jmb

Fermented *Acanthopanax koreanum* Root Extract Reduces UVB- and H₂O₂-Induced Senescence in Human Skin Fibroblast Cells

Min-Ja Park and Young-Seuk Bae*

School of Life Sciences, BK21 Plus KNU Creative BioResearch Group, Kyungpook National University, Daegu 41566, Republic of Korea

The present study assessed the effects of an aqueous extract of *Acanthopanax koreanum* root (AE) and of AE following fermentation by lactic acid bacteria (*Lactobacillus plantarum* and *Bifidobacterium bifidum*) (AEF) on human skin fibroblast HS68 cells exposed to ultraviolet B (UVB) irradiation and oxidative stress. AEF effectively antagonized the senescence-associated β-galactosidase staining and upregulation of p53 and p21$^{Cip1/WAF1}$ induced by UVB or H₂O₂ treatment in HS68 cells. It also exhibited excellent antioxidant activities in radical scavenging assays and reduced the intracellular level of reactive oxygen species induced by UVB or H₂O₂ treatment. The antioxidant and antisenescent activities of AEF were greater than those of non-fermented *A. koreanum* extract. AEF significantly repressed the UVB- or H₂O₂-induced activities of matrix metalloproteinase (MMP)-1 and -3, overexpression of MMP-1, and nuclear factor κB (NF-κB) activation. This repression of NF-κB activation and MMP-1 overexpression was attenuated by a mitogen-activated protein kinase activator, suggesting that this AEF activity was dependent on this signaling pathway. Taken together, these data indicated that AEF-mediated antioxidant and anti-photoaging activities may produce anti-wrinkle effects on human skin.

Keywords: *Acanthopanax koreanum*, anti-photoaging, UV irradiation, lactic acid bacteria fermentation, antioxidant

Introduction

Skin aging is divided into intrinsic aging, which is a natural consequence of physiological change, and extrinsic aging, which is called premature senescence, and occurs owing to exposure to environmental factors. Intrinsic aging is genetically programmed and is highly correlated with the cell-cycle arrest state triggered by telomere attrition [1, 12, 15, 17]. One of the most important environmental aging determinants is ultraviolet B (UVB) radiation (280–315 nm). UVB is a major component of sunlight and causes skin photoaging, which is characterized by pigmentary change, wrinkle formation, decreased elastic properties, and degradation of the extracellular matrix (ECM), including type I collagen, elastin, and fibronectin [26-29]. UVB radiation generates reactive oxygen species (ROS) and induces DNA damage, either directly or indirectly. UVB-induced DNA damage results in stabilization of p53 and

overexpression of p21$^{Cip1/WAF1}$, which inhibits cell cycle progression [2, 4, 7, 24]. In addition, UVB-induced ROS generation upregulates mitogen-activated protein kinase (MAPK) cascades, activating nuclear factor κB (NF-κB) and activator protein 1 (AP-1). Increased NF-κB and AP-1 activities induce matrix metalloproteinase (MMP)-1 and -3 expression; these enzymes play an essential role in ECM degradation. Therefore, MMPs are important mediators of skin wrinkle formation during the process of photoaging induced by UVB irradiation [3, 6, 10, 19].

Acanthopanax species are widely distributed in Korea, China, and Japan. Among them, *Acanthopanax koreanum* is a popular plant in Jeju Island, South Korea. The roots and stems of the *Acanthopanax* genus have been traditionally used in oriental medicine as an energy booster and for the treatment of musculoskeletal pain, rheumatism, hypertension, and diabetes. Many studies have shown that *A. koreanum* has hypotensive, hepatoprotective, antioxidant, anti-allergic,

and anti-inflammatory activities, as well as enhancing the immune response and reducing blood cholesterol levels [8, 20, 22, 30, 31]. However, the anti-photoaging activity of *A. koreanum* extract has not been reported to date.

Fermentation is one of the oldest technological processes and is widely used in diverse fields, including the food, drug, and cosmetic industries. Fermentation sometimes reduces toxicity and increases absorption into the skin by altering the molecular structure of organic materials. Lactic acid bacteria have been reported to enhance antioxidant and anti-aging activities by producing various biomaterials [11, 13, 32]. However, no studies have already considered the use of lactic acid fermentation to improve the functional features of *A. koreanum*. In this study, we investigated for the first time the anti-photoaging effects of a root extract of *A. koreanum* (AE) and of AE that had been fermented with *Lactobacillus plantarum and Bifidobacterium bifidum* (AEF). The results showed that compared with AE, AEF produced more effective suppression of senescent phenotypes (senescence-associated β-galactosidase (SA-β-gal) staining and upregulation of p53 and p21$^{Cip1/WAF1}$), ROS production, NF-κB activation, and MMP-1 expression in UVB- or H$_2$O$_2$-treated skin fibroblast cells. Thus, this study demonstrated that AEF can be considered as a superior functional ingredient of anti-aging skin-care products.

Materials and Methods

Preparation of AE

A. koreanum roots were purchased from an herbal medicine shop located in Jeju Island, South Korea. They were cut, washed several times using distilled water, and air-dried naturally in a cool, dark environment. The dried roots were then boiled in 10 times (w/v) distilled water at 90°C for 4 h. The extracted supernatant was filtered three times using filter paper (Advantec, Japan). The collected extract was evaporated under vacuum, freeze-dried, and stored at −20°C until use.

Preparation of AEF

L. plantarum KCCM 12116 and *B. bifidum* KCCM 12096 were provided by the Korean Culture Center of Microorganisms (KCCM, Korea). The bacterial strains were grown in MRS (de Man, Rogosa, and Sharpe) broth (Difco, USA) at 37°C for 12–16 h. The cultivation media were suspended in sterile distilled water to a final optical density of 0.8 at a wavelength of 600 nm. AE powder was dissolved in water (1% (w/v)) and this solution was inoculated to 5% (v/v) with each inoculum. After fermentation with either *L. plantarum* for 12 h or *B. bifidum* for 60 h, each supernatant was recovered by centrifugation at 5,000 rpm for 10 min, boiled for 10 min, and lyophilized. The freeze-dried powder was stored at −20°C. A mixture of *L. plantarum*-fermented AE and *B. bifidum*-

fermented AE (1:2) was used in this study.

Cell Culture

The human foreskin fibroblast cell line HS68 was obtained from the American Type Culture Collection (ATCC, USA). HS68 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal bovine serum. The cells used in the experiments were between the 15th and 25th passages.

UVB Irradiation and H$_2$O$_2$ Treatment

The UVB-irradiation apparatus was a Spectronics Corporation UVB lamp (USA) with an emission peak at 312 nm. The irradiation dose was monitored using a UVX-31 radiometer (Upland, USA). HS68 cells were washed twice with phosphate-buffered saline (PBS) and then exposed to a 20 mJ/cm^2 UVB dose, with a thin layer of PBS. After irradiation, the cells were treated with 500 μg/ml of AE or AEF in serum-free medium for 48 h. For H$_2$O$_2$ treatment, HS68 cells were incubated with 300 μM H$_2$O$_2$ in serum-free medium for 3 h. The medium was then removed and the HS68 cells were washed twice with PBS prior to treatment with 500 μg/ml of AE or AEF in serum-free medium for 48 h. For some experiments, cells were treated with C2 ceramide (Sigma, USA) for 6 h just before harvesting.

SA-β-Gal Activity Assay

SA-β-gal activity in HS68 cells was measured as described previously [18]. The number of blue-stained cells was counted in at least 10 fields and expressed as the percentage of positive cells.

Cell Viability Assay

HS68 cells were plated at a density of 4×10^3 cells per well in 96-well plates for 48 h and then treated with various concentrations of AE or AEF in serum-free medium for 48 h. Fifty microliters of an 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) solution (Sigma) was then added to the wells for 4 h. The optical density at 495 nm was measured using a microplate reader (Bio-Rad, USA).

Measurement of Intracellular ROS

Intracellular ROS were measured as described previously [18], using the oxidation-sensitive fluorescent probes dichlorofluorescein diacetate (DCFDA; Invitrogen, USA) and dihydroethidium (DHE; Invitrogen, USA).

Preparation of Cell Extracts and Western Blotting

HS68 cell extracts were prepared and western blotting was performed as described previously [18]. Antibodies against p53, p21$^{Cip1/WAF1}$, and α-tubulin were obtained from Santa Cruz Biotechnology (USA). Antibodies against MMP-1, IκBα, p65, and phosphorylated-p65 (p-p65) were obtained from Abcam (USA).

Assay for DPPH Radical Scavenging Activity

Antioxidant activity was determined by measuring the hydrogen donating effect using 1,1-diphenyl-2-picrylhydrazyl (DPPH; Sigma,

USA), according to Blois [5]. Reaction mixtures containing 0.05 ml of 0.2 mM DPPH solution and 0.1 ml of various concentrations of AE or AEF were plated in a 96-well plate at room temperature in the dark for 30 min. After incubation, the absorbance was measured at 517 nm using a microplate reader (Molecular Devices, USA). Butylated hydroxyanisole (BHA; Sigma) was used as a positive control. DPPH radical scavenging activity was expressed as the percentage of the difference in absorbance between the wells containing sample and those with no sample:

DPPH radical scavenging activity (%) = $1 - (A_{sample} / A_{H2O}) \times 100$

where A_{sample} indicates the absorbance of wells containing sample and A_{H2O} indicates the absorbance of wells containing water.

Assay for ABTS Radical Scavenging Activity

Antioxidant activity was also measured using the ABTS radical cation decolorization assay [25]. ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt; Sigma) was dissolved to a concentration of 7 mM in water. ABTS radical cation (ABTS$^+$) was produced by reacting the ABTS stock solution with 2.4 mM potassium peroxodisulfate (Sigma) and allowing the mixture to stand in the dark at room temperature for 12 h. The radical stock solution was diluted to the absorbance value of 0.70 (± 0.02) at 734 nm with 99% ethanol. Reaction mixtures containing 0.1 ml of ABTS solution and 0.1 ml of various concentrations of AE or AEF were plated in a 96-well plate at room temperature in the dark for 7 min. After incubation, the absorbance was measured at 734 nm using a microplate reader. BHA was used as a positive control. ABTS radical scavenging activity was determined using the following equation:

ABTS radical scavenging activity (%) = $1 - (A_{sample} / A_{H2O}) \times 100$

where A_{sample} indicates the absorbance of wells containing sample and A_{H2O} indicates the absorbance of wells containing water.

Zymography

MMP-1 and MMP-3 enzyme activity in cell culture medium was evaluated by collagen zymography. The culture medium was collected and concentrated using a speed vacuum concentrator. The same amount of total protein in each sample was separated by electrophoresis through a 12% (w/v) SDS-polyacrylamide gel containing 0.02% (w/v) collagen (Sigma). The gels were washed twice with washing buffer (50 mM Tris-HCl (pH 7.5), 2.5% Triton X-100) and once with reaction buffer (50 mM Tris-HCl (pH 7.5), 5 mM CaCl₂, 0.15 M NaCl) on an orbital shaker at room temperature for 15 min. After incubation in reaction buffer at 37°C for 24 h, the activity bands were visualized by staining with 0.1% Coomassie brilliant blue R-250, followed by destaining with 50% methanol-10% acetic acid. Collagenase activity was visualized as a light translucent zone on a blue background.

Statistical Analysis

The results are expressed as the mean ± standard deviation. The statistical significance of the data was analyzed by one-way analysis of variance (ANOVA) using the SPSS program (SPSS Inc., USA). ANOVA was followed by Scheffe's protected least significant difference post hoc test to determine the significance of group differences. A p value of <0.05 was considered to be statistically significant.

Results

AEF Protects Dermal Fibroblast Cells from UVB- and Oxidative-Stress-Induced Senescence More Efficiently than AE

We examined the effects of AE and AEF on the viability of dermal fibroblast HS68 cells. As shown in Fig. 1A, AF and AEF showed no cytotoxicity at concentrations of up to 500 μg/ml. Therefore, we concluded that it was safe to administer both AF and AEF to this cell line. Next, we investigated whether AE and AEF protected HS68 cells from senescence induced by UVB irradiation and oxidative stress. Cellular senescence can be characterized by the appearance of SA-β-gal activity [18]. HS68 cells were pretreated with UVB (20 mJ/cm²) or H₂O₂ (300 μM), followed by incubation with AF or AEF (500 μg/ml) prior to SA-β-gal staining. There was a significant increase in SA-β-gal staining in the UVB- or H₂O₂-treated cells, as compared with the untreated control cells (Figs. 1B and 1C). The percentage of SA-β-gal staining in the UVB- or H₂O₂-pretreated cells incubated with AEF was significantly reduced ($p < 0.05$), as compared with that in the UVB- or H₂O₂-pretreated cells that were subsequently exposed to vehicle alone. In contrast, exposure to AE did not produce a significant decrease in SA-β-gal staining ($p > 0.05$), indicating that fermentation of AE by *L. plantarum* and *B. bifidum* enhanced the anti-aging effect of AE on skin fibroblasts. The effect of control ferment (with no AE present) on SA-β-gal staining was negligible (data not shown).

We also identified other senescent characteristics in these dermal fibroblast cells. Activation of p53 and p21$^{Cip1/WAF1}$ is essential for the establishment and maintenance of senescence [2, 24]. Thus, we determined whether the p53 and p21$^{Cip1/WAF1}$ protein levels were changed in HS68 cells pretreated with UVB- or H₂O₂. As shown in Fig. 2, the protein expression levels of p53 and p21$^{Cip1/WAF1}$ were upregulated in UVB- or H₂O₂-pretreated cells. When UVB- or H₂O₂-pretreated cells were incubated with AEF, however, the protein levels of p53 and p21$^{Cip1/WAF1}$ were significantly reduced ($p < 0.05$), as compared with those in the UVB- or H₂O₂-pretreated cells that were subsequently exposed to vehicle alone. Again, the reduction in the levels
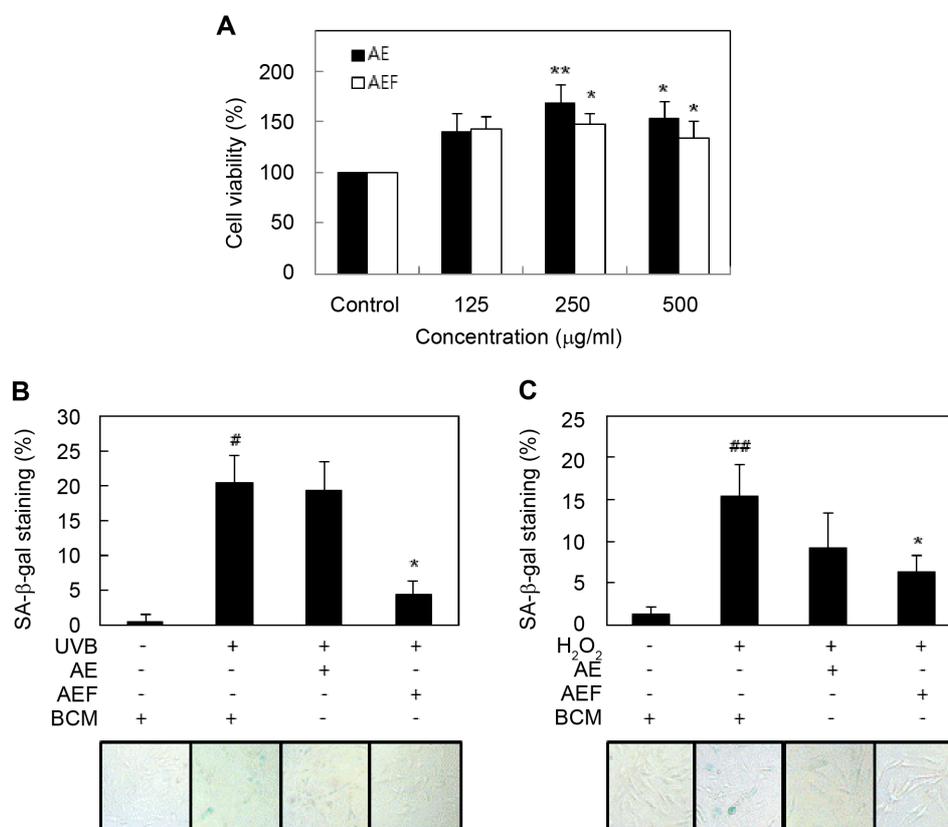
Fig. 1. Fermented *A. koreanum* extract protects dermal fibroblast cells from senescence induced by ultraviolet B (UVB) irradiation and oxidative stress.

(**A**) HS68 cells were treated with various concentrations of *A. koreanum* extract (AE) or fermented *A. koreanum* extract (AEF) for 48 h and cytotoxicity was determined by MTS assay. Bacterial culture medium (BCM) without AE was added for the negative control. Control cells were assigned 100% viability. *$p < 0.05$, **$p < 0.01$, versus control cells. (**B** and **C**) HS68 cells were treated with 20 mJ/cm^2 UVB (**B**) or 300 μM H$_2$O$_2$ for 3 h (**C**), and then further treated with 500 μg/ml of AE or AEF in serum-free medium for 48 h. Cellular senescence was measured by senescence-associated β-galactosidase staining, expressed as the percentage of positively stained cells. #$p < 0.05$, ##$p < 0.01$ versus untreated control cells; *$p < 0.05$ versus UVB- or H$_2$O$_2$-treated cells. AEF was a 1:2 mixture of AE fermented by *L. plantarum* for 12 h and AE fermented by *B. bifidum* for 60 h.

of these proteins observed following AE treatment was not significant ($p > 0.05$). The effect of control ferment (with no AE present) on the levels of p53 and p21$^{Cip1/WAF1}$ was negligible (data not shown). Taken together, the present results suggest that AEF can effectively protect skin fibroblast cells from photoaging and oxidative stress.

AEF Suppresses UVB- or H$_2$O$_2$-Induced ROS Generation in Dermal Fibroblast Cells More Effectively than AE

Since ROS production is one of the upstream mediators of p53 stabilization in senescent cells [4, 7], we tested whether AE and AEF reduced ROS production in UVB- or H$_2$O$_2$-pretreated HS68 cells. To accomplish this, UVB- or H$_2$O$_2$-pretreated cells were incubated with CM-H$_2$DCFDA or DHE, which are indicators of cellular ROS production.

UVB or H$_2$O$_2$ treatment significantly increased ROS production relative to that observed in control cells, as indicated by a rightward shift in fluorescence detected by flow cytometry. Treatment with either AE or AEF strongly suppressed ROS production in UVB- or H$_2$O$_2$-pretreated cells. However, AEF showed a more effective antioxidant activity (Figs. 3A and 3B). The effect of control ferment (with no AE present) on ROS production was negligible (data not shown).

To examine whether the antioxidant effect of AE and AEF in cells was direct or indirect, their antioxidant activities were measured in vitro. DPPH and ABTS radical scavenging activity assays are widely used to evaluate antioxidant activity [5, 25]. BHA was used as a positive control. As shown in Figs. 3C and 3D, both AE and AEF showed concentration-dependent DPPH and ABTS radical
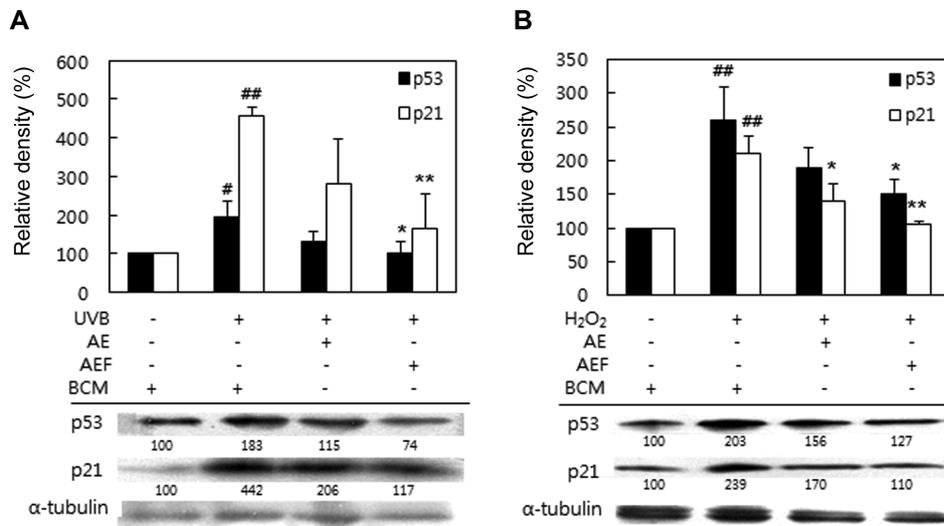
Fig. 2. Fermented *A. koreanum* extract attenuates the ultraviolet B (UVB)- or H_2O_2-induced upregulation of p53 and p21$^{Cip1/WAF1}$ in dermal fibroblast cells.

HS68 cells were treated with 20 mJ/cm^2 UVB (**A**) or 300 μM H_2O_2 for 3 h (**B**), and then treated with 500 μg/ml of *A. koreanum* extract (AE) or fermented AE (AEF) in serum-free medium for 48 h. Bacterial culture medium (BCM) without AE was added for the negative control. Expression of p53 and p21$^{Cip1/WAF1}$ protein was determined by western blotting. α-Tubulin served as the loading control. The densitometry data are expressed as a percentage of the untreated control cell signal (upper panels). Values shown are means ± SD. $^{#}p < 0.05$, $^{##}p < 0.01$, $^{###}p < 0.001$ versus untreated control cells; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus UVB- or H_2O_2-treated cells. Representative western blot images are shown in the lower panels.

scavenging activities. However, the radical scavenging activity of AEF was higher than that of AE (Figs. 3C and 3D). Taken together, the present results indicate that AEF can reduce the ROS level in UVB- or H_2O_2-treated skin fibroblast cells.

AEF Inhibits UVB- or H_2O_2-Induced MMP Activation in Dermal Fibroblast Cells More Strongly than AE

MMP-1 and -3 are UVB-inducible MMPs that play a major role in skin fibroblast photoaging [6, 10]. Collagen zymography showed that pretreatment with UVB or H_2O_2 resulted in stimulation of MMP-1 and -3 activities in HS68 cells; this effect was attenuated by treatment with AE or AEF (Figs. 4A and 4B). Furthermore, the UVB- or H_2O_2-mediated induction of the MMP-1 protein level was also reduced by treatment with AE and AEF (Figs. 4C and 4D). However, treatment with AEF produced greater reduction in both MMP activity and MMP-1 expression, as compared with treatment with AE, suggesting that fermentation may enhance the anti-wrinkle effects of AE on skin fibroblasts.

AEF Inhibits UVB- and H_2O_2-Mediated Upregulation of MMP-1 via an Effect on MAPK Signaling

Expression of MMP-1 is regulated by NF-κB, which is activated by the MAPK signaling cascade [3, 6, 10, 19]. To

examine whether AEF attenuated UVB- or H_2O_2-induced MMP-1 expression in skin fibroblasts via an effect on MAPK signaling, we determined the effects of AEF on the expression of Iκ-Bα and NF-κB in UVB- or H_2O_2-pretreated cells. UVB or H_2O_2 treatment reduced Iκ-Bα expression and increased the levels of p65 (a component of NF-κB) and p-p65 in HS68 cells. This effect was significantly attenuated by treatment with AEF, which increased Iκ-Bα expression and reduced the levels of p65 and p-p65 in UVB- or H_2O_2-treated cells (Figs. 5A and 5B). To investigate whether AEF modulated the levels of Iκ-Bα and NF-κB via the MAPK signaling pathway, the cells were further treated with C2 ceramide, a MAPK activator. This treatment suppressed the effect of AEF on NF-κB activation; Iκ-Bα expression was reduced and the levels of p65 and p-p65 were increased relative to the UVB- or H_2O_2-pretreated cells incubated with AEF. Furthermore, C2 ceramide suppressed the AEF-mediated reduction of MMP-1 expression in cells treated with UVB or H_2O_2. Collectively, these data suggest that AEF suppresses UVB- and H_2O_2-mediated MMP-1 upregulation via an effect on the MAPK signaling pathway.

Discussion

UVB radiation, which leads to ROS production in cells, is

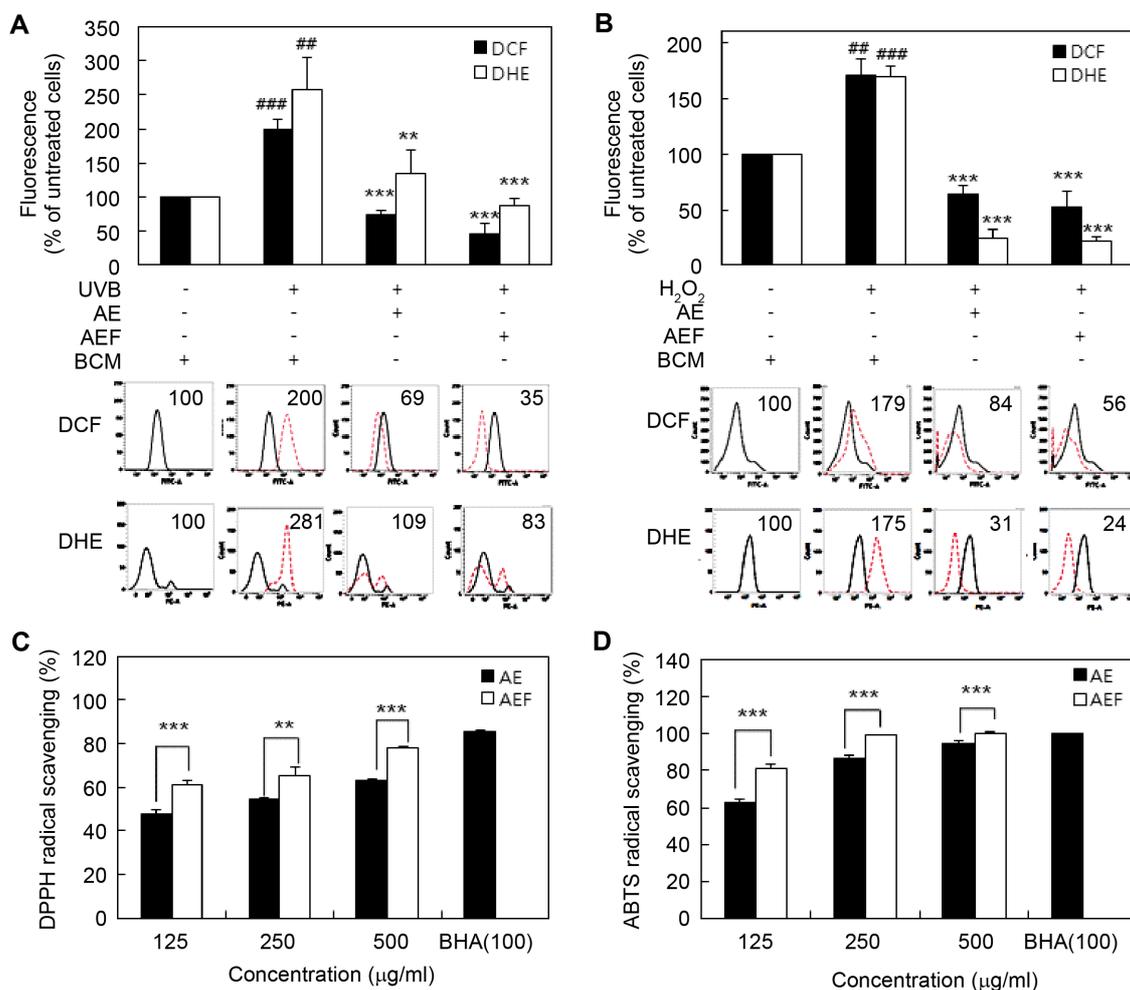**Fig. 3.** Fermented *A. koreanum* extract suppresses ultraviolet B (UVB)- or H_2O_2-induced reactive oxygen species (ROS) generation in dermal fibroblast cells.

(**A** and **B**) HS68 cells were treated with 20 mJ/cm^2 UVB (**A**) or 300 μM H_2O_2 for 3 h (**B**), and then treated further with 500 μg/ml of *A. koreanum* extract (AE) or fermented AE (AEF) in serum-free medium for 48 h. Bacterial culture medium (BCM) without AE was added for the negative control. The cells were incubated with 5 mM CM-H$_2$DCFDA or dihydroethidium (DHE), and fluorescence intensity was determined by flow cytometry analysis. The fluorescence intensity data are expressed as a percentage of the untreated control cell signal (upper panels). Values shown are means ± SD. Representative histograms are shown (lower panels). The fluorescence intensity value is shown in the upper-right corner of the histograms. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ versus untreated control cells; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ versus UVB- or H_2O_2-treated cells. (**C** and **D**) The free radical scavenging activities of AE and AEF were determined by 1,1-diphenyl-2-picrylhydrazyl (DPPH) (**C**) or 2,2′-azino-bis 3-ethylbenzothiazoline-6-sulfonic acid (ABTS) (**D**) radical scavenging assays. $**p < 0.01$, $***p < 0.001$ versus AE.

a well-characterized and major environmental stressor that accelerates skin aging. Oxidative stress also plays an important role in intrinsic skin aging [1, 12, 17, 26–29]. Cellular ROS can be produced by various intracellular systems that include the mitochondrial electron transport chain, cytochrome P450, lipoxygenases, NADPH oxidases, and cyclooxygenases [21]. The use of materials exhibiting antioxidant activity, therefore, can provide a promising approach for the treatment and prevention of both extrinsic

and intrinsic skin aging. The antioxidant capacities of herbal medicine extracts containing phenolic or isoflavonoid components have therefore attracted attention within the cosmetic industry [9, 14, 23]. The present study developed an effective approach to protect skin fibroblasts against UVB irradiation and oxidative stress by using a root extract of *A. koreanum* and characterized the underlying mechanisms involved in its anti-photoaging effect in HS68 cells.
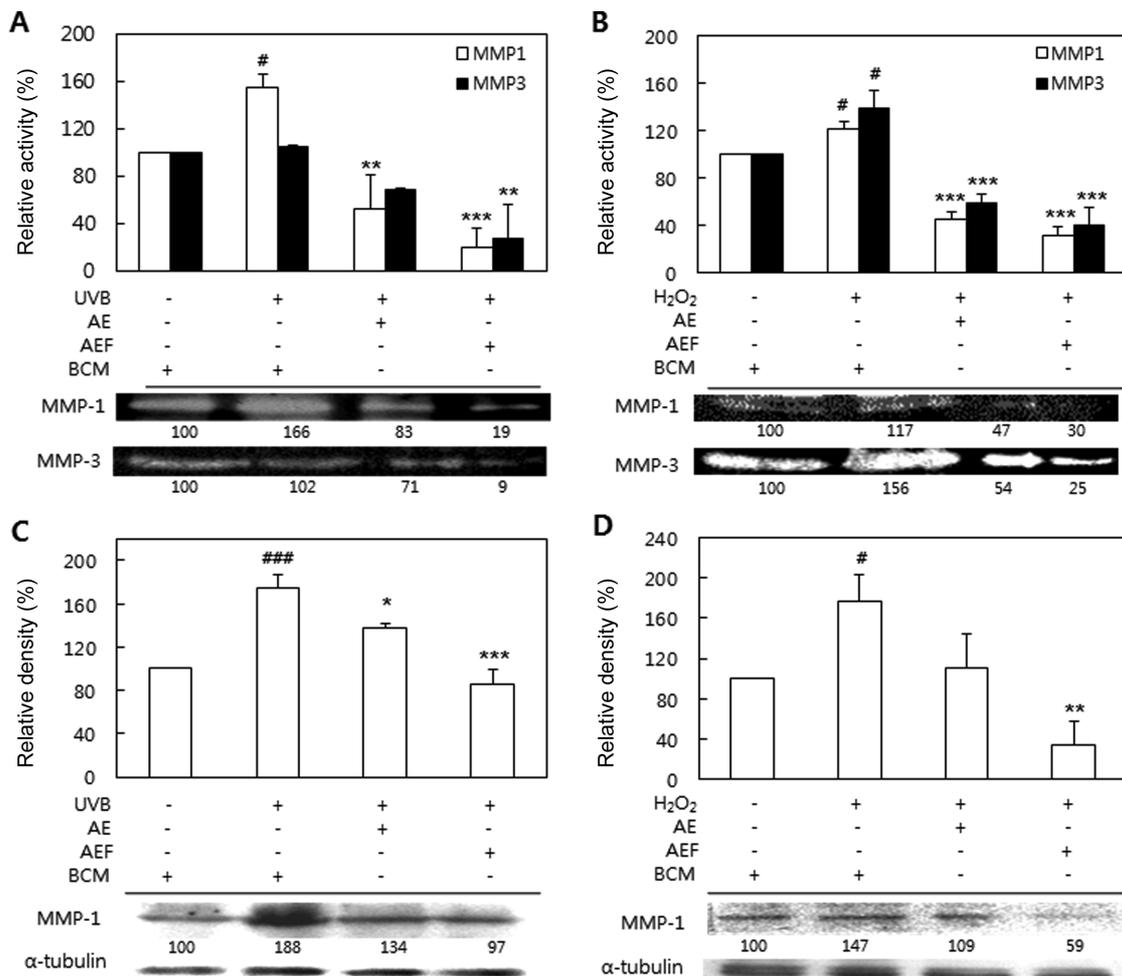
This study showed that both UVB irradiation and H_2O_2

Fig. 4. Fermented *A. koreanum* extract suppresses ultraviolet B (UVB)- or H_2O_2-induced matrix metalloproteinase (MMP) activation in dermal fibroblast cells.

HS68 cells were treated with 20 mJ/cm^2 UVB (**A** and **C**) or 300 μM H_2O_2 for 3 h (**B** and **D**), and then treated further with 500 μg/ml of *A. koreanum* extract (AE) or fermented AE (AEF) in serum-free medium for 48 h. Bacterial culture medium (BCM) without AE was added for the negative control. (**A** and **B**) Enzyme activity of MMP-1 and MMP-3 was determined by collagen zymography. (**C** and **D**) Expression of MMP-1 protein was determined by western blotting. α-Tubulin served as the loading control. The densitometry analysis data are expressed as a percentage of the signal observed in untreated control cells (upper panels). Values shown are means ± SD. #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$ versus untreated control cells; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus UVB- or H_2O_2-treated cells.

treatment produced comparable stimulation of aging-related biological markers, such as an increase in SA-β-gal activity and p53 and p21$^{Cip1/WAF1}$ protein levels, and generated ROS in human dermal fibroblast HS68 cells. We found that AE successfully suppressed the intracellular ROS production (Figs. 3A and 3B) induced by UVB irradiation and H_2O_2 treatment in HS68 cells, but its effects on SA-β-gal activity and expression of p53 and p21$^{Cip1/WAF1}$ were negligible (Figs. 1 and 2). Fermentation by microorganisms has been reported to produce or enhance bioactive substances [11, 13, 32] and we therefore fermented AE with *L. plantarum*

and *B. bifidum*. We found that AEF, a mixture produced by blending the AE fermented by 5% *L. plantarum* for 12 h and the AE fermented by 5% *B. bifidum* for 60 h at a 1:2 ratio, exhibited greater inhibition of SA-β-gal activity (Fig. 1), p53 and p21$^{Cip1/WAF1}$ expression (Fig. 2), and ROS production (Figs. 3A and 3B) in HS68 cells treated with UVB or H_2O_2, as compared with AE. Analysis of DPPH and ABTS radical scavenging capacity also indicated that AEF had greater antioxidant activity than AE (Figs. 3C and 3D). Thus, the antioxidant activities of AE and AEF may be due to their reducing capacities, which allow them to ameliorate oxidative
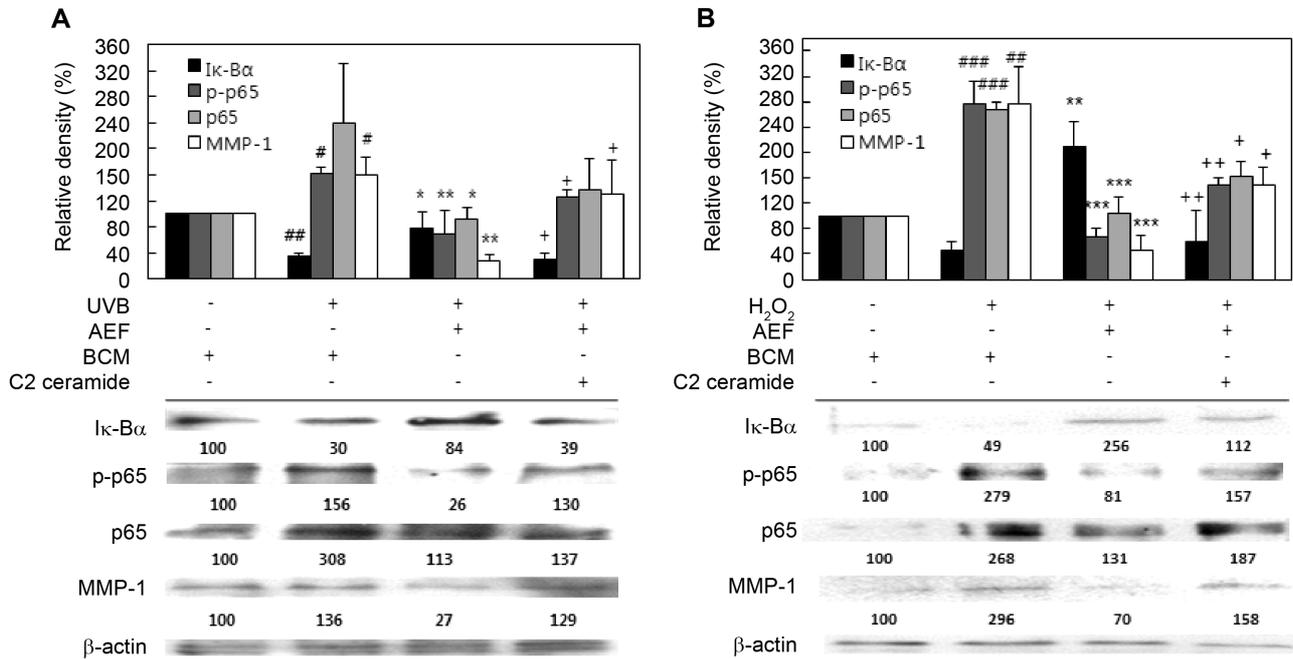
A

B

Fig. 5. Fermented *A. koreanum* extract (AEF) inhibits ultraviolet B (UVB)- and H_2O_2-mediated upregulation of matrix metalloproteinase-1 (MMP-1) via an effect on mitogen-activated protein kinase (MAPK) signaling.

HS68 cells were treated with 20 mJ/cm^2 UVB (**A**) or 300 μM H_2O_2 for 3 h (**B**), and then treated further with 500 μg/ml of AEF for 48 h. Bacterial culture medium (BCM) without AE was added for the negative control. Some cells were treated with C2 ceramide (30 μM) for 6 h immediately prior to harvesting. Levels of Iκ-Bα, the phosphorylated form of p65 (p-p65), p65, and MMP-1 protein were determined by western blotting. β-Actin served as the loading control. The densitometry analysis data are expressed as a percentage of the signal in the untreated control cells. Values shown are means ± SD. $^{#}p < 0.05$, $^{##}p < 0.01$, $^{###}p < 0.001$ versus untreated control cells; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus UVB- and H_2O_2-treated cells; $^{+}p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$ versus UVB- and H_2O_2-treated cells exposed to AEF.

stress. Taken together, these data suggest that fermentation of AE with *L. plantarum* and *B. bifidum* significantly enhanced its antioxidant and anti-aging capacity.

UVB irradiation and oxidative stress upregulate the expression of MMPs in cells [6, 10]. MMP-1 cleaves several types of collagen, whereas MMP-3 activates proMMP-1. MMP-1 and MMP-3 are thus mainly responsible for the decreased elasticity of the dermis attributable to collagen degradation and are used as major markers of UVB-induced wrinkles. In this study, we found that AE suppressed the enhanced activity of MMP-1 and -3 and the overexpression of MMP-1 in dermal fibroblast cells treated with UVB or H_2O_2. Consistently, AEF exhibited greater reduction of UVB- or H_2O_2-induced activation of MMP-1 and -3 and MMP-1 overexpression than did AE (Fig. 4).

The p38 MAPK−NF-κB signaling pathway has been shown to modulate expression of MMPs [3, 6, 10, 19]. The current study found that the expression of Iκ-Bα was downregulated and both p65 and p-p65 were upregulated in HS68 cells treated with UVB or H_2O_2 and that AEF

significantly attenuated these effects, indicating that AEF suppressed UVB- or H_2O_2-induced overexpression of MMP-1 by modulation of NF-κB activation. Finally, this effect of AEF on the protein levels of MMP-1, Iκ-Bα, p65, and p-p65 in HS68 cells treated with UVB or H_2O_2 was attenuated by treatment with a MAPK activator (Figs. 5A and 5B). Taken together, these results suggest that the effect of AEF on UVB- or ROS-mediated MMP-1 upregulation is, at least partly, dependent on the p38 MAPK−NF-κB signaling pathway in dermal fibroblast cells (Fig. 6).

In conclusion, we have identified a powerful agent for the protection of human skin cells from UVB irradiation and oxidative stress. The present study indicated for the first time that AEF exhibited greater antioxidant and anti-photoaging capacities than AE. In addition, AEF reduced UVB- or H_2O_2-mediated MMP-1 overexpression via an effect on the MAPK−NF-κB pathway in skin fibroblast cells. AEF, therefore, could be a valuable candidate for cosmetic anti-photoaging and anti-wrinkle effects. To our best knowledge, no studies have reported the use of lactic
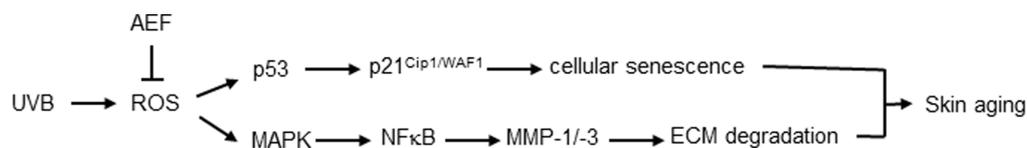
Fig. 6. Possible anti-photoaging mechanism underlying the effects of fermented *A. koreanum* extract (AEF).
Ultraviolet B (UVB) irradiation induces generation of reactive oxygen species (ROS), which results in activation of matrix metalloproteinase (MMP) -1 and -3 through the mitogen-activated protein kinase (MAPK)-nuclear factor-κB (NF-κB) signaling pathway and upregulation of p53 and p21$^{Cip1/WAF1}$ in skin fibroblast cells. This process can be significantly attenuated by AEF-mediated elimination of ROS.

acid fermentation to enhance the functional activities of *A. koreanum*. Previous studies have reported that the roots of *A. koreanum* contain a number of substances, including eleutheroside B, eleutheroside E, sesamin, and acanthoic acid [8, 16, 20, 22, 30, 31]. These and/or other compounds may be responsible for the anti-photoaging activity of AE. However, the mechanism underlying the enhanced anti-photoaging capacity of AEF remains to be elucidated. Studies to isolate and identify the compounds in AEF that exert anti-photoaging effects will be carried out in the future.

Acknowledgments

References

1. Akazaki S, Nakagawa H, Kazama H, Osanai O, Kawai M, Takema Y, Imokawa G. 2002. Age-related changes in skin wrinkles assessed by a novel three-dimensional morphometric analysis. *Br. J. Dermatol.* **147:** 689-695.

2. Al-Mohanna MA, Al-Khodairy FM, Krezolek Z, Bertilsson PA, Al-Houssein KA, Aboussekhra A. 2001. p53 is dispensable for UV-induced cell cycle arrest at late G1 in mammalian cells. *Carcinogenesis* **22:** 573-578.

3. Bell S, Degitz K, Quirling M, Jilg N, Page S, Brand K. 2003. Involvement of NF-κB signalling in skin physiology and disease. *Cell. Signal.* **15:** 1-7.

4. Bickers DR, Athar M. 2006. Oxidative stress in the pathogenesis of skin disease. *J. Invest. Dermatol.* **126:** 2565-2575.

5. Blois MS. 1958. Antioxidant determination by the use of a stable free radical. *Nature* **181:** 1199-1200.

6. Bond M, Baker AH, Newby AC. 1999. Nuclear factor kappaB activity is essential for matrix metalloproteinase-1 and -3 upregulation in rabbit dermal fibroblasts. *Biochem. Biophys. Res. Commun.* **264:** 561-567.

7. Cadet J, Wagner JR. 2013. DNA base damage by reactive oxygen species, oxidizing agents, and UV radiation. *Cold Spring Harb. Perspect. Biol.* **5:** a012559.

8. Cai XF, Lee IS, Shen G, Dat NT, Lee JJ, Kim YH. 2004. Triterpenoids from *Acanthopanax koreanum* root and their inhibitory activities on NFAT transcription. *Arch. Pharm. Res.* **27:** 825-828.

9. Chen L, Hu JY, Wang SQ. 2012. The role of antioxidants in photoprotection: a critical review. *J. Am. Acad. Dermatol.* **67:** 1013-1024.

10. Di Girolamo N, Coroneo MT, Wakefield D. 2003. UVB-elicited induction of MMP-1 expression in human ocular surface epithelial cells is mediated through the ERK1/2 MAPK-dependent pathway. *Invest. Ophthalmol. Vis. Sci.* **44:** 4705-4714.

11. Di Marzio L, Cinque B, Cupelli F, De Simone C, Cifone M, Giuliani M. 2008. Increase of skin-ceramide levels in aged subjects following a short-term topical application of bacterial sphingomyelinase from *Streptococcus thermophilus*. *Int. J. Immunopathol. Pharmacol.* **21:** 137-143.

12. Farage MA, Miller KW, Elsner P, Maibach HI. 2008. Intrinsic and extrinsic factors in skin ageing: a review. *Int. J. Cosmet. Sci.* **30:** 87-95.

13. Fiore R, Miller R, Coffman SM. 2013. *Mycobacterium mucogenicum* infection following a cosmetic procedure with poly-L-lactic acid. *J. Drugs Dermatol.* **12:** 353-357.

14. Fisher GJ, Datta SC, Talwar HS, Wang ZQ, Varani J, Kang S, Voorhees JJ. 1996. Molecular basis of sun-induced premature skin ageing and retinoid antagonism. *Nature* **379:** 335-339.

15. Goldstein S. 1990. Replicative senescence: the human fibroblast comes of age. *Science* **249:** 1129-1133.

16. Hahn DR, Kim CJ, Kim JH. 1985. A study on the chemical constituents of *Acanthopanax koreanum* Nakai and its pharmaco-biological activities. *Yakhak Hoeji* **29:** 357-361.

17. Jenkins G. 2002. Molecular mechanisms of skin ageing. *Mech. Ageing Dev.* **123:** 801-810.

18. Jeon SM, Lee SJ, Kwon TK, Kim KJ, Bae YS. 2010. NADPH oxidase is involved in protein kinase CKII down-regulation-mediated senescence through elevation of the level of reactive oxygen species in human colon cancer cells. *FEBS Lett.* **584:** 3137-3142.

19. Lewis DA, Spandau DF. 2008. UVB-induced activation of NF-kB is regulated by the IGF-1R and dependent on p38 MAPK. *J. Invest. Dermatol.* **128:** 1022-1029.

20. Lin QY, Jin LJ, Cao ZH, Xu YP. 2008. Inhibition of inducible

nitric oxide synthase by *Acanthopnax senticosus* extract in RAW264.7 macrophages. *J. Ethnopharmacol.* **118:** 231-236.

21. Liou GY, Storz P. 2010. Reactive oxygen species in cancer. *Free Radic. Res.* **44:** 479-496.

22. Nhiem NX, Kim KC, Kim AD, Hyun JW, Kang HK, Van Kiem P, *et al.* 2011. Phenylpropanoids from the leaves of *Acanthopanax koreanum* and their antioxidant activity. *J. Asian Nat. Prod. Res.* **13:** 56-61.

23. Poljšak B, Fink R. 2014. The protective role of antioxidants in the defence against ROS/RNS-mediated environmental pollution. *Oxid. Med. Cell. Longev.* **2014:** 671539.

24. Poon RY, Jiang W, Toyoshima H, Hunter T. 1996. Cyclin-dependent kinases are inactivated by a combination of p21 and Thr-14/Tyr-15 phosphorylation after UV-induced DNA damage. *J. Biol. Chem.* **271:** 13283-13291.

25. Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. 1999. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radic. Biol. Med.* **2:** 1231-1237.

26. Rittié L, Fisher GJ. 2002. UV-light-induced signal cascades and skin aging. *Ageing Res. Rev.* **1:** 705-720.

27. Takema Y, Yorimoto Y, Kawai M. 1995. The relationship between age-related changes in the physical properties and development of wrinkles in human facial skin. *J. Soc. Cosmet. Chem.* **46:** 163-173.

28. Tsatsou F, Trakatelli M, Patsatsi A, Kalokasidis K, Sotiriadis D. 2012. Extrinsic aging: UV-mediated skin carcinogenesis. *Dermatoendocrinol.* **4:** 285-297.

29. Watson RE, Gibbs NK, Griffiths CE, Sherratt MJ. 2014. Damage to skin extracellular matrix induced by UV exposure. *Antioxid. Redox Signal.* **21:** 1063-1077.

30. Wu YL, Jiang YZ, Jin XJ, Lian LH, Piao JY, Wan Y, *et al.* 2010. Acanthoic acid, a diterpene in *Acanthopanax koreanum*, protects acetaminophen-induced hepatic toxicity in mice. *Phytomedicine* **17:** 475-479.

31. Yang EJ, Moon JY, Lee JS, Koh J, Lee NH, Hyun CG. 2010. *Acanthopanax koreanum* fruit waste inhibits lipopolysaccharide-induced production of nitric oxide and prostaglandin E2 in RAW 264.7 macrophages. *J. Biomed. Biotechnol.* **2010:** 715739.

32. Yang HS, Choi YJ, Oh HH, Moon JS, Jung HK, Kim KJ, *et al.* 2014. Antioxidative activity of mushroom water extracts fermented by lactic acid bacteria. *J. Korean Soc. Food Sci. Nutr.* **43:** 80-85.