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Anti-photoaging Constituents from *Eurya emarginata* Leaves

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ABSTRACT. *Eurya emarginata* is an evergreen tree growing in the southern regions of Korea including Jeju Island. In this study, antioxidant and anti-photoaging effects were investigated with the extracts of *E. emarginata* leaves. As comparative studies with the extract and five solvent fractions, the EtOAc fraction showed the most effective DPPH and $ABTS^+$ radical scavenging activities. The EtOAc fraction also inhibited matrix metalloproteinase-1 (MMP-1) production most effectively. Phytochemical study on the EtOAC fraction resulted in the isolation of three compounds; eutigoside C (**1**), eutigoside B (**2**) and quercitrin (**3**). Among the isolates (**1**-**3**), quercitrin (**3**) showed excellent antioxidant activities and inhibitory properties on MMP-1 production. Based on these results, it was suggested that extracts of *E. emarginata* leaves could be developed as natural anti-photoaging agents in cosmetic formulations.

Key words: *Eurya emarginata*, Anti-oxidant, Anti-photoaging, Quercitrin

INTRODUCTION

The desire to maintain beautiful and healthy skin is increasing along with improved living standards. In addition, as outdoor leisure activities become more active, people are exposed to more ultraviolet radiation. This has led to an increased interest in researching the effects of sunlight exposure on skin damage and aging.[1-4]

Skin aging is a complex process that occurs in the layers of the epidermis and dermis. Both internal and external factors influence this process. The most important external factor is ultraviolet rays that are linked to skin photoaging. The free radicals formed as a result of the ultraviolet radiation cause the decomposition of unsaturated lipids of the intercellular cement, cracks in the skin lipid barrier leading to transepidermal water loss, and the structural changes in fibrillar proteins–collagen and elastins.[5]

Solar ultraviolet light reaching the earth surface is a combination of UVB (290–320 nm) and UVA (320–400 nm). UVB rays, which have higher energy than UVA, have been recognized as a key hazard factor generated by solar radiation. However, UVA radiation has now proven to play an increasingly more important role than UVB. In fact, it is because UVA is penetrating deeper into the skin reaching to connective tissue inducing more serious damages.[6]

UV rays reduce the amount of collagen and increase the expression and activity of matrix metalloproteinase (MMP) in the skin.[7] Ultraviolet irradiation also accelerates the degradation of extracellular substrates resulting in decrease of skin collagen and increase of MMP-1 expression that ultimately cleave interstitial collagen leading to skin aging.[8,9]

As UVA penetrates deeply through the layers of the epidermis and dermis, it damages cells by increased incidence of reactive oxygen species (ROS). ROS interacts with lipid-rich membranes, enzymes and cellular DNA, causing oxidative stress, DNA damage, apoptosis and autoantigens release from keratinocytes.[10,11] Recently, development of natural substance to prevent photo-aging by inhibition of MMP-1 and ROS production is being paid attention.

Eurya emarginata is a shrub belonging to Theaceae family with evergreen branches, and this plant is distributed along the southern coast of Korea including Jeju Island. There are more than 100 species of *Eurya* genus worldwide, and only two species are described in Korea.[12] *E. emarginata* leaves have traditionally been used as medicine for diuretic, sputum and abscess removal.[13] *E. emarginata* extract has been reported to possess anti-inflammatory[14] and anti-cancer,[15] with studies showing that it can alleviate symptoms of atopic dermatitis in NC/Nga mice[16] and inhibit melanin synthesis in melan-a cells.[17] However there is no report on anti-photoaging property for this

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plant, our study was performed to confirm the efficacy as well as to identify the active chemical compounds.

EXPERIMENTAL

Chemicals and Reagents

The *n*-hexane, chloroform, ethyl acetate and *n*-butanol were purchased from Samchun Pure Chemical (Pyeongtaek, Gyeonggi-do, KR) and OCI (Jung-gu, Seoul, KR). Silica gel 60 and Methanol-d_4 were purchased from Merck (Darmstadt, DE) and Cambridge Isotope Laboratories Inc. (Tewksbury, MA, USA), respectively. The 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) were purchased from Sigma-Aldrich chemical Co. (St. Louis, MO, USA). Human Dermal Fibroblast (HDFn) were purchased from ATCC (American Type Culture Collection) (Manassas, VA, USA). The MMP-1 human ELISA kit was purchased from Abcam (Cambridge, UK). Dulbecco's modified Eagle's medium (DMEM), heat-inactivated fetal bovine serum (FBS) and Ham's F-12 Nutrient Mix were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Dimethyl sulfoxide (DMSO) was purchased from Amresco (Solon, OH, USA).

Extraction and Isolation

E. emarginata leaves were collected in August 2022 on Jeju Island, Korea. To isolate the effective compounds, dried powdered leaves (200.0 g) of *E. emarginata* were extracted with hot water at 100 ℃ for 4 h. The resulting water solution was combined and filtered. The filtrate was concentrated using a rotary evaporator at 37 ℃ to obtain 55.0 g extract. A portion of the extract (50.0 g) was suspended in water, and successively fractionated into *n*-hexane (Hex), chloroform ($CHCl_3$), ethyl acetate (EtOAc), *n*-butanol (BuOH), and water (H_2O) fractions. The EA layer was subjected to normal silica gel CC with $CHCl_3$-MeOH (4:1) to obtain compounds **1** (19.2 mg), **2** (13.4 mg) and **3** (14.0 mg). The structure of the isolated compounds was identified using a nuclear magnetic resonance (NMR) system (JEOL, Tokyo, Japan). The methanol-d_4 was used as NMR solvents, and the samples were dissolved in 0.5 mL of the solvent. The NMR spectra were recorded on 400 and 100 MHz NMR spectrometer for ^1H and ^{13}C NMR, respectively. The chemical shifts were referenced to solvent signals of methanol- d_4 (δ_H 3.31 and δ_C 49.15 ppm).

Total Polyphenol Content Assessment

The total polyphenol content was measured by applying the Folin-Ciocalteu method.[18] 50 μL of Folin-Ciocalteu's phenol agent were added to 50 μL of each sample solution to react at room temperature for 3 min. 100 μL of 7% (w/v) Na_2CO_3 solution and 300 μL of distilled water were added, reacted at room temperature for 1 h, and then absorbance was measured at 725 nm. The total polyphenol content was obtained after preparing a standard test curve according to concentration using gallic acid as standard substance.

Total Flavonoid Content Assessment

The total flavonoid content was measured according to the colorimetric method.[19] 400 μL of distilled water was added to 100 μL of each sample, and then 30 μL of a 5% $NaNO_2$ solution was added. After 5 minutes, a 10% $AlCl_3$ solution of 30 μL was added to stand for 5 minutes, and then 200 μL of the 1 M NaOH solution was added. Distilled water was added so that the total amount of the mixed solution was 1 mL, and then the absorbance was measured at 510 nm using a microplate reader. As for the reference material, a standard calibration curve according to concentration was prepared using (+)-catechin, and then the total flavonoid content was obtained.

DPPH Radical Scavenging Assay

The Blois method[20] was applied to the DPPH radical scavenging activity experiment. 100 μL of the sample solution diluted by concentration and 100 μL of the 0.2 mM DPPH solution were mixed on a 96 well plate, reacted at room temperature for 10 min, and then absorbance was measured at 517 nm. An ascorbic acid was used as a positive control, and DPPH radical scavenging activity was calculated using the following equation.

※ Scavenging rate (%) = [1-(A-C)/(B-D)] × 100
A: Absorbance of samples
B: Absorbance of control
C: Absorbance of samples not treated with DPPH
D: Absorbance of control not treated with DPPH

ABTS Radical Scavenging Assay

ABTS cationic radical scavenging activity was applied by methods such as Re et al.[21] 7.0 mM ABTS and 2.45 mM potassium persulfate were mixed to form $ABTS^+$ radicals at room temperature for 16 h. The prepared ABTS reagent was diluted 30 times in methanol and used. 100 μL of the sample and 100 μL of the $ABTS^+$ solution were mixed on a 96 well plate, reacted at room temperature for 10 min, and then absorbance was measured at 517 nm. An ascorbic acid was used as a positive control, and ABTS radical scavenging activity was calculated using the following equation.

※ Scavenging rate (%) = [1-(A-C)/(B-D)] × 100
A: Absorbance of samples
B: Absorbance of control
C: Absorbance of samples not treated with ABTS
D: Absorbance of control not treated with ABTS

UV-A Irradiation and Cell Culture

Human dermal fibroblast (HDFn) cells were cultured in DMEM/F-12 (3:1), supplemented with 10% FBS and 1% penicillin/streptomycin at 37 ℃ and 5% CO_2 in a humidified atmosphere. Cells were exposed to a Bio-Link UV irradiation system (Vilber Lourmat, Collegien, France) equipped with UV-A sources. The cells were washed with PBS and covered with PBS until slightly submerged. The cells were exposed to UV-A up to 6 J/cm^2. After UVA irradiation, PBS was replaced with fresh serum-free medium and the experiments were conducted.

Cell Viability Assay

HDFn cells were seeded in 24 well-plates at a density of 7×10^4 cells/well and cultured for 24 h. After washing once, the cells were treated with the extract and compounds at the specified concentrations in serum-free media and incubated for 48 h. The supernatant was removed and 500 μL of MTT reagent per well (0.4 mg/mL in serum-free media) was added, and incubated for 4 h. After removing the MTT reagent, the formed formazan crystals were dissolved by adding DMSO, and the absorbance was measured at 570 nm using a microplate reader (Tecan, Salzburg, Austria).

MMP-1 Production Inhibitory Assay

The measurement was performed using Human MMP-1 ELISA Kit (ab215083, Abcam, MA, USA) to confirm inhibition of MMP-1 (Matrix metalloproteinase-1) production of the extract, fractions and compounds. HDFn cells were divided into 24 well-plates at a concentration of 7×10^4 cells/well and then incubated in a 37 ℃ 5% CO_2 incubator for 24 h. A PBS (phosphate buffered saline) buffer (pH 7.4, Thermo Fisher Scientific, MA, USA) was washed once, and then irradiated 6 J/cm^2 UV-A on PBS. Immediately after UV-A irradiation, the sample was treated using serum-free media and cultured for 24 h. A culture supernatant was taken, and MMP-1 produced according to the manufacturers' protocols was quantified. The MMP-1 production rate was expressed as a percentage (%) based on the UV-A treatment group.

Statistical Analysis

The results of all experiments were performed three times and expressed as mean ± standard deviation (mean ± SD). Statistical differences were calculated using one-way analysis of variance (ANOVA) to compare the control and treatment groups. Statistical analysis was performed using Winks statistics software (TexaSoft, Plano, TX, USA).

RESULTS AND DISCUSSION

We conducted the comparative studies with using the plant extract and its solvent fractions to investigate the anti-oxidative and anti-photoaging effects. The dried *E. emarginata* leaves were extracted with hot water, and the hot water extract was fractionated to afford *n*-Hex, $CHCl_3$, EtOAc, *n*-BuOH and H_2O fractions.

First, the total polyphenol and flavonoid contents for the extract and solvent fractions were measured (*Table* 1). The total polyphenol content (TPC) was 113.11 mg/g GAE for the extract, and the highest content was 296.37 mg/g GAE in the EtOAc layer. Similarly, the total flavonoid content (TFC) was 78.83 mg/g CE for the extract, and the highest content was 168.96 mg/g CE in the EtOAc fraction.

In this study, DPPH radical scavenging activity was also comparatively investigated. The highest activity was observed

***Table* 1.** Total polyphenol and flavonoid contents of extract and solvent fractions from *E. emarginata* leaves. TPC: total polyphenol content, TFC: total flavonoid conetent, GAE: gallic acid equivalents, CE: (+)-catecthin equivalents

	TPC (mg GAE/g)	TFC (mg CE/g)
Extract	113.11	78.83
Hex fr.	71.53	49.47
$CHCl_3$ fr.	83.63	65.47
EtOAc fr.	296.37	168.96
BuOH fr.	148.03	101.71
H_2O fr.	51.23	47.04

***Table* 2.** DPPH and ABTS radical scavenging activity of hot water extracts and solvent fractions of *Eurya emarginata*. SC_{50}: concentration of required to scavenging 50% of DPPH and ABTS radical. N.D.: Not detected

	DPPH radical	$ABTS^+$ radical
	SC_{50} value (μg/mL)	
Extract	93.91 ± 7.62	23.57 ± 1.13
Hex fr.	N.D.	11.55 ± 0.32
$CHCl_3$ fr.	N.D.	36.33 ± 2.52
EtOAc fr.	24.33 ± 0.53	5.01 ± 0.07
BuOH fr.	N.D.	22.88 ± 0.67
H_2O fr.	N.D.	95.85 ± 4.96
Ascorbic acid	3.09 ± 0.06	2.80 ± 0.22

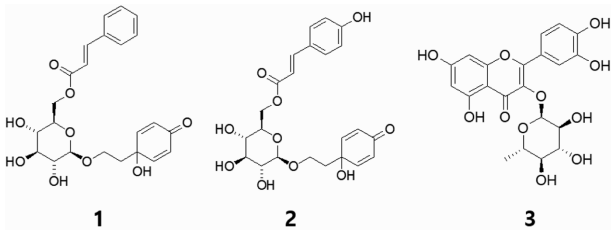

***Figure* 1.** The structures of isolated compounds **1-3** form *E. emarginata* leaves.

in EtOAc fraction with SC_{50} values of 24.33 μg/mL. In addition, in the $ABTS^+$ radical experiment, the EtOAc fraction also showed excellent scavenging activity (SC_{50} 5.01 μg/mL) similar to that of a control material, ascorbic acid (2.80 μg/mL) (*Table* 2).

Inhibition of MMP-1 production was measured by using UV-A stimulated fibroblast cells (*Fig.* 2). The tests were conducted after confirming the appropriate sample concentrations that did not cause cell toxicity. For example, in the case of EtOAc, it was carried out below 80 μg/mL. As shown in *Fig.* 2, the extract and fractions inhibited the production of MMP-1 in dose dependent manner, and the EtOAc fraction was observed to exhibit the most significant activity.

Based on biological activity data, EtOAc layer was chosen for further study to isolate the active compounds. Through chromatographic purification and analyzing NMR spectroscopic data, the compound **1-3** were identified from the fraction. Compound **1** showed 21 signals in the ^{13}C NMR spectrum, where two carbonyl carbons, nine sp^2 carbons, eight oxygen-bearing sp^3 carbons were confirmed. The presence of a glucoside groups was indicated based on the ^1H (4.25, 1H, d, J=7.8 Hz, H-1', and 3.18-4.34 ppm, sugar protons) and ^{13}C NMR signals (64.9-78.0 ppm, sugar carbons). By comparing the obtained data to known previous literature, compound **1** was identified to be eutigoside C.[15] The compound **2** has similar ^{13}C NMR data to those of compound **1**, except for the presence of a signal at 161.5 ppm which was interpreted as phenolic carbon. Therefore, we identified compound **2** as eutigoside B based on previously established values.[15] Compound **3** indicated typical signals of flavonoids in the ^1H-NMR and ^{13}C-NMR spectra. The proton NMR signals at 5.34 and 0.94 ppm, and a carbon NMR signal at 17.8 ppm indicated the presence of a rhamnoside in the structure of **3**. And, the proton signals for B-ring were showed at 6.92-7.34 ppm. The presence of H-6 and and H-8 was indicated by two broad doublet signals (6.20 and 6.37 ppm). By comparing the previous data with the obtained values, compound **3** was identified as a flavonoid, quercitrin.[22]

The anti-oxidant effects of isolated compounds (**1-3**) were measured using DPPH and ABTS radical scavenging methods. As a result, the compound **3** has the best antioxidant effect, whose efficacy was similar to the positive

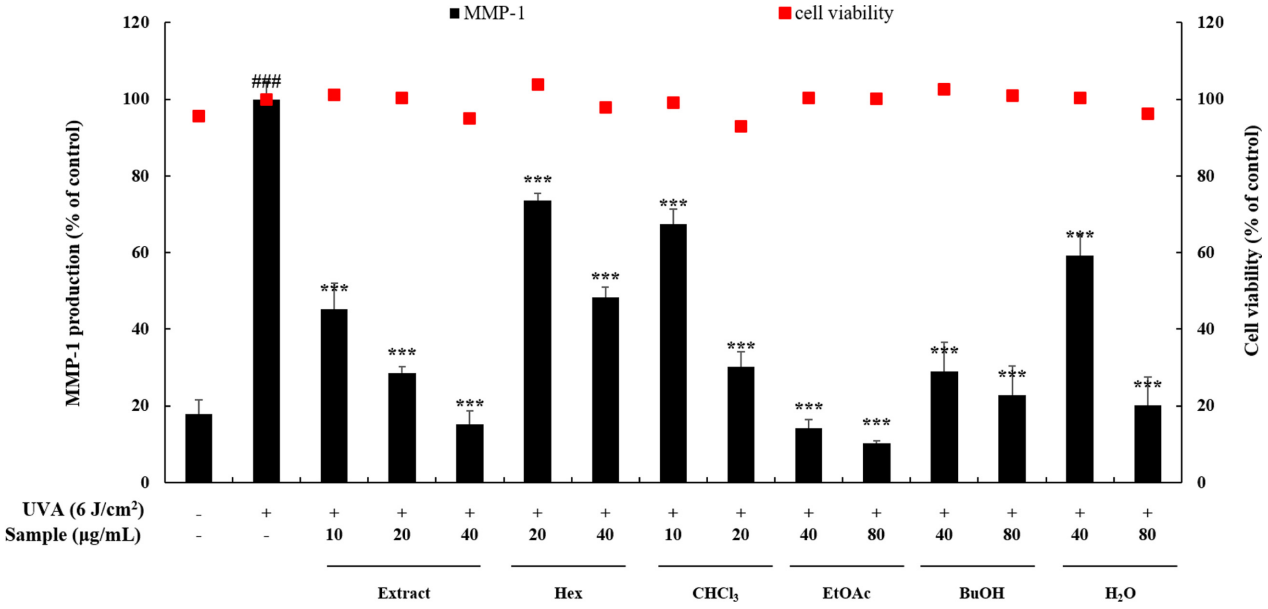

***Figure* 2.** Effects of extract and solvent fractions on MMP-1 production and protein expression in UVA-irradiated HDFn cells. The cells were pretreated with samples for 1 h. After UVA irradiation, cells were incubated in the compounds for an additional 24 h. Data are presented as mean ± SD (n=3). $^{\#\#}p < 0.01$ vs. non-treated control; $^{**}p < 0.01$ vs. UVA-irradiated group.

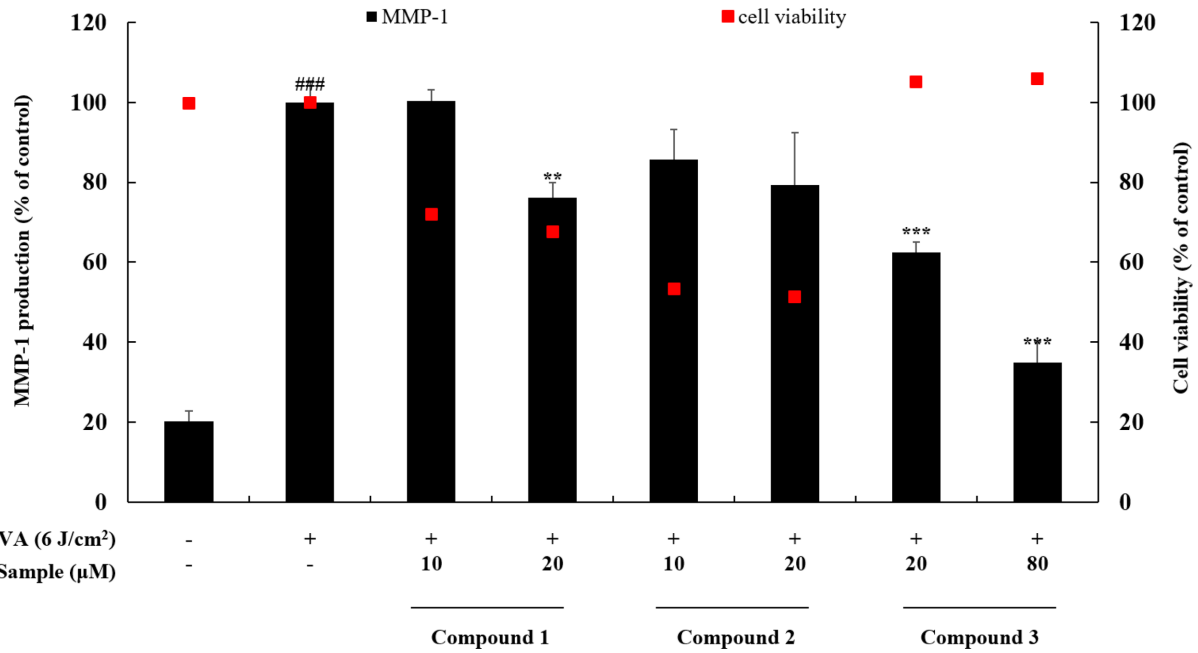

***Figure* 3.** Effects of isolated compounds **1-3** on MMP-1 production and protein expression in UVA-irradiated HDFn cells. The cells were pretreated with samples for 1 h. After UVA irradiation, cells were incubated in the compounds for an additional 24 h. Data are presented as mean ± SD (n=3). $^{\#\#}p < 0.01$ vs. non-treated control; $^{**}p < 0.01$ vs. UVA-irradiated group.

***Table* 3.** DPPH and ABTS radical scavenging activity of isolated compounds **1-3**. IC_{50}: concentration of required to scavenging 50% of DPPH and ABTS radical. N.D.: Not detected

Compound	DPPH radical	$ABTS^{+}$ radical
	IC_{50} value (μM)	
Eutigoside C (1)	N.D.	49.88 ± 10.37
Eutigoside B (2)	N.D.	36.23 ± 2.80
Quercitrin (3)	46.09 ± 1.00	10.10 ± 0.11
Ascorbic acid	38.31 ± 1.55	12.66 ± 0.70

control, ascorbic acid (*Table* 3). In the inhibition test of MMP-1 production, the isolated compound **3** displayed significant effect without causing cytotoxicity at the concentration of up to 80 μM (*Fig.* 3).

The plant *Eurya* genus has previously been investigated in various directions on the utilization of natural materials. For example, *E. japonica* has confirmed its antioxidant and anti-diabetic effects.[23] *E. acuminata* has exhibited anti-cancer and anti-microbial activities.[24] In addition, *E. emarginata* previously has identified its anti-inflammatory, anti-cancer and melanin synthesis inhibitory properties.[14,15,16] Since no research has been published on the MMP-1 in *Eurya* genus, however, this study was conducted in relation to the photo-aging prevention efficacy. The isolated compound quercitrin (**3**) was confirmed as an active ingredient displaying significant activities related to radical scavenge and MMP-1 production, which is in a good agreement with the previous literature results.[25]

CONCLUSION

In this study, the extracts of *E. emarginata* leaves was under investigation to reveal the possibility of use as an anti-oxidant and anti-photoaging materials. In the comparative study, the EtOAc fraction was observed to significantly inhibit MMP-1 production in HDFn cells, and exhibit the highest antioxidant activity. In the additional isolation process for the EtOAc fraction, three compounds (**1-3**) were identified. Among the isolates, quercitrin (**3**) showed strong activities on anti-oxidation as well as inhibition of MMP-1 production. Based on these results, it was suggested that *E. emarginata* could be developed as an effective natural cosmeceutical agent with anti-photoaging effects.

Supporting Information. NMR data information for additional compounds **1-3** can be found in the online version of this article.

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