Cytotechnology (2021) 73:127–138
https://doi.org/10.1007/s10616-020-00449-w

ORIGINAL ARTICLE

Anti-photoaging activities of *Sorbaria kirilowii* ethanol extract in UVB-damaged cells

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Received: 28 July 2020 / Accepted: 6 December 2020 / Published online: 4 January 2021

Abstract *Sorbaria kirilowii* (Regel) Maxim, a plant found in China, Korea, Japan, and east of Europe, is a common herb used for traditional medicinal purposes. However, its ability to prevent photoaging has not been studied. In this study, we investigated the anti-photoaging functions of an ethanol extract (Sk-EE) of *S. kirilowii (Regel) Maxim* using human keratinocytes exposed to UVB. First, we analyzed the cytotoxicity of Sk-EE. Then, we determine the expression of genes related to inflammation, collagen degradation, and moisture retention. We also explored the anti-photoaging mechanism of Sk-EE by determining correlated signaling pathways and target molecules using reporter gene assays and immunoblotting analyses. Sk-EE treatment of cells increased hyaluronic acid synthase (*HAS*), filaggrin (*FLG*), and collagen type I alpha 1 (*COL1A1*) expression. Sk-EE dose-dependently inhibited the UVB-induced expression of matrix metalloproteinases (*MMPs*) *1, 2, 9* and cyclooxygenase (*COX*)-*2* by blocking the activator protein (AP)-1 signaling pathway, in particular the phosphorylation of c-Jun N-terminal kinase (JNK), p38, and extracellular response kinase (ERK). In addition, c-Fos and c-Jun were targeted by Sk-EE. Our results indicate that Sk-EE has anti-inflammatory and skin-protective properties, and could be a candidate to treat signs of photoaging.

Keywords *Sorbaria kirilowii* (regel) maxim · Anti-photoaging · UVB irradiation · AP-1 signaling pathway

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Introduction

Skin is the largest organ in the human body, and functions as our primary environmental barrier in addition to its vital role in maintaining homeostasis. Skin aging is due to endogenous aging and exogenous aging (Puizina-Ivic 2008). Generally speaking, endogenous aging refers to the natural skin aging progress, while exogenous aging refers to aging due to external conditions. Skin aging can negatively affect appearance, and may be associated with various skin diseases (Zouboulis and Makrantonaki 2011) or skin tumors (D'Orazio et al. 2013). Skin aging is due to changes at the molecular level (Gragnani et al. 2014). At the molecular level, DNA is destroyed and there are

decreased levels of several proteins and cytokines including activator protein (AP)-1, matrix metalloproteinases (MMPs), interleukins, and nuclear factor kappa-B (NF-κB). Aged skin tends to be dry and have enlarged pores and wrinkles; in terms of vascular changes, spider veins, red spots and purpura can be present. An example of an aging-related degenerative condition is actinic keratosis.

Numerous external factors can contribute to skin aging, such as smoking, a stressful lifestyle, poor nutrition, excess alcohol, and environmental pollution (Gragnani et al. 2014). A major contributor to skin aging is photoaging. Ultraviolet (UV) radiation is subdivided into three wavelength components, namely UVA, UVB, and UVC (Polefka et al. 2012b). UVC has the shortest wavelength (100–280 nm) and is found in the upper part of the atmosphere; the ozone layer absorbs almost all UVC. UVB has a marginally longer wavelength (280–315 nm) than UVC, and some UVB arrives at the surface of earth. UVA radiation possesses the longest wavelength (315–400 nm) but is less energetic than UVB, and very little is absorbed by the atmospheric layer. Photoaging refers to oxidative damage caused by UVB irradiation of the epidermis, UVA irradiation of the dermis, or infrared A radiation of deeper layers of the skin (Panich et al. 2016). UVB is considered the most harmful type of ultraviolet radiation as it causes various cancers of the skin (Ichihashi et al. 2003; Svobodová et al. 2003).

Many techniques such as laser rejuvenation, plastic surgery and sunscreen creams, are currently available (Ganceviciene et al. 2012), and these in addition to anti-photoaging natural herbal cosmetics are used to treat photoaging (Saewan & Jimtaisong 2015). There is increased interest in using natural plant ingredients in cosmetics. Plant extracts that can scavenge free radicals or inhibit MMP activity may have the potential to reduce photoaging (Korać and Khambholja 2011). Various botanical extracts that claim to reduce skin aging and enhance the health of the skin are commercially available (Argyropoulou et al. 2013; Cavinato et al. 2017; Li et al. 2009; Mukherjee et al. 2011). Some medicinal plants have several constituents with antioxidant properties, such as polyphenols (Nichols and Katiyar 2010), alkaloids (Beak et al. 2004), tannins (Gali-Muhtasib et al. 2000), carotenoids, and flavonoids (Stahl and Sies 2007). *Sorbaria kirilowii* (Regel) Maxim, which is a common plant with a wide distribution, especially in East Asia, is used to beautify the environment and is a component of traditional medicine in the East. Nowadays, some studies of *Sorbaria* genus have revealed that these plants have anti-inflammatory (Jang et al. 2020), anti-melanogenic (Nishi et al. 2020), anti-proliferative (Zhang et al. 2007), and hapatoprotective (Zhang et al. 2004) activities. The skin-protective function of this plant, however, has not previously been investigated. Our aim in this study was to determine if an ethanol extract of *Sorbaria kirilowii* (Sk-EE) had anti-photoaging effects.

Materials and methods

Materials

A 95% ethanol extract of *Sorbaria kirilowii* (Sk-EE) was acquired from the Korean Plant Extract Bank (Cheongju, Korea) (Jang et al. 2020). Sk-EE stock solution (100 mg/mL) was dissolved in DMSO, as reported previously (Jang et al. 2020). HEK293T cells, HaCaT cells, and HDF cells were bought from the American Type Culture Collection (ATCC) (Rockville, MD, USA). Dulbecco's Modified Eagle's medium (DMEM), fetal bovine serum (FBS), phosphate-buffered saline (PBS), reduced serum medium (Opti-MEM), and TRIzol reagent were acquired from Gibco (Grand Island, NY, USA). (3–4–5-dimethylthiazol-2-yl)-2–5-diphenyltetrazolium bromide (MTT), retinol, dimethyl sulfoxide (DMSO), 2,20-azinobis(3ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), polyethyleneimine (PEI), and 1-diphenyl-2-picryl-hydrazyl (DPPH) were acquired from Sigma Chemical Co. (St. Louis, MO, USA). USA). U0126, SP600125, SB203580, and MG132 were acquired from Calbiochem (La Jolla, CA, USA). Antibodies specific for β-actin, rabbit IgG, mouse IgG, as well as the phosphorylated and total forms of MEK1/2, MKK7, ERK, P38, JNK, c-Fos, and c-Jun were acquired from Cell Signaling Technology (Beverly, MA, USA). A UVB lamp was acquired from Bio-Link BLX-312, VILBER LOURMAT Company, France.

Cell culture

Cultured in DMEM supplemented with 1% antibiotics (penicillin and streptomycin) and 10% FBS.

HEK293T cells (human embryonic kidney cells) were cultured in DMEM containing 1% antibiotics and 5% FBS. All cell lines were cultured at 37 °C in a 5% CO_2 incubator.

Cell viability assay

To evaluate the cytotoxicity of Sk-EE, HaCaT cells, HEK293T cells, and HDF cells were seeded in 96-well plates at 2×10^4 cells/well, 1×10^4 cells/well, and 4×10^4 cells/well, respectively, using the appropriate culture medium containing 12.5, 25, 50, and 100 μg/mL Sk-EE. To evaluate the cytotoxicity of Sk-EE, HaCaT cells were plated into 96-well plates at 1×10^5 cells/well in 10% DMEM and then treated with Sk-EE for 30 min. After suctioning off of all of the media, cells were covered with PBS and irradiated with 30 mJ/cm^2 UVB. Before treatment with different doses of Sk-EE for a further 24 h, the remaining liquid was suctioned. Cell viability was tested with the MTT assay. Cell viability was calculated as a percentage using the following formula: cell viability % = $A_1/A_2 \times 100$, A_1: sample mean value; A_2: group mean value.

UVB irradiation

HaCaT cells were irradiated in plates using a UVB lamp with the wavelength set to 312 nm. When the confluence of HaCaT cells was around 70%, HaCaT cells were treated with different doses of Sk-EE (25, 50 and 100 μg/mL) for 30 min. Before UVB irradiation, the remaining medium was exchanged with PBS. After removing the plate lid, HaCaT cells were irradiated with 30 mJ/cm^2 UVB. The PBS was then replaced with 10% DMEM containing 25, 50, or 100 μg/mL Sk-EE and plates were incubated for an additional 12 h.

Morphological changes

To evaluate morphological changes, 0.8×10^5 HaCaT cells were seeded in 35 mm plates. After treatment with 25, 50, or 100 μg/mL of Sk-EE or UVB irradiation for 12 h, cell images were captured using an optical microscope connected to a video camera.

DPPH decolorimetric assay

To determine the oxidant scavenging ability of Sk-EE, a DPPH decolorimetric assay was conducted. One hundred microliters of Sk-EE (12.5–200 μg/mL) or 100 μL ascorbic acid (500 μM) was added to 100 μL of DPPH (250 μM in methanol). Control group cells were treated with 100 μL of PBS and 100 μL of DPPH, respectively. All the steps described above were performed on ice. After incubation in the dark at 37 °C for 30 min, absorbance was measured at 517 nm using a spectrophotometer. DPPH scavenging activity was calculated as follows:

$$\text{DPPH scavenging effect } (\%) = \frac{[A_0 - A_1]}{A_0} \times 100,$$

where A_0 is the absorbance of DPPH alone and A_1 is the absorbance of the sample (Sk-EE or ascorbic acid).

ABTS assay

To determine the oxidant scavenging ability of Sk-EE, an ABTS scavenging assay was performed. In order to make radicalized ABTS solution, 7.4 mM of ABTS and 2.4 mM of potassium persulfate reagent were mixed with 1:1 ratio and the mixture was incubated at 50 °C for 1 h in a dark condition. Then, PBS was used to prepare ABTS solution with an absorbance ranging from 0.72 to 0.74 at 730 nm. One hundred microliters of Sk-EE (12.5–200 μg/mL) or 100 μL of ascorbic acid (500 μM) was added to 100 μL of ABTS. The control group was prepared with 100 μL of PBS and 100 μL of ABTS solution. After incubation in the dark at 37 °C for 30 min, absorbance was measured at 700 nm using a spectrophotometer. ABTS scavenging activity was calculated as follows:

$$\text{ABTS scavenging effect } (\%) = \frac{[A_0 - A_1]}{A_0} \times 100$$

where A_0 is the absorbance of ABTS alone and A_1 is the absorbance of the sample (Sk-EE or ascorbic acid).

Luciferase reporter gene assay

HEK293T cells were plated at a density of 1.25×10^5 cells/well in 24-well plates in 5% DMEM (without antibiotics). When cell confluence was 60–70%, plasmids expressing β-galactosidase, Col1α1-luciferase, and PEI were transfected into cells for 24 h,

as reported previously (Kim et al. 2019). All reagents were prepared in Opti-MEM. After suctioning all reagents, cells were treated with 50 or 100 μg/mL of Sk-EE or 10 μg/mL of retinol prepared in 5% DMEM followed by incubation for 24 h.

Evaluation of messenger RNA levels through reverse transcriptase polymerase chain reaction

To measure mRNA expression levels of *MMP*s and *COX-2*, HaCaT cells were seeded at 2.0×10^5 cells/mL (for detecting moisture-related genes) or 0.4×10^5 cells/mL (for detecting) in 6-well plates. HDF cells were seeded in 6-well plates at a density of 5×10^5 cells/mL to evaluate expression of *COL1A1*. HaCaT cells and HDF cells were treated with 25, 50, or 100 μg/mL Sk-EE for 30 min (HaCaT cells) or 24 h (HDF cells). After UVB irradiation, HaCaT cells were cultured with different doses of Sk-EE for an additional 12 h. Total RNA was then extracted from cells using TRIzol reagent according to the manufacturer's instructions. PCR reactions were performed using a cDNA synthesis kit and the following cycling profile: 5 min at 95 °C for preliminary denaturation; 30 s at 95 °C for denaturation, 15 s at 55–60 °C for annealing, 20 s at 72 °C for extension, and 5 min at 72 °C for final extension for a total of 38 cycles, as reported previously (Lee et al. 2020). Primer sequences and annealing temperatures are provided in Tables 1 and 2, respectively.

Total cell lysate preparation

HaCaT cells were plated in 35 mm culture plates at 0.4×10^5 cells/mL using fresh complete culture medium. When cells were 70% confluent, they were pretreated with 25, 50, or 100 μg/mL of Sk-EE for 30 min. After suctioning all the media, 2 mL warm PBS was added to the cells and they were irradiated with UVB. Before treatment with 25, 50, and 100 μg/mL of Sk-EE, the remaining liquid was removed by suctioning. After a further 12 h incubation, culture plates were placed on ice to stop the reaction. Cells in the supernatant were collected and adherent cells were exfoliated using cold PBS. After centrifugation at 12,000 rpm for 5 min, the supernatant was removed and total cell lysate was obtained by adding cold fresh lysis buffer to the cell pellet followed by a 10-min incubation on ice. After cells were lysed, the mixture was centrifuged at 12,000 rpm for 5 min and the protein-containing supernatant was subjected to Western blot analysis, as reported previously (Choi et al. 2019).

Table 1 Sequences of PCR primers used in this study

Target	Direction	Sequences (5′ to 3′)
MMP1	Forward	GCCTGCGTCCATCAACACT
	Reverse	CCCTCCTCGTCCACCTCAA
MMP2	Forward	ACGACCGCGACAAGAAGTAT
	Reverse	CTGCAAAGAACACAGCCTTCTC
MMP9	Forward	GCCACTTGTCGGCGATAAGG
	Reverse	CACTGTCCACCCCTCAGAGC
COX-2	Forward	CAAAAGCTGGGAAGCCTTCT
	Reverse	CCATCCTTCAAAAGGCGCAG
COL1A1	Forward	CAGGTACCATGACCGAGACG
	Reverse	AGCACCATCATTTCCACGAG
TGM-1	Forward	GAAATGCGGCAGATGACGAC
	Reverse	AACTCCCCAGCGTCTGATTG
FLG	Forward	AGGGAAGATCCAAGAGCCCA
	Reverse	ACTCTGGATCCCCTACGCTT
HAS-1	Forward	CCACCCAGTACAGCGTCAAC
	Reverse	CATGGTGCTTCTGTCGCTCT
HAS-2	Forward	TCCCGGTGAGACAGATGAGT
	Reverse	GGCTGGGTCAAGCATAGTGT
HAS-3	Forward	TATACCGCGCGCTCCAA
	Reverse	GCCACTCCCGGAAGTAAGACT
GAPDH	Forward	GGTCACCAGGGCTGCTTTTA
	Reverse	GATGGCATGGACTGTGGTCA

Table 2 Annealing temperatures used in this study

Name	Annealing temperature (°C)
MMP2	53
MMP9	56
Other genes	55

Immunoblotting

To prepare samples for western blotting, protein absorbance was measured at 570 nm using a spectrophotometer and 40 μg protein was loaded per sample. Membranes were blocked with 5% BSA for 45 min at room temperature and incubated with

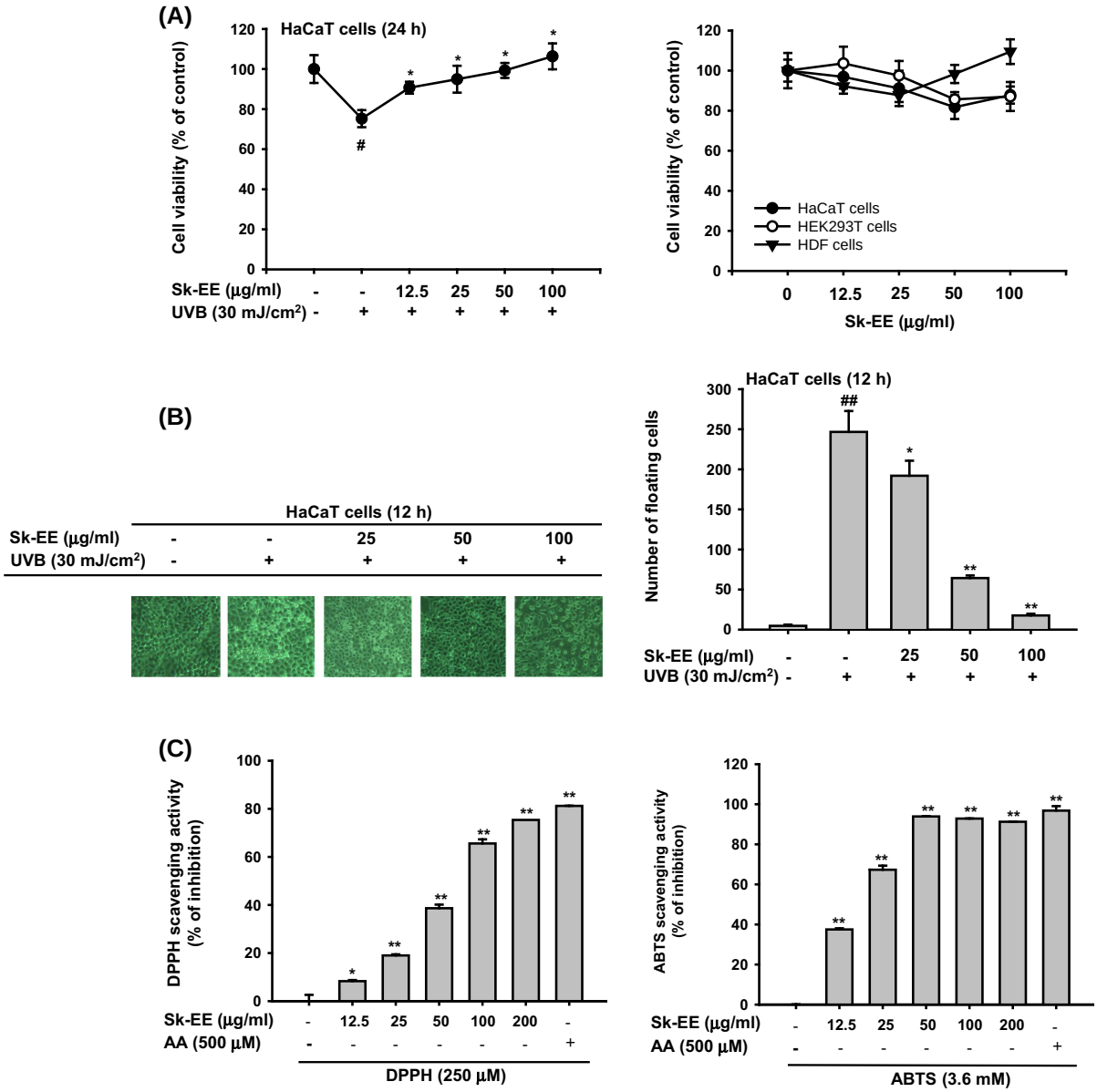
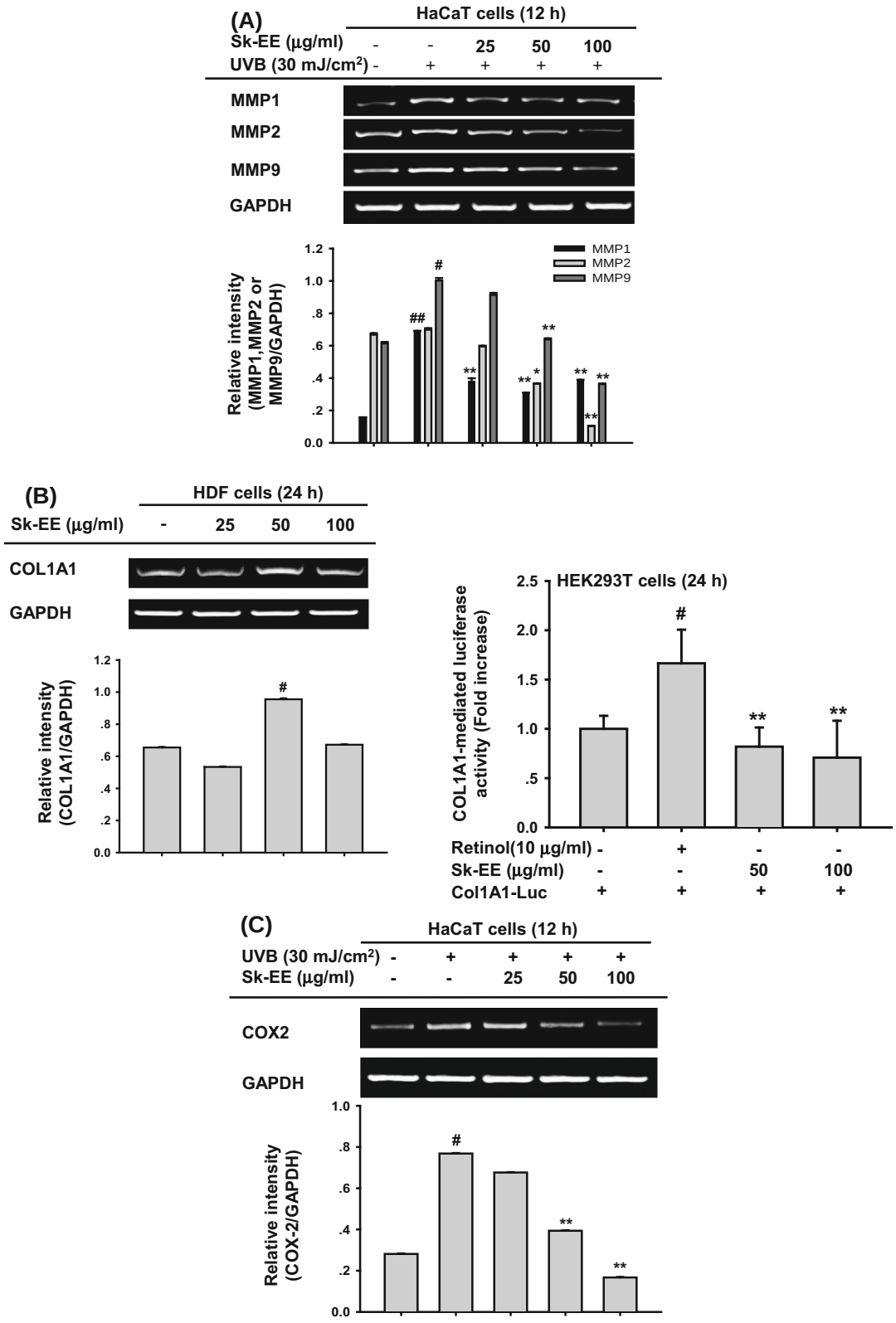


Fig. 1 Anti-oxidant effect of Sk-EE against UVB-induced damage. **a** Viability of three different cells types was evaluated by MTT assay in Sk-EE-treated cells exposed to UVB irradiation (HaCaT cells) (left panel) and those not exposed to UVB irradiation (HaCaT cells, HEK293T cells, and HDF cells) (right panel). **b** Morphology of HaCaT cells after treatment with Sk-EE (0–100 μg/mL) and UVB irradiation was assessed (left panel), and the number of floating cells was analyzed (right panel). **c** The anti-oxidant effect of Sk-EE (12.5, 25, 50, 100, and 200 μg/mL) and ascorbic acid (500 μM) was assessed by DPPH assay (left panel) and ABTS assay (right panel). The data are expressed as the means ± SD of an experiment performed with six (**a**, **b**, and **c**) samples. $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ compared with the normal group, and $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with the control group

primary antibody overnight at − 4 °C. After washing the membranes three times for 10 min, they were incubated with secondary antibody in 5% BSA at room temperature for 4 h, as reported previously (Lee et al. 2019). After an additional three 10-min washes, bands were checked.


(A)
HaCaT cells (12 h)
Sk-EE (μg/ml) - - 25 50 100
UVB (30 mJ/cm2) - + + + +
MMP1
MMP2
MMP9
GAPDH
Relative intensity (MMP1, MMP2 or MMP9/GAPDH)
MMP1
MMP2
MMP9
(B)
HDF cells (24 h)
Sk-EE (μg/ml) - 25 50 100
COL1A1
GAPDH
Relative intensity (COL1A1/GAPDH)
HEK293T cells (24 h)
COL1A1-mediated luciferase activity (Fold increase)
Retinol(10 μg/ml) - + - -
Sk-EE (μg/ml) - - 50 100
Col1A1-Luc + + + +
(C)
HaCaT cells (12 h)
UVB (30 mJ/cm2) - + + + +
Sk-EE (μg/ml) - - 25 50 100
COX2
GAPDH
Relative intensity (COX-2/GAPDH)

◀**Fig. 2** Sk-EE modulates the expression of MMPs, collagen synthesis-related factors, inflammatory factors, and moisturizing factors in HaCaT or HDF cells exposed to UVB irradiation. **a** After UVB irradiation, HaCaT cells were treated with Sk-EE for 12 h and the expression of *MMP1*, *MMP2*, and *MMP9* was measured. **b** Expression of *Col1A1* in HDF cells was measured after treatment of cells with Sk-EE for 24 h (left panel), and COL1A1-mediated luciferase activity in HEK293T cells was measured using a luminometer (right panel). **c** Expression of natural moisturizing-related genes (*FLG*, *TGM-1* and *HAS-1,-2,* and *-3*) was measured after Sk-EE treatment for 24 h. The data are expressed as the means ± SD of an experiment performed with six (**b** right panel) or three (bottom, left or right panels of **a**, **b**, **c**, and **d**) samples. Band intensity (**a**, **b**, **c**, and **d**) was measured and quantified using ImageJ. $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ compared with the normal group, and $*p < 0.05$ and $**p < 0.01$ compared with the control group

Statistical analysis

IC_{50} values were determined using Graphpad Prism 7.0. Data are presented as means ± standard deviations. All data were analyzed using Mann–Whitney *U* tests. $P < 0.05$ was considered statistically significant and $P < 0.02$ was considered strongly statistically significant.

Results

Anti-oxidant effect of Sk-EE against UVB-induced damage of HaCaT cells

To explore the potential ability of Sk-EE to protect skin against UVB irradiation, we pre-treated cells with Sk-EE prior to UV irradiation. Viability of cells increased with concentration of Sk-EE in a dose-dependent manner (Fig. 1a, left panel), indicating that Sk-EE protects against UVB-induced cell death. The cytotoxicity of Sk-EE was assessed by incubating cells with four different concentrations of Sk-EE (12.5, 25, 50 and 100 µg/mL) followed by MTT assay. Sk-EE had no obvious cytotoxicity towards HaCaT cells, HEK293T cells, or HDF cells up to a concentration of 100 µg/mL (Fig. 1a, right panel). To further confirm the protective effect of Sk-EE, the morphology of HaCaT cells was examined after Sk-EE pretreatment and UVB irradiation (Fig. 1b, left panel). As expected, the number of floating dead cells was dramatically higher after UVB irradiation than in the control group. Sk-EE pretreatment decreased the proportion of dead cells, especially in the groups treated with 50 and 100 µg/mL Sk-EE (Fig. 1b, right panel). To determine the antioxidant effects of Sk-EE in vitro, two different assays were performed. Ascorbic acid is a strong scavenger and was used as the positive control in both experiments. In the DPPH assay, the scavenging level in the 50 and 100 µg/mL Sk-EE groups was 39% and 65% respectively (Fig. 1c, left panel). Similarly, Sk-EE demonstrated a strong ability to remove ABTS

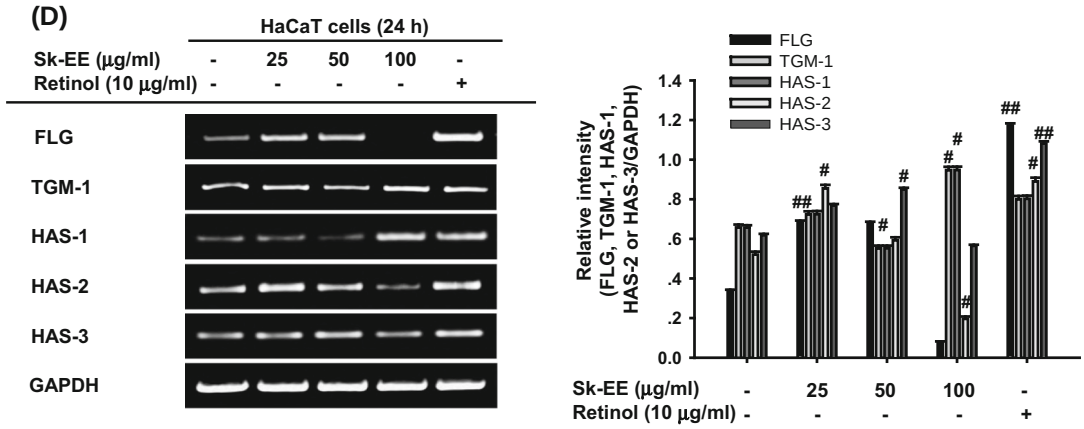
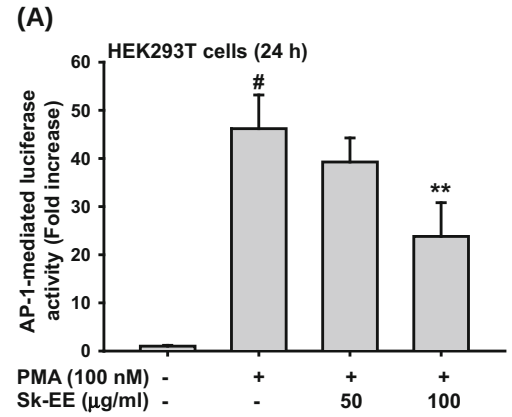
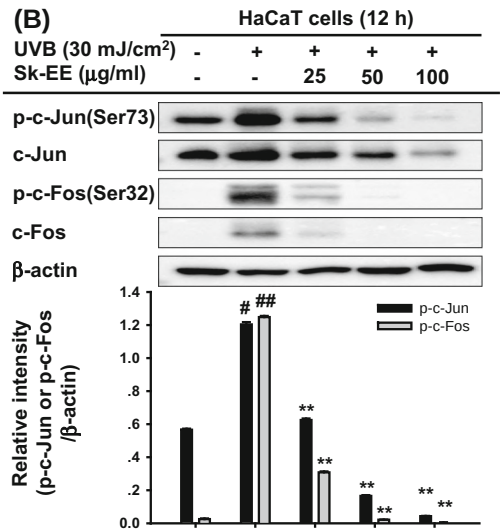
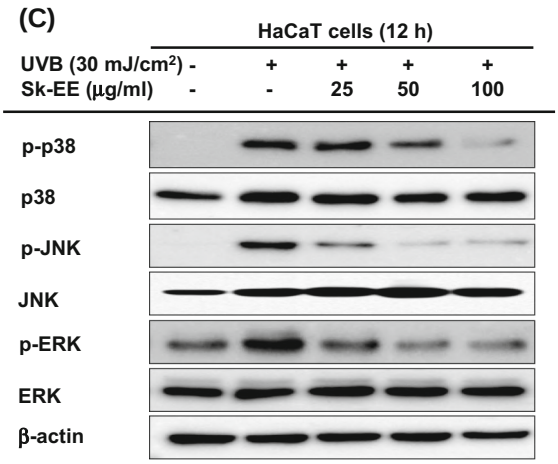
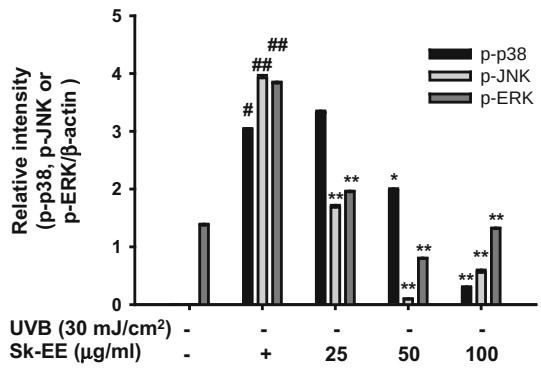


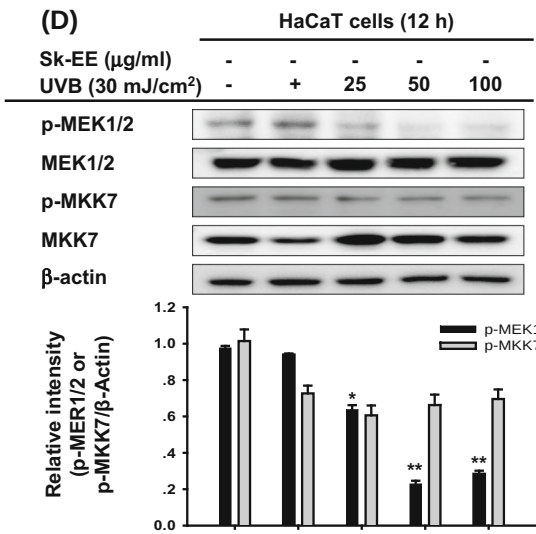
Fig. 2 continued

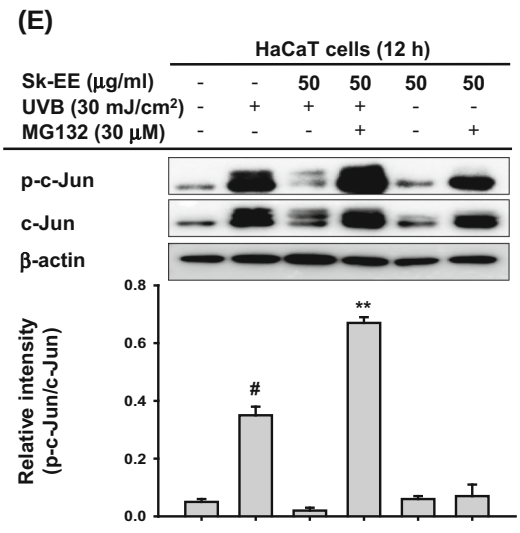

(A)
HEK293T cells (24 h)
AP-1-mediated luciferase activity (Fold increase)
PMA (100 nM)
Sk-EE (μg/ml)


(B)
HaCaT cells (12 h)
UVB (30 mJ/cm²)
Sk-EE (μg/ml)
p-c-Jun(Ser73)
c-Jun
p-c-Fos(Ser32)
c-Fos
β-actin
Relative intensity (p-c-Jun or p-c-Fos /β-actin)
p-c-Jun
p-c-Fos


(C)
HaCaT cells (12 h)
UVB (30 mJ/cm²)
Sk-EE (μg/ml)
p-p38
p38
p-JNK
JNK
p-ERK
ERK
β-actin


Relative intensity (p-p38, p-JNK or p-ERK/β-actin)
p-p38
p-JNK
p-ERK
UVB (30 mJ/cm²)
Sk-EE (μg/ml)


(D)
HaCaT cells (12 h)
Sk-EE (μg/ml)
UVB (30 mJ/cm²)
p-MEK1/2
MEK1/2
p-MKK7
MKK7
β-actin
Relative intensity (p-MER1/2 or p-MKK7/β-Actin)
p-MEK1/2
p-MKK7


(E)
HaCaT cells (12 h)
Sk-EE (μg/ml)
UVB (30 mJ/cm²)
MG132 (30 μM)
p-c-Jun
c-Jun
β-actin
Relative intensity (p-c-Jun/c-Jun)

◀**Fig. 3** The effect of Sk-EE on the activation of AP-1 and MAPKs in HaCaT cells exposed to UVB radiation. **a** AP-1-mediated Luc and β-gal plasmids were transfected into HEK293 cells by PEI for 24 h, and then cells were treated with Sk-EE (50 and 100 μg/ml) for an additional 24 h. **b–d** The expression of phospho- and total forms of c-Fos, c-Jun, p38, JNK, ERK, MEK1/2, MKK7, and β-actin in whole cell lysates were measured in Sk-EE-treated HaCaT cells exposed to UVB irradiation. **e** The expression of c-Jun and p–c-Jun in whole cell lysates was measured in the presence or absence of UVB, Sk-EE (50 μg/ml), and MG132 (30 μM) for 12 h. **f** Expression of *COX-2* at the mRNA level was measured after treatment of UVB-irradiated HaCaT cells with MAPK inhibitors [SB203580 (p38 inhibitor, 20 μM), SP600125 (JNK inhibitor, 20 μM) and U0126 (ERK inhibitor, 20 μM)]. The data are expressed as the means ± SD of an experiment performed with six (**a**) or three (bottom or right panels of **b**, **c**, **d**, **e**, and **f**) samples. Band intensity (**b**, **c**, **d**, **e**, and **f**) was measured and quantified using ImageJ. $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ compared with the normal group, and $*p < 0.05$ and $**p < 0.01$ compared with the control group

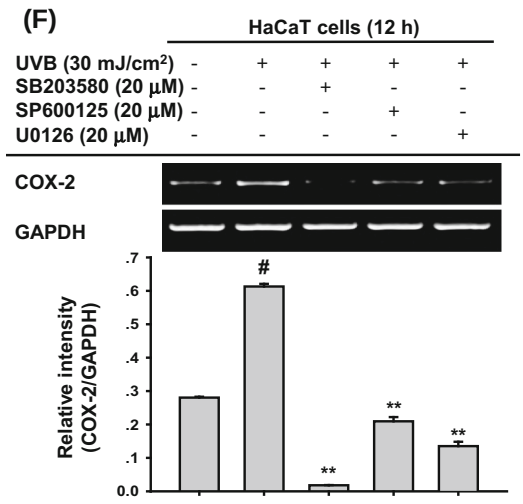


Fig. 3 continued

radicals (Fig. 1c, right panel). Half maximal inhibitory concentration (IC_{50}) of Sk-EE in the DPPH and ABTS assays was 73.64 and 12.89 μg/mL, respectively.

The protective effect of Sk-EE against UVB irradiation via downregulation of extracellular matrix degrading and inflammatory genes and increased expression of moisture-retention related genes.

To explore the possible skin protective effect of Sk-EE against UVB irradiation condition, mRNA levels of several different genes were measured. First, MMP proteins involved in collagen degradation were analyzed. After UVB irradiation, *MMP1*, *MMP2* and *MMP3* gene expression was upregulated compared to the control group. The Sk-EE treatment groups showed reduced expression of those genes, especially the groups treated with 100 μg/mL Sk-EE (Fig. 2a). Second, expression of *COL1A1* was examined in HDF cells that were not irradiated with UVB (Fig. 2b, left panel). The expression of *COL1A1* in the group treated with 50 μg/mL Sk-EE was significantly higher than in the control group. To examine this further, a COL1A1-containing luciferase plasmid was transfected with PEI into HEK293T cells. However, Sk-EE did not induce COL1A1-mediated luciferase activity (Fig. 2b, right panel). Third, we measured the expression of the pro-inflammatory gene *COX-2*. *COX-2* expression in HaCaT cells was induced by UVB irradiation. Sk-EE largely reversed the UVB-induced increase in *COX-2* expression in dose-dependent manner. After treatment with Sk-EE, the expression of *COX-2* decreased by 50% in the 50 μg/mL Sk-EE group, and *COX-2* expression in the 100 μg/mL Sk-EE group was even lower than that in the control group (Fig. 2c). Finally, we assessed the expression of natural moisturizing factor (NMF) synthesis-related genes. Sk-EE increased the expression of moisture retention-related genes such as *FLG*, *HAS-2*, and *HAS-3* as shown in Fig. 2d. However, as for *TGM-1* and *HAS-1*, the increase in expression of these genes was significant only in the group treated with 100 μg/mL Sk-EE.

Effect of Sk-EE on AP-1 activation

MAPK signaling can be switched on by UVB irradiation, resulting in recruitment of c-Jun and c-Fos to the nucleus and activation of the AP-1 complex (Xu & Fisher 2005). We therefore performed an AP-1-mediated luciferase expression assay to investigate if Sk-EE affected AP-1 signaling. As shown in Fig. 3a, the AP-1 signaling pathway was induced by PMA, and its activity was suppressed by Sk-EE treatment. Expression of AP-1 subunits (c-Fos and c-Jun) was also assessed (Fig. 3b). Interestingly, expression of these proteins and their phosphorylated forms was induced by UVB irradiation and decreased by Sk-EE treatment. Because MAPKs can regulate proteins downstream of AP-1, we also examined the protein expression of MAPKs like P38, JNK, and ERK (Fig. 3c, left panel). Consistent with published results,

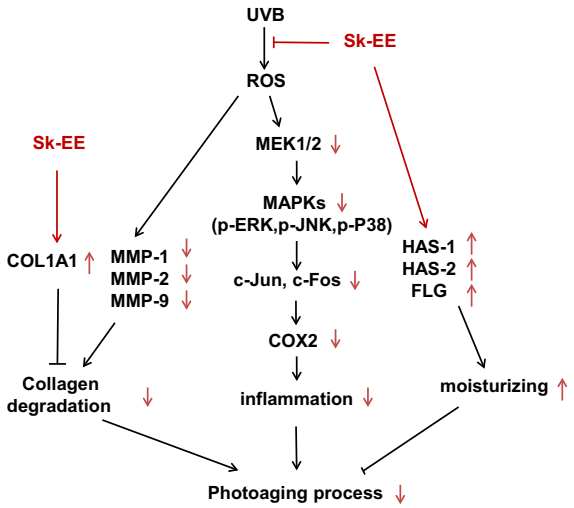


Fig. 4 Summary of pathways regulated by Sk-EE related to its anti-photoaging effects

p-p38, p-JNK, and p-ERK levels were dramatically increased by UVB irradiation. Sk-EE treatment, however, decreased expression of these proteins, particularly in the 50 and 100 μg/mL Sk-EE treatment groups. The expression levels of p-p38 and p-JNK were 3–fourfold lower in the group treated with 100 μg/mL Sk-EE than in the UVB irradiation group. Therefore, expression of additional upstream proteins (MEK1/2 and MKK7) was assessed to explore proteins targeted by Sk-EE. There was no obvious difference in p-MKK7 expression between Sk-EE treatment groups and the control group (Fig. 3d). However, Sk-EE inhibited the UVB-induced increase in p-MEK1/2 expression.

To further investigate the decrease in levels of phosphorylated and total forms of c-Jun and c-Fos, the proteasome inhibitor MG132 was added to cells treated with 50 μg/mL Sk-EE as this concentration of Sk-EE reduced expression of these proteins and their phosphorylated forms appreciably. Addition of MG132 blocked the ability of Sk-EE to decrease levels of c-Fos and c-Jun. To explore whether the decrease in *COX-2* expression was mediated by the MAPK pathway, cells were treated with specific inhibitors of MAPKs. As shown in Fig. 3f, *COX-2* expression was inhibited by U0126 (an ERK inhibitor), SB203580 (a p38 inhibitor), and SP600125 (a JNK inhibitor).

Discussion

This study aimed to explore the ability of Sk-EE to prevent the skin damage caused by UVB irradiation. We investigated the potential skin protective functions of Sk-EE by evaluating the expression of genes related to collagen degradation, retention of moisture, and inflammation.

UVB irradiation of skin induces the generation and accumulation of ROS (Polefka et al. 2012b). As a consequence, signaling pathways that result in photoaging are triggered. We found that Sk-EE had a strong oxidant radical scavenging effect in a cell free system, suggesting that Sk-EE might have the similar function in vitro. What is more, Sk-EE clearly protected against UVB-induced cell death (Fig. 1a).

After treating cells with relatively non-cytotoxic doses of Sk-EE (25, 50, and 100 μg/mL), RT-PCR was performed to evaluate the specific mechanisms by which Sk-EE protected against UVB-induced cell damage. Sk-EE promoted the synthesis of *HAS-1, 2* and *FLG*; these proteins are constituents of the cornified cell envelope. Through quantification of MMP family genes (Fig. 2a), we found that mRNA expression of *MMP* genes was increased by UVB irradiation but decreased dramatically in response to Sk-EE treatment in a dose-dependent manner. What is more, Sk-EE also increased the expression of *COL1A1*, suggesting that Sk-EE stimulates the synthesis of type I collagen. Surprisingly, however, Sk-EE did not stimulate *COL1A1*-mediated luciferase activity. As mentioned previously, MMP proteins play a large role in degrading extracellular matrix components and remodeling tissues (Wen et al. 2012). Collagens, by contrast, support and strengthen various tissues like tendon, bone, cartilage, and skin, with Type I collagen the most abundant collagen in the human body. According to our study results (Fig. 2), Sk-EE may protect against collagen degradation by downregulating enzymes that degrade collagen. Sk-EE is rich in various types of flavonoids, which have been shown to have anti-inflammatory functions (Jang et al. 2020). COX-2 is an enzyme that results in inflammation and pain. Normally, pharmaceutical inhibition of COX can offer relief from the symptoms of inflammation and pain (Brune and Patrignani 2015). Sk-EE dramatically decreased UVB-induced *COX-2* expression in a dose-dependent manner (Fig. 3c).

These results indicate that Sk-EE might be a potential anti-inflammatory candidate drug.

Previous research has demonstrated that the UVB irradiation-induced increase in expression of *MMP*s and *COX-2* is mediated via the AP-1 signaling pathway (Cho et al. 2005; Jeon et al. 2011; Polefka et al. 2012b). To clarify the mechanism by which Sk-EE protected against UVB, the effects of Sk-EE on AP-1 transcriptional activity were assessed by performing luciferase reporter assays. As expected, Sk-EE downregulated PMA-induced AP-1-mediated luciferase expression without UVB stimulation (Fig. 3a). In addition, we examined the expression of p-c-Fos and p-c-Jun after UVB treatment. Expression of both the phosphorylated forms and total forms of these proteins was strongly blocked by Sk-EE treatment (Fig. 3b). Therefore, we examined the effect of the proteasome inhibitor MG132 on expression of those proteins (Fig. 3e). MG132 rescued the Sk-EE-induced decrease in expression of both phosphorylated and total forms of c-Fos and c-Jun. UVB can induce synthesis of c-Fos and c-Jun; however Sk-EE can block this synthesis. We found that c-Jun itself was degraded under control conditions, because levels of this protein increased after addition of MG132. However, Sk-EE could block the synthesis of c-Jun. Sk-EE induced an increase in degradation of c-Jun under UVB irradiation. Expression of c-Fos and c-Jun can be triggered by MAPKs, so we evaluated the expression of specific MAPKs (p38, JNK and ERK) at the protein level (Fig. 3c and d). Immunoblotting results showed that Sk-EE was able to inhibit the expression of these upstream activators of AP-1. We further evaluated the expression of MAPKs such as MEK1/2 and MKK7. The ability of Sk-EE to decrease expression of AP-1 in response to UVB damage was due to inhibition of MEK1/2 rather than MKK7. Next, cells were treated with specific MAPK inhibitors (U0126, SB203580 and SP600125) to investigate whether the ability of Sk-EE to inhibit COX-was mediated by the MAPK pathway (Fig. 3f). All of these inhibitors strongly blocked the UVB-induced increase in *COX-2* expression, especially the p38 inhibitor SB203580. These findings indicate that Sk-EE suppresses the UVB-induced increase in *COX-2* gene expression by regulating MAPK signaling.

Conclusions

To sum up, we demonstrated that Sk-EE has potential anti-photoaging activity as evidenced by its ability to increase the expression of genes related to moisture retention, decrease the expression of genes involved in collagen degradation, and decrease the expression of the inflammatory mediator COX-2 (Fig. 4). Sk-EE regulated *COX-2* gene expression by modulating the activity of MAPKs (ERK, JNK, and p38), thereby affecting AP-1 expression. Sk-EE could therefore potentially be used to protect against photoaging caused by UVB irradiation. So far, SK-EE has been also reported to suppress TLR4/LPS-induced inflammatory responses and ameliorate HCl/EtOH-induced gastritis (Jang et al. 2020). Therefore, these findings strongly suggest that SK-EE could be developed as anti-inflammatory and anti-photoaging herbal medicine.

Acknowledgments This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF), the Ministry of Education (Grant Number: 2017R1A6A1A03015642), and by National Institute of Biological Resources, the Ministry of Environment (Grant Number: NIBR202006202), Republic of Korea. We acknowledge International Biological Material Research Center, Korea Research Institute of Bioscience and Biotechnology (Daejeon, Republic of Korea) for providing Sk-EE (ethanol extract of dried and refined leaves and twigs of *Sorbaria kirilowii*).

Author contributions HC: conceived and designed the experiments, performed the experiments, analyzed the data, and wrote the paper. JJ, SRK, and KY: performed the experiments. JY: participated in revising this paper and provided research grant. JYC: conceived and designed the experiments, analyzed the data, and wrote the paper.

Compliance with ethical standards

Conflicts of interest The authors declare that they have no conflict of interest.

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