a pivotal role in regulating the production of proinflammatory cytokines, such as TNFα (16). Inhibitor κB kinase (IKK) is activated by proinflammatory cytokines and then phosphorylates IκB and leads to its degradation. In addition, NF-κB enhances MMP-1 production and increases the gene expression levels

of proinflammatory cytokines by translocating into the nucleus (17). Consequently, these pathways result in skin wrinkles by promoting the synthesis of MMP-1 in fibroblasts, which degrade collagen (18). For these reasons, herbal products have been investigated as candidates for anti-aging agents, as a means to regulate the production of MMP-1 without toxicity.

Previous studies have reported the effects of *Artemisia capillaris* regarding hepatitis, obesity, inflammation, antimicrobial activity, antioxidant effects, hemostasis, pyrexia, hypertension, cytoprotection, and choleretic action (19-22). Several compounds have been isolated from *A. capillaris*, including coumarin derivatives such as esculetin, scoparone, and scopoletin (23), and flavonoid derivatives such as quercetin (24), hyperoside (25), isorhamnetin (26), and isoquercitrin (27). Scopoletin (7-hydroxy-6-methoxychromen-2-one) is naturally derived from coumarin and phytoalexin (28). Scopoletin has been reported to inhibit acetylcholinesterase (29), to have antioxidant properties (30) and anti-inflammatory effects (31), and to reduce insulin resistance (32). However, no study has investigated the effects and related mechanisms for *A. capillaris* ethanol extract (ACE) with the active compound, scopoletin, in fibroblasts. The present study evaluated the inhibition of MMP-1 protein expression and the underlying mechanisms for scopoletin in fibroblasts treated with conditioned medium from UVB-exposed-HaCaT cells.

Materials and methods

Chemicals and antibodies. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Anti-MMP-1 (cat. no. ab52631, 1:1,000) antibody was purchased from Abcam (Cambridge, UK). ERK1/2 (cat. no. 4377; 1:1,000), phosphorylated (p-) ERK1/2 (cat. no. 9101; 1:1,000), stress-activated protein kinase (SAPK)/JNK (cat. no. 9252; 1:1,000), p-SAPK/JNK (cat. no. 9251; 1:1,000), p38 MAPK (cat. no. 8690; 1:1,000), p-p38 MAPK (cat. no. 9215; 1:1,000), IκBα (cat. no. 2859; 1:1,000), p-IκBα (cat. no. 2078; 1:1,000), NF-κB p65 (cat. no. 9609; 1:1,000), p-NF-κB p65 (cat. no. 4887; 1:1,000), and β-actin (cat. no. 4967; 1:1,000) antibodies were obtained from Cell Signaling Technology, Inc., (Danvers, MA, USA). p38 inhibitor (cat. no. SB203580, 1:1,000) was purchased from Calbiochem (Merck KGaA).

Cell culture. HaCaT human keratinocytes were provided by Professor Moon Je Cho (Department of Biochemistry, National University, Cheju, Korea). Fibroblasts as human primary dermal cells were purchased from American Type Culture Collection (Manassas, VA, USA; cat. no. PCS-201-012TM). HaCaT cells and fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM; Hyclone; GE Healthcare Lifesciences, Logan, UT, USA) with 10% fetal bovine serum (FBS; PEAK, Colorado, USA) and 1% penicillin/streptomycin (10,000 U/100 μg/ml; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) in a 5% CO_2 humidified atmosphere incubator at 37˚C. HaCaT cells were maintained until 80% confluence and then cultured for 24 h in medium without FBS. The cell medium was then replaced with 5 ml PBS and the cells were exposed to UVB light. The UVB doses were determined by irradiating the HaCaT cells with various doses of UVB (0, 20, 40, 60, 80 and 100 mJ/cm^2) and optimizing the UVB light as 40 mJ/cm^2. Cells were cultured in DMEM medium containing 10% FBS. At 24 h post-irradiation, HaCaT-conditioned medium was collected and added on the fibroblasts. After 24 h incubation, the culture medium was collected. Fibroblasts were treated with various concentrations of scopoletin for 24 h in HaCaT-conditioned medium. Vehicle control was serum-free medium-treated fibroblasts.

To determine the effects of scopoletin on the NF-κB signaling pathway, fibroblasts were pretreated with scopoletin for 6 h, and then treated with HaCaT-conditioned medium (40 mJ/cm^2) containing scopoletin (0, 30, 100 and 300 μM) for 15 min prior to western blot analyses. When examining the signaling pathway activation in fibroblasts, generally the phosphorylation reaction time is short, therefore pretreatment with scopoletin was performed in order to provide the required time to act on the fibroblasts.

Isolation of active compound and structure determination. A. capillaris was purchased from Hwasun-bul-minari Company (Hwasun, Korea). Dried *A. capillaris* (1,475 g) was extracted with 100% ethanol for 3 days at room temperature. The filtered extract was concentrated with a vacuum evaporator (EYELA Rotary evaporator, Tokyo, Japan) and was freeze-dried. The ethanol (EtOH) extract of *A. capillaris* (71.343 g) was dissolved in H_2O, and extracted with ethyl acetate (EtOAc). The EtOAc layer (38.56 g) was evaporated to dryness under vacuum and partitioned with 90% methanol (MeOH) and n-Hexane. The 90% MeOH layer (24.19 g) was fractionated by Waters MPLC system (Waters, Milford, MA, USA) using a YMC-DispoPackAT (SIL-25; 40 g) eluted with EtOAc and n-Hexane mixture in a gradient mode (EtOAc:n-Hexane, 2:8 to 10:0 in 60 min). The flow rate was 30 ml/min and the elution was monitored at UV 254 nm. The MMP-1 expression levels were evaluated in order to determine the effects of the fraction layers on HaCaT-conditioned medium-treated fibroblasts. Among the nine fractions eluted, the fraction with inhibitory activity on MMP-1 protein expression was further purified using a Waters prep-HPLC system using a YMC ODS A column (YMC-pack ODS-A; 5 μm, 20x250 mm). The column was eluted with 40% MeOH containing 0.2 mM ammonium acetate at a flow rate of 10 ml/min. The compounds were monitored by ultraviolet absorbance at 254 nm. The scopoletin structure was confirmed via nuclear magnetic resonance (NMR, 1D, 2D). ^{1}H- and ^{13}C-NMR spectra were obtained on an Advance DPX 500 MHz NMR spectrometer (Bruker Corporation, Billerica, MA, USA), recorded in a deuterated chloroform (CDCl3) solution (33). Scopoletin was dissolved in dimethyl sulfoxide (DMSO) and then diluted in serum-free medium for *in vitro* studies.

Cell viability. Cell viability was determined by MTT assay. Fibroblasts were seeded at 1.5x10^4/well in a 24-well plate. After 24 h of incubation, the cell medium was replaced with serum-free medium and incubated for an additional 24 h. The cells were treated with scopoletin for 24 h in serum-free medium. MTT solution (5 mg/ml) was added to each of the wells, and the cells were incubated for 3 h at 37˚C. The supernatants were removed, and DMSO was added to dissolve

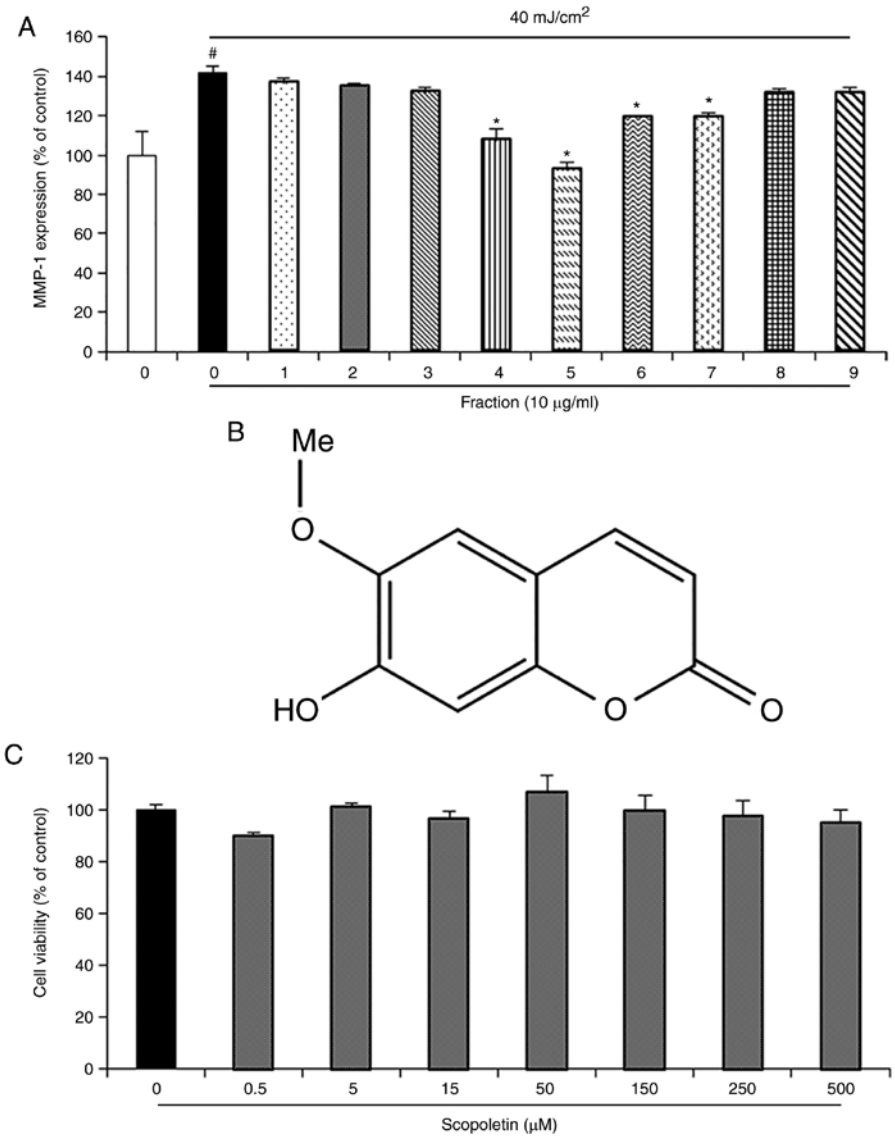

Figure 1. Isolation of the active substance from the ACE and cell viability of scopoletin-treated fibroblasts. (A) Effect of 9 fraction layers from ACE on MMP-1 protein expression levels. (B) Chemical structure of scopoletin. (C) Cell viability of fibroblasts treated with various concentrations (0, 0.5, 5, 15, 50, 150, 250 and 500 μM) of scopoletin for 24 h, was assessed MTT assay. Results are presented as the mean ± standard deviation of triplicate independent experiments. $^{\#}$P<0.05 compared with the vehicle control; *P<0.05 compared with the HaCaT-conditioned medium (40 mJ/cm^2) alone-treated control. ACE, *Artemisia capillaris* ethanol extract; MMP-1, metallopeptidase-1.

the formazan crystals. Absorbance at 570 nm was measured using an ELISA plate reader (Tecan group Ltd., Mannedorf, Switzerland).

Western blot analysis. Fibroblasts were lysed in RIPA buffer (Sigma-Aldrich, Merck KGaA) containing protease inhibitors and phosphatase inhibitors. The lysates were centrifuged at 3,000 x g for 15 min at 4˚C, and the protein concentrations were determined using a BCA assay. The proteins (40 μg) were separated by 10% SDS-PAGE and transferred to poly-vinylidene fluoride membranes (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The membranes were initially blocked with 5% skimmed milk in Tris-buffered saline containing 0.1% Tween-20 (TBS-T) for 30 min at room temperature and then incubated with primary antibodies at 4˚C overnight. The membranes were washed with TBS-T and incubated with goat anti-rabbit antibody conjugated to horseradish peroxidase (cat. no. 1662408edu, 1:2,500, Bio-Rad Laboratories, Inc.) at room temperature for 1 h. The immunoreactive bands were visualized using Clarity Western ECL Substrate (cat no. 1705060, Bio-Rad Laboratories, Inc.) and quantified with Image J software (version 1.49v; National Institutes of Health, Bethesda, MD, USA) (34).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Total RNA samples from treated cells were isolated using an RNeasy Mini kit (Qiagen GmbH, Hilden, Germany). The cDNA samples were then synthesized using a Primescript 1st Strand cDNA Synthesis kit (Takara Bio, Inc., Otsu, Japan) following the manufacturer's protocol. The reaction conditions were 42˚C for 60 min and 95˚C for 5 min. qPCR was performed with a SYBR Green Master Mix (Bio-Rad Laboratories, Inc.) and the following primers: IL-1α, forward 5'-CGCCAATGACTCAGAGGAAGA-3' and reverse

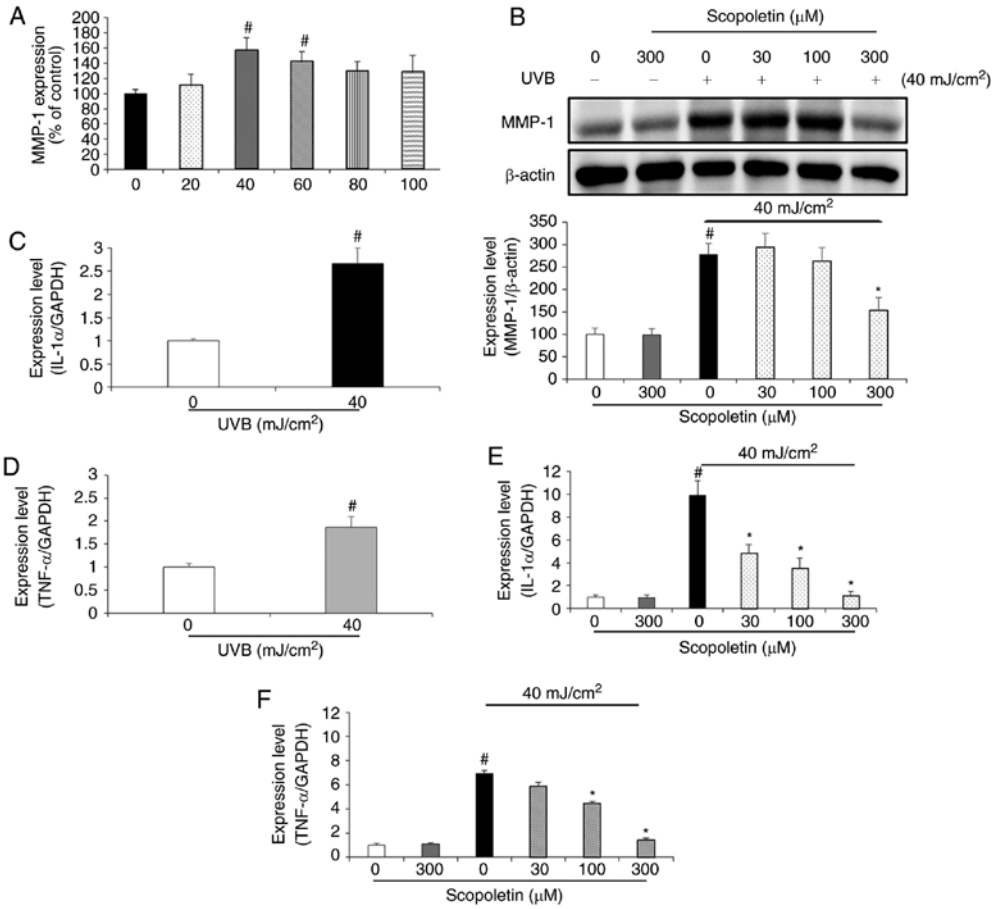

Figure 2. Effects of scopoletin on MMP-1 and proinflammatory cytokine expression in fibroblasts treated with conditioned medium from UVB-exposed HaCaT cells. (A) HaCaT cells were exposed to different doses of UVB irradiation (0, 20, 40, 60, 80 and 100 mJ/cm^2), and the conditioned media was collected. The effect of the different conditioned media on the fibroblast MMP-1 expression was assessed. (B) Effects of scopoletin on MMP-1 protein expression in fibroblasts. Fibroblasts were treated with HaCaT-conditioned medium containing scopoletin (0, 30 100 and 300 μM) for 24 h. MMP-1 expression levels were assessed by western blot analysis. (C) IL-1α and (D) TNFα mRNA expression levels were assessed in UVB-exposed HaCaT cells. (E) Effect of scopoletin on IL-1α and (F) TNFα mRNA expression levels in fibroblasts. Results are presented as the mean ± standard deviation of triplicate independent experiments. $^{\#}$P<0.05 compared with the vehicle control; *P<0.05, compared with the HaCaT-conditioned medium (40 mJ/cm^2) alone-treated control. MMP-1, metallopeptidase-1; UVB, ultraviolet B; IL-1α, interleukin-1α; TNFα, tumor necrosis factor α.

5'-AGGGCGTCATTCAGGATGAA-3' (120 bp); TNFα. Forward 5'-TCTTCTCGAACCCCGAGTGA-3' and reverse 5'-CCTCTGATGGCACCACCAG-3' (151 bp); GAPDH, forward 5'-TGCCACCAGAAGACTGTGG-3' and reverse 5'-AGCTTCCCGTTCAGCTCAGG-3'. The thermocycling conditions were as follows: 10 min at 94˚C, followed by a total of 45 cycles of 15 sec at 94˚C and 1 min at 60˚C. The expression levels of the genes presented as the quantification cycle (Cq) value was measured using the $2^{-\Delta\Delta Cq}$ relative quantitative analysis method (35), as automatically determined using the LightCycler 96 Software 1.1 (Roche Diagnostics, Basel, Switzerland).

Statistical analysis. All experiments were repeated at least three times, and results were presented as the mean ± standard deviation from three individual experiments. Statistical significance between two groups was examined with two-tailed Student's t-test using the SPSS 19.0 software package (IBM Corps, Armonk, NY, USA). Multiple-group comparisons were performed using one-way analysis of variance followed by Dunnett's post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

Isolation of the active substance from the ACE and cell viability of scopoletin-treated fibroblasts. To investigate the effects of the ACE fractions on MMP-1 protein expression inhibition, fibroblasts were treated with the 9 fractions. The highest inhibition activity of MMP-1 protein expression was observed in the Fraction 5 (Fig. 1A). A bioassay-guided fraction aided in the isolation of a single compound from Fraction 5 that exhibited inhibition of MMP-1 protein expression. The NMR spectrum of this compound was identical to scopoletin, which has been reported to be isolated from ACE in a previous study (23). The structure of this compound is shown in Fig. 1B. To examine whether scopoletin may exhibit cytotoxicity, the cell viability of fibroblasts treated with scopoletin was evaluated using an MTT assay. The results indicated that scopoletin had no cytotoxicity, as tested at the doses of 0.5, 5, 15, 50, 150, 250 and 500 μM for 24 h (Fig. 1C).

Effects of scopoletin on MMP-1 protein expression in fibroblasts treated with conditioned medium from UVB exposed HaCaT cells. To determine the optimal irradiation conditions, HaCaT

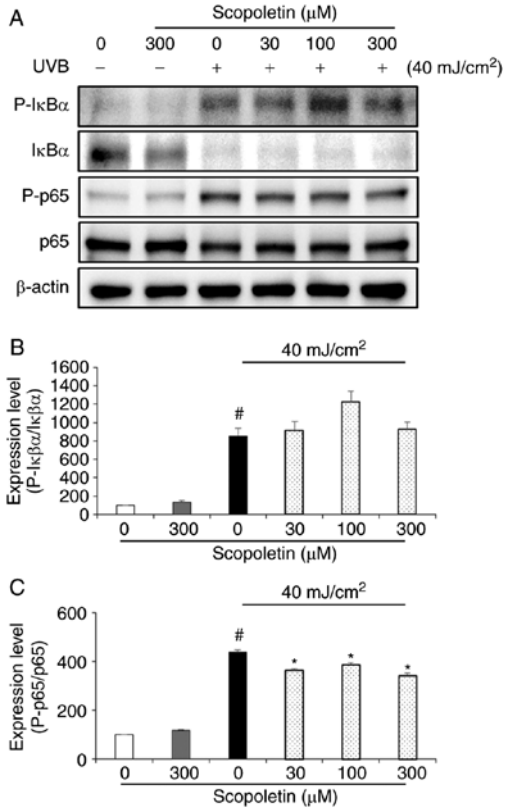

Figure 3. Effects of scopoletin on the NF-κB signaling pathway. Fibroblasts were pretreated with scopoletin for 6 h and then treated with HaCaT-conditioned medium (40 mJ/cm^2) containing scopoletin (0, 30, 100 and 300 μM) for 15 min. At 15 min of treatment, NF-κB signaling was assessed by western blot analysis. (A) Representative blots. β-actin was used as internal control. (B) Quantification of IκBα phosphorylation levels. (C) Quantification of p65 phosphorylation levels. Results are presented as the mean ± standard deviation of triplicate independent experiments. $^{\#}$P<0.05 compared with the vehicle control; *P<0.05 compared with the HaCaT-conditioned medium (40 mJ/cm^2) alone-treated control. NF-κB, nuclear factor-κB; IκBα, inhibitor of κB; UVB, ultraviolet B; p-, phosphorylated.

cells were irradiated with various doses of UVB (0, 20, 40, 60, 80 and 100 mJ/cm^2). After 24 h, HaCaT-conditioned medium was collected and then added to the fibroblasts. HaCaT-conditioned medium from 40 mJ/cm^2 irradiation produced the highest level of MMP-1 overexpression in fibroblasts (Fig. 2A), therefore 40 mJ/cm^2 was used in subsequent experiments. To investigate the effects of scopoletin on MMP-1 protein expression, fibroblasts were treated with various concentrations of scopoletin in conditioned medium from UVB-irradiated HaCaT cells. The protein expression levels for MMP-1 were determined by western blot analysis. MMP-1 protein expression was not altered when the fibroblasts were treated with serum-free medium containing scopoletin (300 μM; Fig. 2B). However, MMP-1 expression was significantly increased in fibroblasts treated with HaCaT conditioned medium. Scopoletin treatment decreased the MMP-1 protein expression levels with an inhibition rate of 44.84% at a concentration of 300 μM in the fibroblasts (Fig. 2B).

Effects of scopoletin on proinflammatory cytokine mRNA expression in fibroblasts treated with conditioned medium from UVB-exposed HaCaT cells. To examine whether UVB causes an increase in the mRNA expression of proinflammatory cytokines, the mRNA expression levels of IL-1α and TNFα were analyzed by RT-qPCR in HaCaT cells exposed to UVB for 24 h. The results demonstrated that 40 mJ/cm^2 UVB enhanced the mRNA levels of IL-1α and TNFα, by 2.66- and 1.85-fold, respectively (Fig. 2C and D). After collecting this conditioned medium, it was added on fibroblasts together with various concentrations of scopoletin (0, 30, 100 and 300 μM) for 24 h. In the fibroblasts treated with conditioned medium from the UVB-exposed HaCaT cells, the mRNA levels of IL-1α and TNFα were significantly increased compared with the control-exposed fibroblasts (Fig. 2E and F). Scopoletin inhibited this effect in a dose-dependent manner, in the range of 50-88.5% for IL-1α, and 15-80% for TNFα, compared with untreated, conditioned media-exposed fibroblasts (Fig. 2E and F).

Effects of scopoletin on NF-κB activation in fibroblasts treated with conditioned medium from UVB-exposed HaCaT cells. To determine the effect of scopoletin on NF-κB activation, the phosphorylation levels of IκBα and p65 were examined by western blot analysis (Fig. 3). No phosphorylation of IκBα and p65 was observed when fibroblasts were treated with serum-free medium containing scopoletin (300 μM; Fig. 3A). However, IκBα and p65 phosphorylation levels were increased when the fibroblasts were treated with conditioned medium from UVB-exposed HaCaT cells (Fig. 3). While IκBα phosphorylation was unaffected (Fig. 3B), p65 phosphorylation was significantly inhibited by scopoletin treatment (Fig. 3C). The reduction of p65, in the rate of 11.54-21.92%, was observed at scopoletin concentration of 300 μM (Fig. 3C).

Effects of scopoletin on MAPK activation in fibroblasts treated with conditioned medium from UVB-exposed HaCaT cells. To determine whether scopoletin inhibited MMP-1 expression by blocking MAPK signaling, the phosphorylation levels of ERK, JNK, and p38 were examined by western blot analysis. No phosphorylation of ERK, JNK, and p38 was observed in fibroblasts with serum-free medium containing scopoletin (300 μM; Fig. 4A and B). By contrast, the phosphorylation of ERK, JNK, and p38 was markedly increased in the fibroblasts treated with conditioned medium from UVB-exposed HaCaT cells (Fig. 4). Scopoletin treatment significantly inhibited p38 phosphorylation, with a rate of 17.67-28.33% observed at the scopoletin concentration of 300 μM (Fig. 4A and C). No effect on reducing phosphorylation of ERK and JNK was observed following scopoletin treatment in fibroblasts treated with conditioned medium from UVB-exposed HaCaT cells (Fig. 4B, D and E).

Effects of SB203580 and scopoletin on MMP-1 protein expression in fibroblasts treated with conditioned medium from UVB-exposed HaCaT cells. First, the potential cytotoxicity of SB203580 was tested on the fibroblasts by MTT assay. The results indicated that SB203580 had no cytotoxicity at the concentration of 10 μM (Fig. 5A). Then, the inhibition effect of scopoletin on p38 phosphorylation and MMP-1 expression was confirmed by western blotting (Fig. 5B and C). To further explore this pathway, the effect of SB203580, a well-known p38 inhibitor (36-40), was assessed. The results demonstrated that treatment with SB203580 and scopoletin significantly inhibited the phosphorylation of p38 by 37.31 and 33.45%

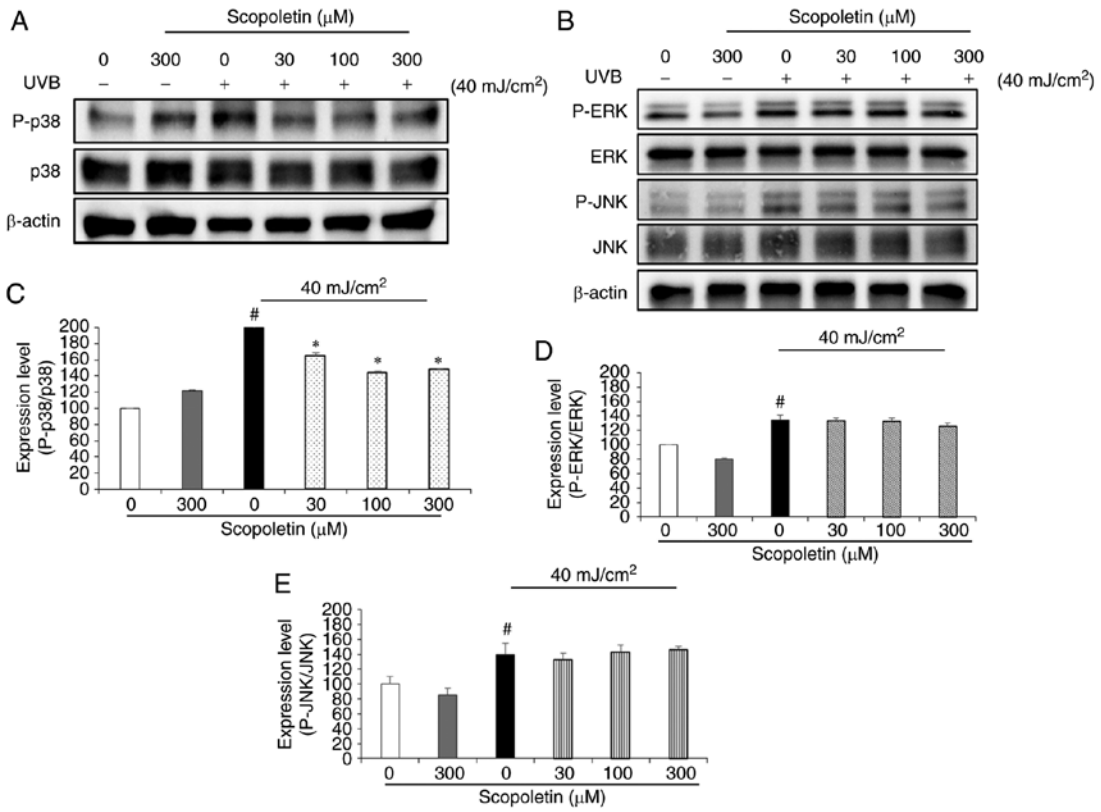

Figure 4. Effects of scopoletin on the MAPK signaling pathway. Fibroblasts were pretreated with scopoletin for 6 h and then treated with HaCaT-conditioned medium (40 mJ/cm^2) containing scopoletin (0, 30, 100 and 300 μM). At 15 min of treatment, MAPK signaling was assessed by western blot analysis. (A) Representative blots of p38 and of (B) ERK and JNK evaluation. β-actin was used as internal control. (C) Quantification of p38, (D) ERK and (E) JNK phosphorylation levels. Results are presented as the mean ± standard deviation of triplicate independent experiments. $^{\#}$P<0.05 compared with the vehicle control; *P<0.05 compared with the HaCaT conditioned medium (40 mJ/cm^2) alone-treated control. MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; UVB, ultraviolet B; p-, phosphorylated.

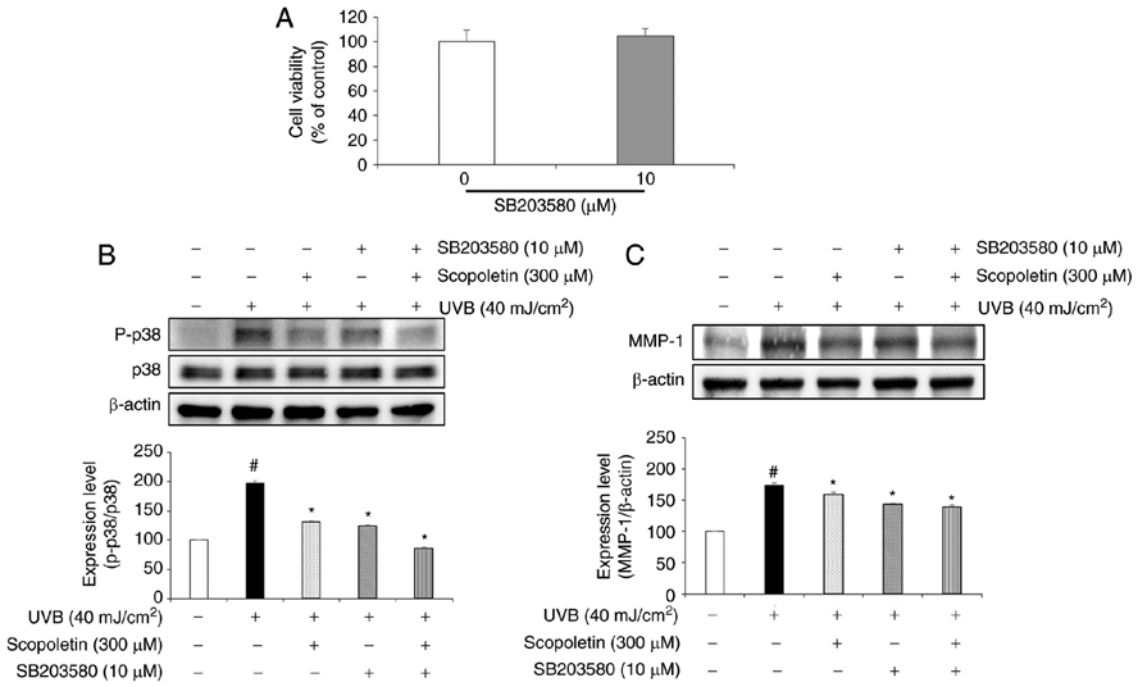

Figure 5. Effects of scopoletin and SB203580 on MMP-1 protein expression. (A) Fibroblasts were treated with 10 μM SB203580 for 24 h, and cell viability was then measured by MTT assay. (B) Fibroblasts were pretreated with SB203580 and scopoletin for 6 h and then treated with HaCaT-conditioned medium (40 mJ/cm^2) containing SB203580 and scopoletin at the concentration of 10 μM and 300 μM, respectively. Phosphorylation of p38 was evaluated at 15 min of treatment, while (C) MMP-1 protein expression levels were evaluated at 24 h of treatment. Results are presented as the mean ± standard deviation of triplicate independent experiments. $^{\#}$P<0.05 compared with the vehicle control; *P<0.05 compared with the HaCaT-conditioned medium (40 mJ/cm^2) alone-treated control. MMP-1, matrix metallopeptidase-1; UVB, ultraviolet B; p-, phosphorylated.

(Fig. 5B) and decreased the MMP-1 protein expression by 17.39 and 8.94%, respectively (Fig. 5C).

Discussion

Several studies have demonstrated that UVB is the most dangerous light, causing skin cancer (4). Furthermore, UVB irradiation is responsible for epidermal thickness and degradation of extracellular matrix (ECM), leading to damage in skin tissue integrity, formation of wrinkles, and inflammation (41). Therefore, protecting the skin from UVB irradiation may prevent the processes of wrinkle formation, photoaging, and inflammatory reactions of the skin (42).

In many studies, herbal products have been investigated and extensively used as candidates for traditional medicine without toxicity. Among these, *A. capillaris* has been reported to possess several biological effects, including hepatoprotective, antibacterial, antioxidant, antiobesity, and health properties. *A. capillaris* contains several compounds, including coumarin derivatives and flavonoid derivatives (23). However, no study has investigated the effects and related mechanisms of *A. capillaris* ethanol extract (ACE) with the active compound, scopoletin, in fibroblasts.

When UVB irradiation reaches the skin, it does not penetrate deeply into the dermis and damages keratinocyte cells in the epidermis (43). Damaged keratinocyte cells secrete proinflammatory cytokines, including IL-1α, IL-1β, IL-6, IL-8, and TNFα (5-9). Based on this knowledge, the present study used an *in vitro* model where HaCaT cells were irradiated with UVB to produce an environment similar to that of human skin, and then the conditioned medium containing proinflammatory cytokines released from the HaCaT cells was collected and added on fibroblasts (12). Proinflammatory cytokines, such as IL-1α and TNFα, that were secreted from UVB-exposed keratinocyte cells stimulate fibroblasts to express MMP-1 protein, a member of the collagenase subfamily of MMPs. MMP-1 has a major role in skin photoaging, by degrading the ECM to maintain the dermal skin layers (44,45). In the present study, MMP-1 protein expression was demonstrated to be significantly increased in fibroblasts that were treated with HaCaT-conditioned medium (40 mJ/cm^2). Scopoletin inhibited the MMP-1 protein overexpression in fibroblasts treated with HaCaT-conditioned medium. In addition, the mRNA levels of IL-1α and TNFα were increased in fibroblasts treated with HaCaT-conditioned medium, and this effect was reversed by scopoletin treatment. IL1α and TNFα are known to induce phosphorylation of MAPKs and NF-κB in fibroblasts. NF-κB, a regulator of gene expression associated with inflammatory responses, is activated by IL-1α and TNFα. NF-κB activation occurs by phosphorylation and degradation of IκBα and translocation of NF-κB p65 (46). In addition, MAPK signaling pathways serve a central role in regulating cell proliferation, cell motility, MMP gene expression, cell survival and death. Three major MAPK subfamilies in mammalian cells include ERK, JNK and p38. The activation of p38 MAPK leads to the induction of many proteins that are key to the inflammatory process, including a further induction of cytokine secretion. The p38 MAPK signaling pathway has a pivotal role in regulating the production of proinflammatory cytokines, such as TNFα (16). When these two pathways, NF-κB and MAPKs, are activated, MMP-1 protein and proinflammatory cytokine mRNA are expressed in fibroblasts (47). Proinflammatory cytokines, such as IL-1α and TNFα, are then secreted from fibroblasts to further activate the MAPK and NF-κB pathways in an autocrine action (48). Phosphorylation of p65 decreased slightly following scopoletin treatment of fibroblasts stimulated with HaCaT conditioned medium. In addition, HaCaT conditioned medium induced phosphorylation of ERK, JNK, and p38 MAPKs in fibroblasts. The phosphorylation of p38 MAPK decreased significantly in fibroblasts treated with scopoletin, but no effect was observed on the phosphorylation of ERK and JNK. In summary, the present study demonstrated that scopoletin inhibited the phosphorylation of p38 MAPK and decreased MMP-1 protein expression in fibroblasts. To evaluate whether the inhibition of MMP-1 protein expression in the fibroblasts is due to a reduction in the phosphorylation of p38 MAPK, we treated fibroblasts with a p38 inhibitor (SB203580). The results demonstrated that phosphorylation of p38 was inhibited following treatment with SB203580. Notably, treatment with SB203580 also reduced the levels of MMP-1. These findings indicated that scopoletin inhibited the expression of IL-1α and TNFα mRNA by reducing the phosphorylation of p38 MAPK, thereby decreasing the expression of MMP-1 protein in fibroblasts treated HaCaT-conditioned medium.

In conclusion, although there is no antiwrinkle effect of scopoletin in mice, it has been reported that *A. capillaris* extract alleviates atopic dermatitis in mice (49,50). Based on these results, it is expected that the inhibitory effect on MMP-1 protein expression may be tested by treating with scopoletin in UVB-irradiated mice. Future studies will also investigate the effect of scopoletin treatment on the expression and cellular distribution of cytokines by immunofluorescence analysis, as well as its effects on cellular morphology. In addition, future studies will investigate whether the observed changes in MMP-1 levels are due to alterations at the transcriptional, translational, or post-translational levels. Although further studies are necessary to fully explore the use of scopoletin in humans, the present results suggest a possible role of scopoletin as a potential preventing factor against skin photoaging.

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Availability of data and materials

The analyzed datasets generated during the study are available from the corresponding author on reasonable request.

Authors' contributions

HLK was analyzed the experimental data and was a major contributor in writing the manuscript. SMW optimized the

HaCaT cell cultures. WRC and HSK performed the cell viability assays. CY and JC isolated the active compounds and determined the chemical structure. KHK and SHY performed the western blot analysis. JWS performed the RT-PCR analysis. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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