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Antioxidants and Inhibitor of Matrix Metalloproteinase-1 Expression from Leaves of *Zostera marina* L.

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In order to develop new anti-photoaging agents, we examined the antioxidative activity and the inhibition effect of matrix metalloproteinase-1 (MMP-1) on the extracts of a marine product, *Zostera marina* L., which is known for its potent activity. Three compounds (compounds **1**, **2**, and **3**) were isolated from an ethyl acetate (EtOAc) soluble fraction of the product; they were identified as apigenin-7-*O*-β-D-glucoside (**1**), chrysoeriol (**2**), and luteolin (**3**). These compounds were found to scavenge radicals and reactive oxygen species (ROS) and were measured to have SC_{50} values of 0.18 mM, 0.68 mM, and 0.01 mM against the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical and 0.04 mM, 0.03 mM, and 0.01 mM against the superoxide radical in the xanthine/xanthine oxidase system, respectively. Compound **3** suppressed the expression of MMP-1 by up to 44% at 4.0 μM and inhibited the production of interleukin 6 (IL-6), which is known as a cytokine that induces MMP-1 expression. From these results, compound **3** and the other compounds were determined to have antioxidative activity and to inhibit MMP-1 expression. Thus, the three compounds are expected to be useful for preventing the photoaging of skin.

Key words: *Zostera marina* L., Antioxidant, MMP-1, Apigenin-7-*O*-β-D-glucoside, Chrysoeriol, Luteolin

INTRODUCTION

The aging of human skin has two elements: changes that occur in all individuals with the passage of time and changes that occur in varying degrees to different individuals as a result of repeated environmental exposure. The primary environmental factor is UV irradiation from the sun (Gilchrest *et al.*, 1994), which produces free radicals and related reactive oxygen species (ROS). These injure the DNA and extracellular matrix (ECM) and cause peroxidation of membrane lipids in skin cells (Brenneisen *et al.*, 1998; Girotti, 1998; Ji, 1999). In addition, UV irradiation has been shown to stimulate the overexpression of genes of matrix metalloproteinases (MMPs) by cytokines, such as interleukins, through DNA damage or the generation of ROS (Wlaschek *et al.*, 1994; Scharffetter-Kochanek *et al.*, 1993). Researches have shown that MMP-1 derived from fibroblasts is induced dose-dependently *in vitro* and *in vivo* by UV irradiation, and this induction was at least partly mediated by interleukins (IL) IL-1α/β and IL-6 (Bauer *et al.*, 1983; Wlaschek *et al.*, 1994).

MMPs constitute a large family of proteases that have been identified and classified in more than 20 species (Hoekstra *et al.*, 2001). These enzymes can degrade most components of the ECM such as collagens, laminins, fibronectins, elastins (Leung *et al.*, 2000; Netzel-Arnett *et al.*, 2002). Histological studies have revealed that the major alterations in photoaged skin are localized in the connective tissue (Fisher *et al.*, 2000). Collagen constitutes about 90% (in dry weight) of skin connective tissue, and MMP-1 is a specific enzyme to collagens. Thus, UV irradiation may cause damage by disorganizing of collagen

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fibrils by MMP reaction, especially MMP-1 (Scharffetter-Kochanek *et al.*, 2000). Since collagen fibrils with elastin are responsible for the strength and resiliency of skin, their disarrangement causes wrinkles and skin aging.

In order to develop anti-photoaging agents, we focused on the ability to scavenge free radicals and ROS and suppress MMP expression and related cytokine production. In the course of screening for anti-photoaging agents in marine plants, we found the ethyl acetate (EtOAc) soluble fraction of *Zostera marina* L. leaves to show significant activity. *Z. marina* is a seagrass that lives in the nearshores of East Asia, Europe, and North America (Lee *et al.*, 2000).

This paper discusses the isolation, structure determination, and biological activity of three compounds isolated from *Z. marina* against DPPH, superoxide radicals, and MMPs.

MATERIALS AND METHODS

General procedures

Z. marina leaves were collected at the western shores of Korea in February 2002. ^{1}H- and ^{13}C-NMR spectra were recorded with Unity INOVA 500 (Varian, USA), and DPX 300 (Bruker, Germany). Chemical shifts were given in δ (ppm) from TMS. EIMS was measured on JMS 700 (JEOL, Japan). TLC was performed on the pre-coated Kieselgel 60 F_{254} (Art. 1.05554. and 1.13895.) and RP-18 F_{254} (Art. 1.05559.) plate (Merck, Germany). Sephadex LH-20 was purchased from Sigma (USA). The organic solvents and chemicals were obtained from Sigma, Bio Whittaker (USA), and Gibco BRL (USA), and purified by the appropriated methods before use.

Extraction and isolation

Dried *Z. marina* leaves (250 g) were refluxed with 70% aqueous ethanol and the extract was evaporated. The extract (75 g) was suspended in water and the suspension was partitioned with hexane (24.0 g), CH_2Cl_2 (11.7 g), EtOAc (2.9 g), and butanol (9.38 g), consecutively. The EtOAc extract (2.8 g) was chromatographed on a Sephadex LH-20 column (3.1×45 cm, 40 → 100% MeOH) to afford 18 subfractions (I~XVIII). Compounds **1** (40 mg) and **3** (90 mg) were obtained by recrystallyzation in MeOH from the subfractions VII and XVII, respectively. Purification of subfraction XIII on a TLC plate (kieselgel 60 F_{254}, CH_2Cl_2: MeOH = 10:1.5) yielded compound **2** (35 mg).

Apigenin-7-*O*-β-D-glucoside (1)

Amorphous pale yellowish powder; $FeCl_3$ Positive; $C_{21}H_{20}O_{10}$ (M. w. 432.39); EIMS *m/z*: 432 $[M]^+$; ^{1}H-NMR (500 MHz, DMSO-d_6): 7.97 (2H, d, *J*=9.0 Hz, H-2', 6'), 6.95 (2H, d, *J*=9.0 Hz, H-3', 5'), 6.87 (1H, s, H-3), 6.84 (1H, d, *J*=2.0 Hz, H-8), 6.45 (1H, d, *J*=2.0 Hz, H-6), 5.08 (1H, d, *J*=7.5 Hz, H-1"), 3.73 (1H, m, H-6"), 3.51 (1H, m, H-6"), 3.45~3.2 (3H, m, H-2", 3", 4"); ^{13}C-NMR (75 MHz, DMSO-d_6) δ: Table I.

Chrysoeriol, luteolin 3'-methyl ether (2)

Amorphous pale yellowish powder; $FeCl_3$ Positive; $C_{16}H_{12}O_6$ (M. w. 300.27); EIMS *m/z*: 300 $[M]^+$; ^{1}H-NMR (500 MHz, DMSO-d_6): 7.58 (1H, d, *J*=9.0 Hz, H-6'), 7.57 (1H, s, H-2'), 6.95 (1H, d, *J*=9.0 Hz, H-5'), 6.90 (1H, s, H-3), 6.52 (1H, d, *J*=1.5 Hz, H-6), 6.20 (1H, d, *J*=1.5 Hz, H-8), 3.90 (3H, s, OCH_3); ^{13}C-NMR (75 MHz, DMSO-d_6) δ: Table I.

Luteolin (3)

Amorphous pale yellowish powder; $FeCl_3$ Positive; $C_{15}H_{10}O_6$ (M. w. 286.24); EIMS *m/z*: 286 $[M]^+$; ^{1}H-NMR (500 MHz, DMSO-d_6): 7.43 (1H, dd, *J*=2.0, 8.0 Hz, H-6'), 7.40 (1H, d, *J*=2.0 Hz, H-2'), 6.90 (1H, d, *J*=8.0 Hz, H-5'), 6.67 (1H, s, H-3), 6.45 (1H, d, *J*=2.0 Hz, H-6), 6.20 (1H, d, *J*=2.0 Hz, H-8); ^{13}C-NMR (75 MHz, DMSO-d_6) δ: Table I.

Table I. ^{13}C-NMR data of compounds **1**, **2**, and **3** isolated from *Zostera marina* L.[a]

No.	^{13}C (δ) in DMSO- d_6		
	1	**2**	**3**
2	164.74	164.89	164.88
3	103.59	104.46	103.64
4	182.48	182.56	182.42
5	161.84	162.19	162.24
6	100.00	99.57	99.59
7	163.44	164.42	164.66
8	95.32	94.80	94.60
9	157.42	158.09	158.05
10	105.82	103.97	104.50
1'	121.51	122.27	122.27
2'	129.09	111.00	114.12
3'	116.47	151.47	146.49
4'	161.59	148.78	150.45
5'	116.47	116.51	116.77
6'	129.09	121.12	119.75
1"	100.38		
2"	73.57		
3"	76.91		
4"	70.03		
5"	77.65		
6"	61.08		
Methoxy-C		56.72	

[a]TMS was used as internal standard; the data of compounds **1**, **2**, and **3** were obtained at 75 MHz. DMSO-d_6 was used as the solvent.

DPPH radical scavenging effect

DPPH radical scavenging effect was evaluated according to the method of Blois, *et al.* with minor modifications (Blois 1958; Hatano *et al.*, 1988). DPPH solution (0.1 mM in MeOH) was added to the same volume of sample solution and allowed to react for 10 min at room temperature, after which the optical density was measured at 565 nm using a microplate autoreader (ELX800, Bio-TEK, USA).

Measurement of superoxide radical scavenging

The scavenging activity on the ROS was measured by monitoring the reduction of nitroblue tetrazolium (NBT) (Furuno *et al.*, 2002). Briefly, the sample, Na_2CO_3 buffer (0.05 M, pH 10.2), xanthine (3 mM in Na_2CO_3 buffer), ethylene diamine tetraacetic acid (3 mM), NBT (0.75 mM), and bovine serum albumin solution were mixed and the reactant was incubated at 25°C for 10 min. Xanthine oxidase (0.25 u/mL) enzyme solution was then added and the sample was further incubated at 25°C for 25 min. The reaction was quenched with $CuCl_2$ (6 mM). The scavenging activity was calculated by comparing the OD at 565 nm of the control with that of the sample.

Cell culture

Human skin fibroblasts (Hs68) and keratinocyte cell lines (HaCaT) were purchased from the American Type Culture Collection. The cells were cultured in Dulbeccos Modified Eagles Medium (DMEM) with 10% fetal bovine serum (FBS) and incubated in a humidified 5% CO_2 incubator at 37°C.

Cytotoxicity assay (MTT assay)

Cell survival was measured by the level of mitochondrial respiration in cells after treating the samples, which was determined by the reduction of the tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), into a blue formazan precipitate. The cells were plated at a density of approximately 1×10^4 cells/well in 96-well flat-bottomed microplates. After sample treatment for 24 h, the cells were incubated in MTT solution (0.5 mg/mL) for 4 h at 37°C. The blue formazan produced was solubilized in 0.4 mL of acid-isopropanol (0.04 N HCl in isopropanol), and the optical density was read at 570 nm. Only cells with functional mitochondria are capable of cleaving MTT to generate the dark purple formazan. The results were expressed in percentages relative to the control (Mosmann *et al.*, 1983).

UVA irradiation and detection of MMP-1 by enzyme linked immunosorbent assay (ELISA)

The expression of MMP-1 induced by UVA irradiation was estimated by modifying the reported methods (Petersen *et al.*, 1992; Dunsmore *et al.*, 1996; Chi *et al.*, 2002). The cells (Hs 68, 5×10^4 cells/well) were seeded into 48 well plates and cultured overnight. Prior to UVA irradiation, the cells were washed twice with phosphate buffered saline (PBS). The cells were irradiated by 4.2 J/cm^2 of UVA source (UVA F15T8.BLB, Sankyo Denki, Japan) emitting wavelengths in the 315~400 nm range. Subsequently, the serum-free DMEM containing the test samples was added and the cells were further incubated for 24 h. The culture medium was then collected and used for the MMP-1 ELISA. All media samples were kept at -20 until assayed. The culture medium was transferred into a 96 well plate and incubated overnight at 37°C. The coated well was washed with PBS-T (PBS containing 0.1% Tween20), followed by blocking with 3% BSA in PBS-T for 2 h at 37°C. After washing, primary antibody diluted to 1/2000 (Mouse monoclonal anti-MMP-1, Sigma) in PBS-T was added into each well and incubated for 1.5 h. Subsequently, secondary antibody diluted to 1/1000 (Goat anti-mouse IgG conjugated with alkaline phosphatase, Sigma) in PBS-T was added into each well and further incubated for 1.5 h. After washing, the substrate solution containing *p*-nitrophenyl phosphate was added. The enzyme reaction was quenched with 3 N NaOH after 30 min, and the optical density was measured at 405 nm using a microplate autoreader.

UVB irradiation and cytokines expression by ELISA

The cells (HaCaT cell) were irradiated by 10 mJ/cm^2 of UVB source (UVB G15T8E, Sankyo Denki, Japan) emitting wavelengths in the 280~360 nm range. Subsequently, the serum-free DMEM containing the test samples was added and the cells were further incubated for 5 h. The culture medium was then collected and used for the cytokines (IL-1α and IL-6) ELISA. The culture medium were transferred into a 96 well plate and incubated overnight. The coated well was washed with PBS-T, followed by blocking with 3% BSA in PBS-T for 2 h at 37°C. After washing, primary antibody (Rabbit anti-human IL-1α/anti-human IL-6, Sigma) diluted in PBS-T was added to each well and incubated for 1.5 h. Subsequently, secondary antibody (Goat anti-rabbit IgG conjugated with peroxidase, Sigma) diluted in PBS-T was added to each well and further incubated for 1.5 h. After washing, the substrate solution containing *o*-phenylene diamine was added. The enzyme reaction was quenched with 2 N H_2SO_4 after 30 min, and the optical density was measured at 490 nm using a microplate autoreader.

Statistical analysis

The results of ELISA assay of MMP-1, IL-1α, and IL-6 were expressed as means±S.D. from three separate experiments. The Student's *t*-test was used to evaluate

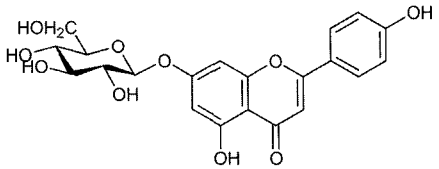
the differences of the means between the control and the samples, accepting $p<0.05$ as significant.

RESULTS AND DISCUSSION

The activity-guided purification of EtOAc soluble fraction was afforded three compounds-compounds **1**, **2**, and **3**. Compound **1** was obtained as an amorphous pale yellowish powder, positive to $FeCl_3$, and showed $[M]^+$ at *m/z* 432 in EIMS spectrum. In ^{1}H-NMR spectrum, two doublet signals at δ 7.97 (d, *J*=9.0 Hz, H-2', 6') and δ 6.96 (d, *J*=9.0 Hz, H-3', 5') suggested the typical A_2B_2 splitting pattern of flavonoid ring B. The singlet signal of 3-H appeared at 6.87, and the *meta*-coupled H-8 and H-6 were detected at δ 6.84 (d, *J*=2.0 Hz) and δ 6.45 (d, *J*=2.0 Hz), respectively. Sugar protons were found at δ 5.08 (1H, d, *J*=7.5 Hz, H-1"), δ 3.73 (1H, m, H-6"), and δ 3.51 (1H, m, H-6"). In ^{13}C-NMR spectrum, carbonyl carbon and C-1" were shown at 182.48 and 100.38, respectively, along with fourteen aromatic carbons (δ 95.32 to 164.74) and five sp^3 carbons (δ 61.08 to 77.65) of the sugar moiety. From these data, compound **1** was postulated to be apigenin-7-*O*-β-D-glucoside. The structure was verified by the reported NMR data (Harborn and Mabry 1982; Jensen *et al.*, 1998; Ersöz *et al.*, 2002).

Compound **2** was positive to $FeCl_3$ and showed $[M]^+$ at *m/z* 300 in EIMS spectrum. In ^{1}H-NMR spectrum, the signals at δ 7.58 (1H, d, *J*=9.0 Hz, H-6'), δ 7.57 (1H, s, H-2'), and δ 6.95 (1H, d, *J*=9.0 Hz, H-5') indicated that flavonoid ring B had a catechol moiety. Two *meta*-coupled protons, which could be assigned to the protons of flavonoid ring A, were observed at δ 6.52 (1H, d, *J*=1.5 Hz, H-6) and δ 6.20 (1H, d, *J*=1.5 Hz, H-8). A methoxyl resonance was observed at δ 3.90. A carbonyl carbon (δ 182.56), and fourteen aromatic carbons (δ 94.80 to 164.89) were detected with a methoxyl carbon (δ 56.72, C-3') in the ^{13}C-NMR spectrum. From the above data, compound **2** was assumed to be chrysoeriol (luteolin 3'-methyl ether). This was finally confirmed by comparing its NMR data with those in the reported references (Brieskorn and Riedel 1977; Harborn and Mabry 1982).

Compound **3** was positive to $FeCl_3$ and $[M]^+$ was detected at *m/z* 286 in EIMS spectrum. The ^{1}H-NMR data were very similar to those of compound **2** except for the absence of methoxyl protons. The proton signals at δ 7.43 (1H, dd, *J*=2.0, 8.0 Hz, H-6'), δ 7.40 (1H, d, *J*=2.0 Hz, H-2'), and δ 6.90 (1H, d, *J*=8.0 Hz, H-5') showed the ABX splitting pattern of the catechol ring. The ^{13}C-NMR spectrum was similar to that of compound **2**. However, the signal of C-3' at B ring was shifted further down field than that of compound **2**. The final structure was identified as luteolin by referring the reported data (Brieskorn and Riedel 1977; Harborn and Mabry 1982; Jensen *et al.*, 1998).



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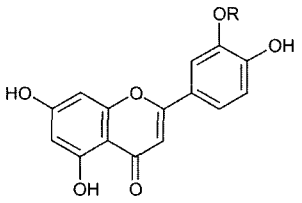

2: R = CH_3
3: R = H

Fig. 1. Structures of compounds **1**, **2**, and **3** isolated from *Zostera marina* L.

The structures of compounds **1**, **2**, and **3** are presented in Fig. 1, and ^{13}C-NMR data are listed in Table I.

The three compounds isolated from *Z. marina* showed strong antioxidative activity. The SC_{50} (the concentration of the sample required for 50% of the free radicals to be scavenged) values of compounds **1**, **2**, and **3** against the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical were 0.18 mM, 0.68 mM, and 0.01 mM, respectively. As summarized in Table II, the SC_{50} of compound **3** was stronger than that of vitamin C (0.06 mM), vitamin E (0.03 mM), and 3-*t*-butyl-4-hydroxyanisole (BHA; 0.08 mM). The SC_{50} values of the three compounds against superoxide radical in the xanthine/xanthine oxidase system were 0.04 mM, 0.03 mM, and 0.01 mM, respectively. Their activities were stronger than that of BHA, which was used as a positive control (Table II).

Table II. Radical scavenging activity of compounds **1**, **2**, and **3** isolated from *Zostera marina* L. (against DPPH and superoxide radical)

Compounds	SC_{50} values (mM) [a]	
	DPPH [b]	Superoxide radical [c]
1	0.18	0.04
2	0.68	0.03
3	0.01	0.01
BHA [d]	0.08	0.18
Vitamin C	0.06	ND [e]
Vitamin E	0.03	ND

[a]SC_{50} value, sample concentration to scavenge reactive oxygen species by 50%. [b]DPPH, 1,1-diphenyl-2-picrylhydrazyl radical. [c]Superoxide radical was produced from xanthine/xanthine oxidase oxidation reaction. [d]BHA, 3-*t*-butyl-4-hydroxyanisole. [e]ND, Not determined.

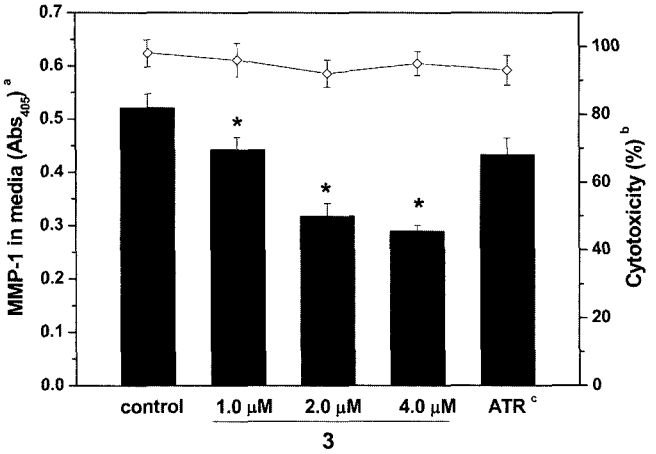

Fig. 2. The suppression activity of compound **3**, isolated from *Zostera marina* L., on the expression of matrix metalloproteinase-1. [a]MMP-1, matrix metalloproteinase-1 (■). The MMP-1 contents of culture media were determined by ELISA as detailed under the Experimental Section. [b]Cytotoxicity was measured by MTT assay (-◇-). The viability of cells was expressed as a percentage. [c]ATR, all-*trans*-retinol. The concentration of all-*trans*-retinol was 4.0 μM. Means±S.D. and t-test significance levels were calculated on the relative values and are presented when the number of wells was 3. **p*<0.05 compared with control.

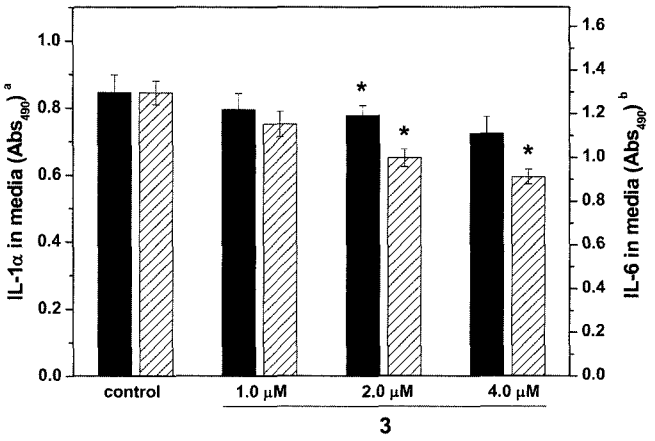

Fig. 3. The suppression activity of compound **3**, isolated from *Zostera marina* L., on interleukins secretion. [a]IL-1α, interleukine 1 (■). [b]IL-6, interleukine 6 (▨). The cytokine content of the culture media was determined by ELISA as detailed under the Experimental Section. Means±S.D. and *t*-test significance levels were calculated on the relative values and are presented when the number of wells was 3. **p*<0.05 compared with control.

Of the three compounds, compound **3** exhibited the most potent antioxidative activity, and the effects of compound **3** on MMP-1 suppression was measured and compared with that of retinoid, a positive control. The inhibitory activity of compound **3** was 44% at 4.0 μM, while that of all-*trans*-retinol was 17% at the same concentration in human skin fibroblasts (Fig. 2). Retinol (Vitamin A), a parent molecule of retinoids, is commonly used as an anti-aging agent in cosmetics (Ramos-e-Silva *et al.*, 2001). According to the previous reports, the inhibitory effects of retinol and various types of retinoic acid were about 50% at 3-6 μM in normal and recessive dystrophic epidermolysis bullosa fibroblasts and human fetal lung fibroblasts (Bauer *et al.*, 1983; Zhu *et al.*, 2001). In this study, compound **3** was more effective than retinol in suppressing MMP-1 in human skin fibroblasts.

In addition, since interleukins are known to stimulate the expression of MMP-1, we tested the effect of compound **3** on them. Compound **3** inhibited the suppression of IL-6 expression by 30% at 4.0 μM, but IL-1α was weak (Fig. 3). Therefore, the inhibitory activity of compound **3** on interleukin production may have an effect on suppressing MMP-1.

Photoaging is due to UV irradiation and related oxidative stress and is characterized by quantitative and qualitative alteration of the dermal connective tissue (Fisher *et al.*, 1996; Fisher *et al.*, 1997). Because collagen is the major structural component of the dermis, degradation of collagens causes resilience reduction and wrinkle formation in the skin. MMP-1 is a specific enzyme to collagens, and therefore its suppressor remains a logical target for protection of ECM degradation (Scharffetter-Kochanek *et al.*, 2000). Accumulating evidence from *in vitro* and *in vivo* studies indicates antioxidative materials, such as quercetin, β-carotene, vitamin A, C, and E, inhibit the expression of MMPs (Greul *et al.*, 2002; Song *et al.*, 2001; Offord *et al.*, 2002). In addition, IL-1α and IL-6 were induced by UV irradiation and were documented to stimulate the synthesis of MMP-1 and other matrix-degrading enzymes in dermal tissue (Bauer *et al.*, 1983; Wlaschek *et al.*, 1994). Therefore, Antioxidants and inhibitors of interleukin secretion are expected to suppress MMP-1 expression, and these can be probably applied to protect collagen degradation and wrinkle formation.

In conclusion, three compounds isolated in this study had strong antioxidative activity. In particular, compound **3** suppressed the expression of MMP-1 because of its antioxidative effect and by inhibiting IL-1α and IL-6 production. Therefore, these compounds are expected to be useful in protecting skin aging from UV irradiation.

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