



## Antioxidant Activity and Anti-wrinkle Effects of *Aceriphyllum rossii* Leaf Ethanol Extract

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We evaluated the antioxidant activity and anti-wrinkle effects of *Aceriphyllum rossii* leaf ethanol extract (ARLEE) *in vitro* using human dermal fibroblasts. The total polyphenol and flavonoid contents of ARLEE were 578.6 and 206.3 mg/g, respectively. At a concentration of 250 µg/mL, the electron-donating ability of ARLEE was 87.1%. In comparison with the vehicle, ARLEE treatment at 100 µg/mL significantly increased type I procollagen synthesis ( $p < 0.01$ ) by 50.7%. *In vitro* ARLEE treatment (10 mg/mL) inhibited collagenase and elastase activity by 97.1% and 99.2%, respectively. Compared with the control, ascorbic acid treatment at 100 µg/mL significantly decreased matrix metalloproteinase (MMP)-1 protein expression ( $p < 0.01$ ) by 37.0%. ARLEE treatment at 50 µg/mL significantly decreased MMP-1 protein expression ( $p < 0.01$ ) by 46.1%. Ascorbic acid and ARLEE treatments at 100 µg/mL significantly decreased MMP-1 mRNA expression ( $p < 0.01$ ) by 26.1% and 36.1%, respectively. From these results, we conclude that ARLEE has excellent antioxidant activity and even better anti-wrinkle effects than ascorbic acid in human dermal fibroblasts. These results suggest that ARLEE could be used in functional cosmetics for the prevention or alleviation of skin wrinkles induced by ultraviolet rays.

**Key words:** *Aceriphyllum rossii*, Antioxidant activity, Collagenase activity, MMP-1, Type I procollagen

### INTRODUCTION

Skin aging is commonly associated with increased wrinkling, sagging, and skin laxity (1). Chronic sun exposure especially causes photoaging of human skin (2). Ultraviolet (UV) light is well known to cause various types of skin damage that lead to premature aging. Collagen accounts for roughly 90% of the extracellular matrix (ECM) in human dermis; type I collagen accounts for at least 80% of the total collagen, and alterations in its structure have been considered to be a primary cause of skin aging and wrinkle formation (3).

Matrix metalloproteinases (MMPs) are a family of zinc-dependent endoproteinases that are capable of degrading almost all of the components of the ECM. They have been classified in more than 20 species (4), and as neutral endo-

peptidases, are produced by various cell types such as fibroblasts, macrophages, endothelial cells, and keratinocytes (5). The expression of MMPs in UV-irradiated fibroblasts is known to be initiated by reactive oxygen species (ROS) (6). MMPs play a pivotal role in dynamic remodeling of the ECM, including degradation of ECM proteins. Ascorbic acid has been shown to increase ECM synthesis as well as assist in the maintenance of ECM proteins, while transforming growth factor (TGF)-β1 may induce the synthesis of type I collagen in the fibrin-based matrix as well as other natural matrices (7). Among the MMPs, MMP-1 plays a major role in the enhanced degradation of dermal type I collagen during UV-induced skin photoaging (8). TGF-β is a potent growth factor that stimulates the production of collagen types I, II, III, IV, V, and VII, elastin, and fibronectin (9), but suppresses the synthesis of MMP-1 (10).

*Aceriphyllum rossii* Engler (family Saxifragaceae), a species endemic to Korea, is a perennial herb that grows on damp rocks along valleys, usually to a height of 30 cm. The main chemical constituents of *A. rossii* are pentacyclic triterpenoids (11). In previous studies, anticarcinogenic (12), antioxidant, anti-inflammatory, antiobestic, antidiabetic (13), and antimicrobial (14) activities of *A. rossii* have been reported. However, its inhibitory efficacy on skin wrinkling

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has been poorly investigated.

In this study, the antioxidant activity and inhibitory effects of *A. rossii* leaf ethanol extract (ARLEE) against skin wrinkling were investigated. The anti-wrinkle effects of ARLEE were evaluated with regard to collagen synthesis, collagenase and elastase activity, and MMP-1 mRNA and protein expression in human dermal fibroblasts.

## MATERIALS AND METHODS

**Reagents and apparatus.** 1,1-Diphenyl-2-picryl hydrazyl (DPPH), tannic acid, Folin-Ciocalteu's phenol reagent, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), elastase from porcine pancreas, N-succinyl-(L-Ala)<sub>3</sub>-*p*-nitroanilide, collagenase, and 4-phenylazobenzyloxy-carbonyl-Pro-Leu-Gly-Pro-D-Arg were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), Roswell Park Memorial Institute (RPMI)-1640 medium, fetal bovine serum (FBS), and penicillin/streptomycin (P/S) were purchased from Lonza Company (Cascade, USA).

An inverted microscope (CKX41, Olympus, Japan) was used for observing cell growth, and a CO<sub>2</sub> incubator (MCO-17AC, SANYO electric, Japan) was used for cell culture.

**Preparation of *Aceriphyllum rossii* ethanol extracts.** The leaf, stem, and root of *A. rossii* were collected from Yanggu, Gangwon, Korea. Pulverized samples (50 g) were put into a flask and extracted with 500 mL of 80% ethanol 3 times for 24 hr each at 25°C. The extract was filtered with filter papers and concentrated using a rotary vacuum evaporator (BÜCHI R-205, BÜCHI Labortechnik AG, Switzerland) followed by lyophilization (yield: leaf 22.8%, stem 12.0%, and root 8.6%).

**Total polyphenol content.** The Folin-Denis assay (15) was performed to determine the total polyphenol content of ARLEE, *A. rossii* stem ethanol extract (ARSEE), and *A. rossii* root ethanol extract (ARREE). One milliliter each of ARLEE, ARSEE, ARREE, and Folin reagent was placed in a test tube and allowed to stand for 3 min before adding 1 mL of 10% Na<sub>2</sub>CO<sub>3</sub> and shaking vigorously. The tubes were incubated for 1 hr at room temperature before measuring the absorbance at 725 nm. A standard curve was prepared using tannic acid.

**Total flavonoid content.** The total flavonoid content of ARLEE, ARSEE, and ARREE was determined using a modified Davies method (16). ARLEE, ARSEE, and ARREE (100 µL) were placed in a test tube before adding 1 mL of diethylene glycol reagent and 100 µL of 1 N NaOH. The mixture was shaken vigorously and incubated at 37°C for 1 hr before measuring the absorbance at 420 nm. A standard curve was prepared using rutin.

**Electron donating ability.** Electron-donating ability was evaluated as previously described (17). One milliliter of ARLEE, ARSEE, and ARREE was dissolved in distilled water at 250, 500, or 1,000 µg/mL and placed in a test tube before adding 4 mL of 0.4 mM DPPH. The mixture was shaken vigorously and incubated for 10 sec at 60°C before measuring the absorbance at 525 nm. Ascorbic acid was used as the positive control.

**Collagenase activity inhibition.** Calcium chloride (4 mM) dissolved in 0.1 M Tris-HCl buffer (pH 7.5) was used as the final buffer with substrate 4-phenylazo-benzyloxy-carbonyl-Pro-Leu-Gly-Pro-D-Arg (0.15 mg/mL) dissolved in the buffer. The substrate solution (250 µL) and 100 µL of an adequate concentration of the sample were injected into the tube. *Clostridium histolyticum* collagenase was dissolved in the final buffer to achieve a concentration of 0.2 mg/mL, and 150 µL of it was added. After incubation at 37°C for 30 min, the reaction was stopped by adding citric acid (6%). The reaction mixture was separated by adding ethyl acetate. The absorbance of the supernatant was measured at 324 nm, with ascorbic acid as the positive control.

**Elastase activity inhibition.** In order to evaluate the inhibition of elastase activity, the amount of released *p*-nitroaniline, which is hydrolyzed from the substrate N-succinyl-(L-Ala)<sub>3</sub>-*p*-nitroanilide by elastase, was read with an absorbance at 410 nm. In brief, 2.9 mM N-succinyl-(L-Ala)<sub>3</sub>-*p*-nitroanilide was prepared in 100 mM Tris-HCl buffer (pH 8.0), and this solution was added to the stock sample. Each sample solution was diluted to a final concentration of 1.25, 2.5, 5, or 10 mg/mL. The solutions were mixed thoroughly by tapping before an elastase (0.2 unit/mL) stock solution was added. Solutions were incubated for 10 min at 37°C, and the absorbance was measured at 410 nm.

**Cell culture and morphological observation.** The human dermal fibroblasts used in this study were obtained from Amore Pacific Company (Korea). The cells were grown in DMEM supplemented with 10% FBS and 1% P/S in a humidified 5% CO<sub>2</sub> atmosphere at 37°C for 72 hr. Human dermal fibroblasts were treated with 25~200 µg/mL ARLEE, and the morphology of the cells observed following 48 hr of growth at 37°C and 5% CO<sub>2</sub>. Following incubation, the medium was changed and the cells observed under an inverted microscope.

**UVA irradiation and sample treatment.** Human dermal fibroblasts were cultivated in a culture dish until approximately 80% confluency at 200 nm. After removing the medium, the cells were washed with PBS. The samples were treated to the cells (1.5 × 10<sup>5</sup> cells/mL) in the DMEM without FBS before irradiation with 6.3 J/cm<sup>2</sup> UVA. This was followed by cultivation for 24 hr and quantification of

MMP-1 expression.

**Cell viability assay.** Cell viability was assessed using the MTT assay to determine the maximum permissible level (MPL) of ascorbic acid and ARLEE in human dermal fibroblasts. Human dermal fibroblasts were placed in a 96-well plate ( $1 \times 10^4$  cells/well) and incubated in a humidified 5% CO<sub>2</sub> atmosphere at 37°C for 24 hr. ARLEE (200 µL) was diluted with phenol red-free DMEM (PRF-DMEM) to concentrations of 25, 50, 100, and 200 µg/mL before addition to the wells; the plate was then incubated for another 48 hr. MTT (0.5 mg/mL) was added to each well and the cells were incubated for a further 3 hr. The plate was centrifuged at 1,000 rpm for 10 min, the supernatant was removed, and 200 µL of DMSO was added to each well. After dissolving the cells for 15 min with a plate-shaker, the absorbance at 540 nm was measured with an ELISA reader (680, Bio-Rad Laboratories, Japan).

**Type I procollagen synthesis.** Human dermal fibroblasts were treated with ARLEE at concentrations of 12.5, 25, 50, and 100 µg/mL, and the supernatants were removed and analyzed with an EIA kit (Takara, Japan). The absorbance at 450 nm was used to calculate the type I procollagen protein yield as described (18), with TGF-β1 (5 ng/mL) used as the positive control.

Human dermal fibroblasts were stabilized in DMEM containing 10% FBS and 1% P/S (DMEM-FBS-P/S), and incubated in a humidified 5% CO<sub>2</sub> atmosphere at 37°C for 24 hr. The cells were harvested and divided into the wells of a 6-well plate in a 500 µL volume with  $0.7 \times 10^5$  cells/well and incubated for 24 hr. After removing the medium, the cells were washed twice with PBS, and 1 mL of PRF-DMEM with 1% P/S was added before incubating for a further 24 hr. The cells were washed with PBS, and 3 mL of PRF-DMEM containing ARLEE at concentrations of 12.5, 25, 50, and 100 µg/mL was added. The cells were incubated for 24 hr, and the supernatants were obtained by centrifugation for 10 min at 12,000 rpm for use as the sample for procollagen protein quantitative analysis. The antibody-peroxidase-conjugate solution (100 µL) was placed into the wells of a 96-well EIA kit and 20 µL of the 4-fold diluted samples were added. The kit was wrapped in foil and incubated at 37°C for 3 hr.

The cells were washed 4 times with PBS; 100 µL of the substrate solution was added, and the foil-wrapped kit was incubated at room temperature for 15 min. The stop solution (100 µL of 1 N H<sub>2</sub>SO<sub>4</sub>) was added, and absorbance at 450 nm was measured 3 times with an ELISA reader. The procollagen protein yield was separated from the protein content as described (19), and quantified with bovine serum albumin (BSA) standards. Cell lysis buffer (80 µL; CellLytic™ B Cell Lysis Reagent, Sigma, USA) was added to the plate; the plate was frozen (-20°C) and thawed 3 times before removing the mixture. The protein content of 1/10 dilution of the freeze-thawed mixture was quantified using the BSA standard curve.

**Western blotting.** Cell lysates treated with 12.5, 25, 50, and 100 µg/mL ARLEE were prepared by sonicating human dermal fibroblasts in 0.1 M Tris-HCl (pH 7.2) buffer containing 1% Nonidet P-40, 0.01% SDS, and a protease inhibitor cocktail (Roche, Mannheim, Germany). The protein concentration of cell lysates was measured using the Pierce Protein Assay Kit (Pierce Biotechnology, Inc., Rockford, USA), with BSA as the standard. Equal amounts of protein (10 µg) were loaded into each lane, and separated by electrophoresis on a 10% polyacrylamide gel. Following transblotting onto nitrocellulose, membranes were incubated with ab 137332 (1:1,000, anti-MMP-1; Abcam, UK) and anti-rabbit IgG (1:1,000; Santa Cruz Biotechnology, USA). Immunoreactive bands were detected by chemiluminescence using electrochemical luminescence reagents (Amersham, Bucks, UK). Band intensity was measured using the Image J program (NIH, Bethesda, USA), with β-actin as an internal control.

**Reverse transcription-polymerase chain reaction (RT-PCR).** The total RNA from the human dermal fibroblasts was extracted with 1 mL of TRIzol (Invitrogen, CA) per the manufacturer instructions, and 5 µg samples were reverse-transcribed in 40-µL of a reaction mixture containing 8 µL of 5 × Molony murine leukemia virus reverse transcriptase (M-MLV RT) buffer, 3 µL of dNTPs (10 mM), 0.45 µL of RNasein (40 U/µL), 0.3 µL of M-MLV reverse transcriptase (200 U/µL; Promega, USA), and 1.5 µL of oligo dT (50 pM/µL; Bioneer, Korea). Single-stranded cDNA was amplified by PCR using 4 µL of 5 × green Go Taq Flexi

**Table 1.** The primer sequences for target genes used for PCR in human dermal fibroblasts

Genes	Primers	Expected size (bp) <sup>1)</sup>
MMP-1 <sup>2)</sup>	Forward (5' → 3')	CGACTCTAGAAACACAAGAG
	Reverse (5' → 3')	AAAGTTAGCTTACTGTCCACACGTT
β-actin	Forward (5' → 3')	ACCGTGAAAAGATGACCCAG
	Reverse (5' → 3')	TACGGATGTCAACGTCACAC

<sup>1)</sup>bp: basepair.

<sup>2)</sup>MMP-1: matrix metalloproteinase-1.

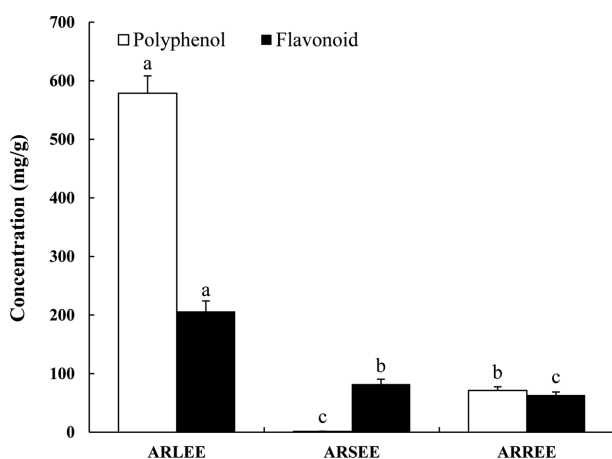
buffer, 0.4  $\mu\text{L}$  of dNTPs (10 mM), 0.1  $\mu\text{L}$  Taq polymerase (5 U/ $\mu\text{L}$ ), 1.2  $\mu\text{L}$  of  $\text{MgCl}_2$  (25 mM; Promega, USA), and 0.4  $\mu\text{L}$  each of (20 pM/ $\mu\text{L}$ ) specific sense and anti-sense primers for MMP-1 or  $\beta$ -actin. The PCR primer sequences are described in Table 1. The PCR conditions were as follows: 28 cycles of 94°C for 60 sec, 50°C for 60 sec, and 72°C for 60 sec. The PCR products were run on a 1.2% agarose gel, with  $\beta$ -actin acting as an internal control to evaluate the relative expression of MMP-1.

**Statistical analysis.** Statistical analysis was performed using SPSS 21.0 (SPSS Inc., Chicago, IL, USA). Differences between the groups were assessed using either one-way analysis of variance (ANOVA) followed by Duncan's multiple range test for a post-hoc comparison or Student's *t*-test. *P* values < 0.05 were considered to be statistically significant.

## RESULTS

**Antioxidant activity.** The total polyphenol contents of ARLEE, ARSEE, and ARREE were 578.6, 1.3, and 71.2 mg/g, respectively, according to the tannic acid standard curve (Fig. 1). The total flavonoid contents of ARLEE, ARSEE, and ARREE were 206.3, 82.3, and 63.5 mg/g, respectively, according to the rutin standard curve (Fig. 1). Electron-donating abilities of ARLEE, ARSEE, and ARREE at 1,000  $\mu\text{g}/\text{mL}$  were 88.7%, 85.1%, and 86.5%, respectively. These values were similar to that of the control ascorbic acid at the same concentration (90.2%) (Fig. 2).

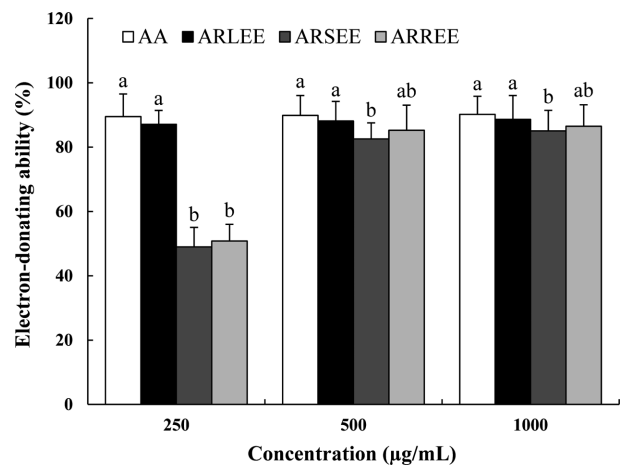
**Collagenase and elastase activity inhibition.** The collagenase inhibitory efficacy of ARLEE at 10 mg/mL was



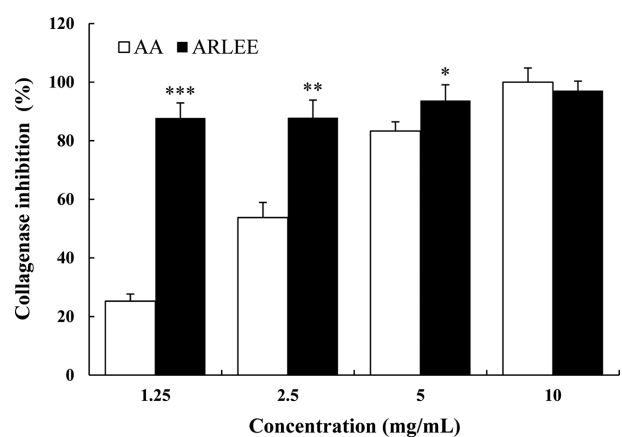
**Fig. 1.** Total polyphenol and flavonoid contents of *Aceriphyllum rossii* ethanol extracts. Values represent the mean  $\pm$  SD of three independent measurements. <sup>a,b,c</sup>Values with different superscripts indicate significant differences ( $p < 0.05$ ) for each polyphenol or flavonoid content, by ANOVA and Duncan's multiple range tests.

97.1%. This value was similar to that of the control, ascorbic acid, at the same concentration (100%) (Fig. 3). ARLEE elastase inhibitory efficacy at 10 mg/mL was 99.2%. This value was significantly ( $p < 0.001$ ) higher than that of the control at the same concentration (51.6%) (Fig. 4).

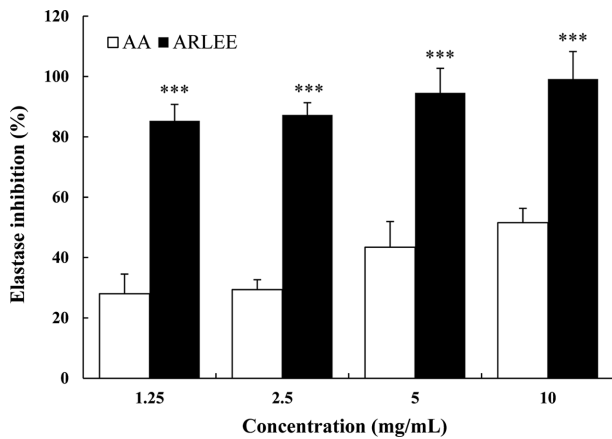
**Cell viability and morphological observation.** Ascorbic acid treatment at concentrations of 100 and 200  $\mu\text{g}/\text{mL}$



**Fig. 2.** Electron-donating ability of *Aceriphyllum rossii* ethanol extracts relative to the control ascorbic acid at the indicated concentrations. Each substance was evaluated on its ability to provide electrons to the free radical DPPH. AA: ascorbic acid, ARLEE: *A. rossii* leaf ethanol extract, ARSEE: *A. rossii* stem ethanol extract, ARREE: *A. rossii* root ethanol extract. Values represent the mean  $\pm$  SD of three independent measurements. <sup>a,b</sup>Values with different superscripts indicate significant differences ( $p < 0.05$ ) for each given concentration, by ANOVA and Duncan's multiple range tests.

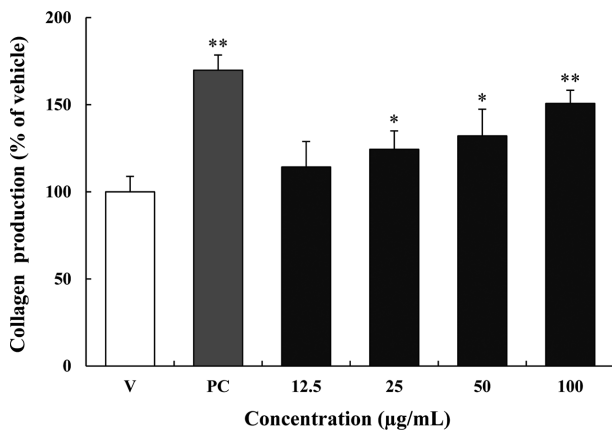


**Fig. 3.** Collagenase activity inhibition of ARLEE relative to the control ascorbic acid. AA: ascorbic acid, ARLEE: *A. rossii* leaf ethanol extract. Values are the mean  $\pm$  SD of three independent measurements. The value with an asterisk is significantly different from the AA group by Student's *t*-test (\*;  $p < 0.05$ , \*\*;  $p < 0.01$ , \*\*\*;  $p < 0.001$ ).



**Fig. 4.** Elastase activity inhibition of ARLEE relative to the control ascorbic acid. AA: ascorbic acid, ARLEE: *A. rossii* leaf ethanol extract. Values are the mean  $\pm$  SD of three independent measurements. The value with an asterisk is significantly different from the AA group by Student's *t*-test (\*\*\*;  $p < 0.001$ ).

reduced cell viability by 6.5% and 30.2%, respectively, compared to vehicle-treated cells. Thus, the MPL for ascorbic acid application to human dermal fibroblasts was 100  $\mu$ g/mL (data not shown). ARLEE treatment at concentrations of 100 and 200  $\mu$ g/mL reduced cell viability by 6.0% and 31.6%, respectively, compared to vehicle-treated cells, exhibiting an MPL of 100  $\mu$ g/mL (data not shown). Fibroblasts treated with low or intermediate concentrations of ascorbic acid maintained the flat, spindle-like shape observed in vehicle-treated cells. However, at a concentration of 200  $\mu$ g/mL, the cells had an irregular or



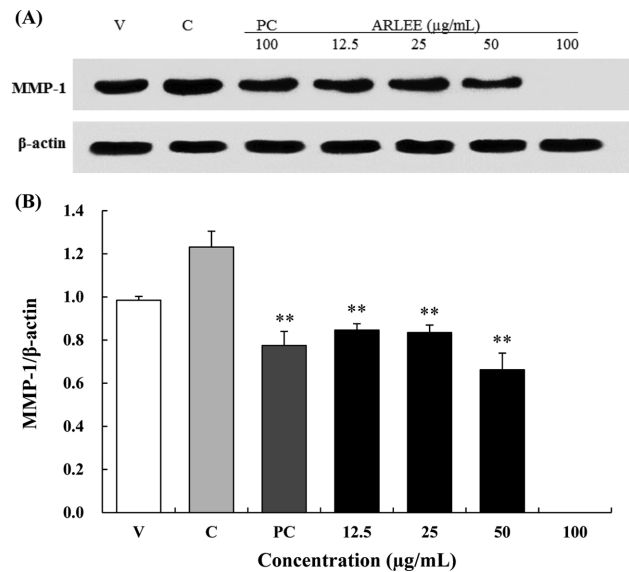
**Fig. 5.** Effect of ARLEE on collagen production in human dermal fibroblasts. Cells were treated with the vehicle (V), 5 ng/mL TGF- $\beta$ 1 (PC), or *A. rossii* leaf ethanol extract (ARLEE) at the indicated concentrations, and the production of procollagen was measured by ELISA. Values are the mean  $\pm$  SD of three independent measurements. The value with an asterisk is significantly different from the vehicle group by Student's *t*-test (\*;  $p < 0.05$ , \*\*;  $p < 0.01$ ).

rounded shape, and cell density was decreased. In contrast, fibroblasts treated with 25~200  $\mu$ g/mL ARLEE maintained the flat, spindle-like shape observed in vehicle-treated cells.

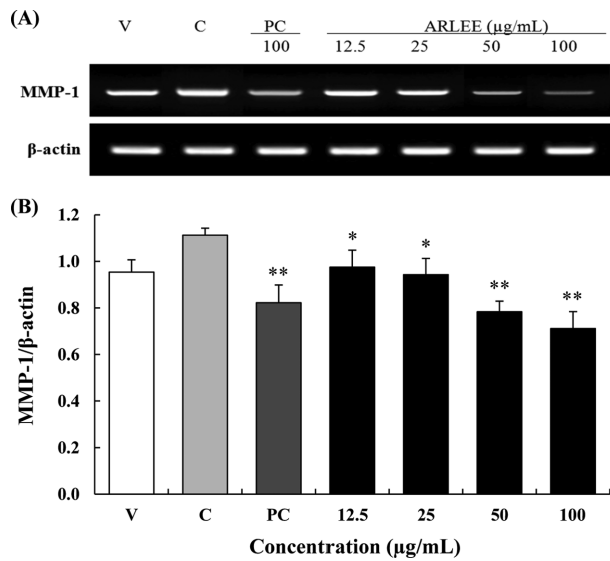
**Effect of ARLEE on type I procollagen synthesis.** In comparison with the vehicle treatment, ARLEE treatment at 12.5, 25, 50, and 100  $\mu$ g/mL increased collagen production by 14.2%, 24.4% ( $p < 0.05$ ), 32.1% ( $p < 0.05$ ), and 50.7% ( $p < 0.01$ ), respectively, in a dose-dependent manner (Fig. 5).

**Effect of ARLEE on MMP-1 protein expression.** ARLEE treatment of cells at 12.5, 25, and 50  $\mu$ g/mL reduced MMP-1 protein expression by 31.2% ( $p < 0.01$ ), 32.2% ( $p < 0.01$ ), and 46.1% ( $p < 0.01$ ), respectively, compared to that in UVA-irradiated control cells (Fig. 6).

**Effect of ARLEE on MMP-1 mRNA expression.** ARLEE treatment of cells at 12.5, 25, 50, and 100  $\mu$ g/mL reduced MMP-1 mRNA expression in a dose-dependent manner by 12.4% ( $p < 0.05$ ), 15.3% ( $p < 0.05$ ), 29.6% ( $p < 0.01$ ), and 36.1% ( $p < 0.01$ ), respectively, compared to that in UVA-irradiated control cells (Fig. 7).



**Fig. 6.** Effect of ARLEE on MMP-1 protein expression in human dermal fibroblasts. (A) MMP-1 protein levels decreased upon treatment with *A. rossii* leaf ethanol extract (ARLEE) compared to UVA-irradiated control cells, as determined by western blotting. Expression was normalized to  $\beta$ -actin levels. (B) Quantification of MMP-1 protein expression in cells treated with vehicle (V), 6.3 J/cm<sup>2</sup> UVA radiation (C), 6.3 J/cm<sup>2</sup> UVA radiation + 100  $\mu$ g/mL ascorbic acid (PC), or 6.3 J/cm<sup>2</sup> UVA radiation + ARLEE at the indicated concentrations. Values are the mean  $\pm$  SD of three independent measurements. The value with an asterisk is significantly different from the control group by Student's *t*-test (\*\*;  $p < 0.01$ ).



**Fig. 7.** Effect of ARLEE on MMP-1 mRNA expression in human dermal fibroblasts. (A) MMP-1 transcript levels decreased upon treatment with *A. rossii* leaf ethanol extract (ARLEE) in a dose-dependent manner compared to UVA-irradiated control cells, as determined by RT-PCR. Expression was normalized to  $\beta$ -actin levels. (B) Quantification of MMP-1 transcript expression in cells treated with the vehicle (V), 6.3 J/cm<sup>2</sup> UVA radiation (C), 6.3 J/cm<sup>2</sup> UVA radiation + 100  $\mu$ g/mL ascorbic acid (PC), or 6.3 J/cm<sup>2</sup> UVA radiation + ARLEE at the indicated concentrations. Values are the mean  $\pm$  SD of three independent measurements. The value with an asterisk is significantly different from the control group by Student's *t*-test (\*;  $p < 0.05$ , \*\*;  $p < 0.01$ ).

## DISCUSSION

Antioxidants protect against potentially damaging oxidative stress, which is a result of an imbalance between the formation of ROS and the body's antioxidant defense. UVA rays absorbed by the skin lead to the generation of ROS in dermal fibroblasts (20). ROS cause oxidative damage to major cellular components and play an important role in different molecular pathways (21).

Phenolic compounds are known to have antioxidant activity due to their capacity to scavenge free radicals and chelate metal ions, along with an ability to prevent free radical formation (22). The DPPH radical has been widely used as a model system to investigate the scavenging activities of several natural compounds including phenolic compounds, flavonoids, and crude mixtures such as ethanol or water extracts of plants (23). In this study, the leaves of *A. rossii* had much higher polyphenol and flavonoid contents than its stems and roots. Therefore, we used ARLEE to evaluate anti-wrinkle effects. The total polyphenol and flavonoid contents of ARLEE were 578.6 and 206.3 mg/g, respectively. These values are higher than the values reported by Lim *et al.* (307.6 and 119.0 mg/g, respectively) (13) and even higher than those for epigallocatechin gallate, a well-

known antioxidant agent (435 and 180 mg/g, respectively) (24). The electron-donating ability of ARLEE at 1,000  $\mu$ g/mL was 88.7%. Lim *et al.* reported its IC<sub>50</sub> value at 549.9  $\mu$ g/mL (13).

Fibroblasts play an important role in production of elastin, collagen, and ground substances such as glycosaminoglycans and proteoglycans (25). The reduction of collagen is either owing to a reduction in the synthetic activity of dermal fibroblasts or an increased rate of degradation by collagenase. Elastin, an insoluble fibrous protein, occupies only 2~4% of the dermis matrix, but plays an important role in maintaining the elasticity of the skin (26). In the current study, ARLEE (1.25~10 mg/mL) strikingly inhibited collagenase and elastase activity in a concentration-dependent manner, exhibiting 97.1% and 99.2% inhibition at 10 mg/mL, respectively, even better than those of ascorbic acid (100.0% and 51.6%).

UV irradiation impairs ongoing collagen synthesis, primarily through the down-regulation of type I procollagen expression (27). MMP-1 is primarily responsible for dermal collagen degradation during the process of skin aging. In this study, levels of carboxy-terminal propeptide of type I procollagen were measured in the medium as a marker of extracellular type I collagen synthesis. In the present investigation of type-I procollagen synthetic ability using human dermal fibroblasts, ARLEE treatment at 100  $\mu$ g/mL significantly ( $p < 0.01$ ) increased type-I procollagen synthesis by 50.7% in comparison with the vehicle control. In addition, ARLEE treatment at 12.5~100  $\mu$ g/mL significantly decreased the protein and mRNA expression levels of MMP-1 in a dose-dependent manner ( $p < 0.05$  or  $p < 0.01$ ).

In conclusion, our study demonstrated that ARLEE strikingly inhibits collagenase and elastase activity, and has high efficacy in the promotion of collagen synthesis and in the inhibition of its degradation in human dermal fibroblasts, even superior to those of ascorbic acid. The potential mechanism of action underlying the anti-wrinkle effects of ARLEE is hypothesized to be due to its outstandingly high contents of phenolic compounds that can scavenge free radicals and prevent free radical formation, along with its ability to chelate MMPs induced by UV rays. Thus, ARLEE could be a useful functional cosmetic in the prevention or alleviation of skin-wrinkling induced by UV rays.

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