Antioxidant and Skin Anti-Aging Effects of Marigold Methanol Extract

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Abstract

The objective of this study was to evaluate the antioxidant and anti-aging effects of marigold methanol extract (MGME) in human dermal fibroblasts. Total polyphenolic and flavonoid contents in MGME were 74.8 mg TAE (tannic acid equivalent)/g and 85.6 mg RE (rutin equivalent)/g, respectively. MGME (500 μg/mL) increased 1,1-diphenyl-2-picryl hydrazyl (DPPH) and 2,2′-azino-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical-scavenging, and superoxide dismutase (SOD)-like antioxidant activities by 36.5, 54.7, and 14.8%, respectively, compared with the control. At 1,000 μg/mL, these activities increased by 63.7, 70.6, and 20.6%, respectively. MGME (100 μg/mL) significantly increased the synthesis of type 1 procollagen by 83.7% compared with control treatment. It also significantly decreased Matrix Metalloproteinase-2 (MMP-2) activity and *MMP-1* mRNA expression by 36.5% and 69.5%, respectively; however, it significantly increased *laminin-5* mRNA expression by 181.2%. These findings suggest that MGME could protect human skin against photo-aging by attenuating oxidative damage, suppressing MMP expression and/or activity as well as by stimulating collagen synthesis.

Key words: Antioxidant activity, Marigold methanol extract, MMP-1 mRNA, MMP-2 activity, Skin anti-aging effects, Type I procollagen synthesis

INTRODUCTION

Clinically, photoaging is characterized by wrinkles, laxity, a leathery appearance, increased fragility, blister formation and impaired wound healing. The Matrix Metalloproteinase (MMP)s are a family of zinc-dependent endopeptidases. Although MMP-1 (interstitial collagenase) cleaves collagen type I, MMP-2 is able to degrade elastin as well as basement membrane (BM) compounds including collagen type IV and type VII. MMP-1 is primarily responsible for dermal collagen degradation during the aging process (1), and MMP-2 and MMP-9 degrade the extracellular matrix (ECM) proteins that influence skin thickness and wrinkle formation (2).

In vitro studies with cultured human dermal fibroblasts have shown that there is an increase in reactive oxygen species (ROS) production and a reduction in type 1 collagen synthesis when they are exposed to ultraviolet radiation (3). ROS are thought to be a major factor in the destruction of collagen, which is a hallmark of photo-aging (4). The maintenance of the structural integrity of the connection between the epidermis and dermis, called the dermal-epidermal junction, is important to prevent skin aging (5). The BM is a complex multi-molecular structure which firmly attaches the epidermis to the underlying dermis. Laminins are a family of ECM proteins that are localized mainly in BM, and they regulate various cellular functions such as adhesion, motility, growth, differentiation, and apoptosis through interaction with integrins, their specific cell surface receptors (6). Laminin-5 supports cell adhesion and migration more efficiently than other laminins (7).

Retinol, known as vitamin A, has been the focus of attention as an alternative long-term anti-aging agent (8). How-

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ever, it can't be used in general cosmetic formulations due to its instability upon exposure to light, oxygen, heat, lipid-peroxidation, or water (9). Retinol also has limited application as a major component of medications and cosmetics because it is fat-soluble and a skin-irritant, causing side-effects such as skin dryness (10). Consequently, there is increasing interest in plant-derived extracts that can induce skin anti-aging effects (11,12).

Tagetes erecta L. is a widespread garden plant that is commonly known as the marigold, and it is widely used as a medicinal herb for its anti-inflammatory, analgesic, and anti-edematous properties, which are important for phytotherapeutic, dermatological and cosmetic applications (13,14). Marigold essential oil has been shown to be an effective free radical scavenger, and the ethanol extract is reportedly effective against parakeratosis (15,16).

In this study, we evaluate the oxidant and anti-aging effects of marigold methanol extract (MGME) by examining collagen synthesis and its degradation, as wells as by examining laminin synthesis in human dermal fibroblasts.

MATERIALS AND METHODS

Reagents and apparatus. Dimethyl sulfoxide (DMSO), 1,1-diphenyl-2-picryl hydrazyl (DPPH), 2,2′-azino-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), tannic acid, ascorbic acid, Folin-Ciocalteu's phenol reagent, and 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) were obtained from Sigma-Aldrich (St. Louis, MO, UK). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and penicillin/streptomycin (P/S) were obtained from Lonza (Cascade, MD, USA).

UV irradiation was performed with a UVA sunlamp (Sankyo Denki, Tokyo, Japan) using a UV-radiometer (HD 9021, Delta Ohm, Padova, Italy) to measure the amount applied. Morphological changes were examined and photographed with an Olympus BX51 microscope (Olympus, Tokyo, Japan) and ProRes® C14 plus charge-coupled device camera (Jenoptik Optical Systems, Tokyo, Japan).

Plant material. Methanol extract of the marigold plant (*Tagetes erecta* L.) was obtained from the Korea Plant Extract Bank (Daejeon, Korea). Whole *T. erecta* plants were collected at late autumn from Hyeongyeong-myeon, Muan-gun, Jeollanam-do, Korea. Pulverized samples (100 g) were added to a flask and subjected to extraction thrice for 24 hr each time with 1 L of 100% methanol at 45°C. The extract was filtered through filter paper, concentrated using a rotary vacuum evaporator, and dried. The yield of the *T. erecta* methanol extract was 5.05%. This specimen was dissolved in DMSO before use.

Total polyphenol and flavonoid contents. The Folin-Denis assay (17) was performed to determine the total polyphenol content of the MGME. One milliliter each of the MGME and Folin reagent were placed in a test tube and allowed to stand for 3 min before adding 1 mL of 10% Na_2CO_3 and shaking vigorously. The tubes were incubated for 1 hr at 25°C before measuring the absorbance at 725 nm. A standard curve was prepared using tannic acid. The total flavonoid content of the MGME was determined by a modified Davis method (18). The MGME (100 μL) was 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. The electron donating ability was evaluated as previously described (19). One hundred microliter of MGME dissolved in 0.1% DMSO at 250, 500, or 1,000 μg/mL was placed in a 96-well plate before adding 100 μL of 0.2 mM DPPH. The mixture was shaken vigorously and incubated for 10 sec at 37°C before measuring the absorbance at 517 nm. Catechin was used as the positive control. The DPPH radical scavenging activity of each solution was calculated as the percent of inhibition.

ABTS radical scavenging ability. The ABTS cation decolorization assay (20) was performed with modifications to compare the antioxidant activity of MGME to that of catechin. The ABTS radical cation (ABTS•$^+$) was produced by reacting a 7 mM ABTS solution with 2.4 mM potassium peroxodisulfate at room temperature in the dark for 12 hr, after which it was diluted with ethanol to an absorbance of 0.7 ± 0.02 at 734 nm. The samples were diluted to concentrations of 31.25~1,000 μg/mL, and 3 replicates of each were deposited in the wells of a 96-well plate. An equal volume of ABTS•$^+$ solution was added, and the reaction proceeded for 7 min in the dark at 25°C before measuring the absorbance at 734 nm, which was used to calculate.

Superoxide dismutase (SOD)-like activity. The SOD-like activity of MGME and catechin were determined as previously described (21) for solutions of 31.25~1,000 μg/mL. The solution (0.2 mL) was mixed with 2.6 mL of Tris-HCl buffer (50 mM Tris, 10 mM EDTA, pH 8.5) and incubated at 25°C for 10 min, after which 0.1 mL of 1 M HCl was added stop the reaction. The absorbance at 420 nm was measured to determine the amount of oxidized pyrogallol.

Cell culture. The human dermal fibroblasts (passages 10-12) used in this study were kindly provided by the Amore Pacific Co (Yongin, Korea). The cells were grown in DMEM supplemented with 10% FBS and 1% P/S in a

humidified 5% CO_2 atmosphere at 37°C for 48 hr.

UVA irradiation and sample treatment. The human dermal fibroblasts were cultivated in a culture dish until to being a confluency of approximately 80%. After removing the medium, the cells were washed with PBS. The samples were treated to the cells (1.5×10^5 cells/mL) in the DMEM without FBS before irradiation with 6.3 J/cm^2 UVA followed by cultivation for 24 hr, and used for MMP-2 activity and expression of MMP-1 and laminin-5.

MTT assay. Human dermal fibroblasts were placed in the wells (0.5×10^4 cells/well) of a 96-well plate and incubated in a humidified 5% CO_2 atmosphere at 37°C for 24 hr. The MGME (200 μL) diluted with phenol red-free DMEM (PRF-DMEM) to concentrations of 25~200 μg/mL was added to the wells, and the plate was incubated for another 48 hr. The MTT (0.5 μg/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.

Morphological observation. The human dermal fibroblasts were treated with MGME at concentrations of 12.5~200 μg/mL, and the cells incubated as described above for 48 hr. After changing the medium, the cells were observed microscopically and photographed.

Type I procollagen synthesis assay. Human dermal fibroblasts were treated with MGME at concentrations of 12.5~100 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 1 procollagen protein yield as described (22), with TGF-β1 (5 ng/mL) used as the positive control.

The 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_2 atmosphere at 37°C for 48 hr. The cells were harvested and divided into the wells of a 12-well plate in a 500 μL volume with 0.7×10^5 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 a further 24 hr. The cells were washed with PBS-P/S, and 1.5 mL of PRF-DMEM containing 0.1% DMSO and MGME at concentrations of 12.5~100 μg/mL was added. The cells were incubated for 48 hr, and the supernatants were obtained by centrifugation for 10 min at 12,000 rpm for use as the sample for the 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 1N H_2SO_4) was added and the absorbance at 450 nm was measured 3 times with an ELISA reader. The procollagen protein yield was separated from the protein content as described (23) and quantified with bovine serum albumin (BSA) standards. Cell lysis buffer (80 μL; CelLytic^{TM} B Cell Lysis Reagent, Sigma-Aldrich, St. Louis, MO, USA) was added to the plate, and frozen (−20°C) and thawed 3 times before removing the mixture from the plate. The protein content of 1/10 dilution of the freeze-thawed mixture was quantified using the BSA standard curve.

MMP-2 activity. The samples were adjusted to contain the same amount of protein and mixed with an equal volume of 2× sample buffer to be a 30 μL of composites, incubated at the room temperature for 10 min, and loaded onto a 10% zymogram gel (Invitrogen, Carlsbad, CA, USA). After electrophoretic separation, the gel was incubated with Novex zymogram renaturing buffer for 30 min, Novex zymogram developing buffer for 30 min, and with fresh developing buffer for 24 hr at 37°C. The gel was incubated with Simply Blue^{TM} Safe Stain (Invitrogen), washed with sterile distilled water, and MMP-2 activity was quantified with an image analyzer.

MMP-1 expression. The total RNA from human dermal fibroblasts was extracted with 1 mL of TRIzol (Invitrogen, USA) per the manufacturer instructions, and 5 μg samples were reverse transcribed in 40 μL of reactions containing 8 μL of 5× M-MLV RT (Murine Leukemia Virus Reverse Transcriptase) buffer, 3 μL dNTPs (10 mM), 0.45 μL RNasein (40 U/μL), 0.3 μL M-MLV reverse transcriptase (200 U/μL; Promega, Madison, WI, USA), and 3.75 μL of oligo dT (20 pmol/μL; Bioneer, Korea). Single stranded cDNA was amplified by PCR using 4 μL of 5× Green Go-Taq® Flexi reaction buffer, 0.4 μL dNTPs (10 mM), 0.1 μL Taq polymerase (5 U/μL), 1.2 μL MgCl$_2$ (25 mM; Promega, USA), and 0.4 μL each (20 pmol/μL) of specific sense and anti-sense primers for MMP-1 or β-actin. The PCR primer sequences were as follows: MMP-1 forward, 5′-AAA GGG AAT AAG TAC TGG GC-3′, and reverse, 5′-AAT TCC AGG AAA GTC ATG TG-3′; and β-actin forward, 5′-ATG CAG AAG GAG ATC ACT GC-3′, and reverse, 5′-CTG CGC AAG TTA GGT TTT GT-3′. The expected size of the amplified MMP-1 and β-actin products were 237 and 248 base pairs, respectively. The PCR conditions were 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 β-actin acting as an internal control to evaluate the relative expression of MMP-1.

Laminin-5 expression. The total RNA was isolated from human dermal fibroblasts, reverse transcribed with specific sense and anti-sense primers for laminin-5 or β-actin, and amplified as described above. The PCR primer sequences were as follows: laminin-5 forward, 5'-GAC TGC CTG CTG TGC CAG C-3', and reverse, 5'-GGG GTA GCC ATG AAA GCC CG-3'; and β-actin forward, 5'-ATG CAG AAG GAG ATC ACT GC-3', and reverse, 5'-CTG CGC AAG TTA GGT TTT GT-3'. The expected size of the amplified laminin-5 and β-actin products were 195 and 248 base pairs, respectively. The PCR condition was 35 cycles of 94°C for 45 sec, 60°C for 45 sec, and 72°C for 60 sec. The amplification products were run on a 1.5% agarose gel and visualized with ethidium bromide staining. The DNA band densities were evaluated with the KODAK Gel Logic 100 image analysis system (Eastman Kodak Co., Rochester, NY, USA).

Statistical analysis. The difference in values between groups was statistically evaluated with one-way analyses of variance (ANOVA) followed by post-hoc Duncan multiple range tests using SPSS 18.0 statistical software (SPSS Inc., Chicago, IL, USA). Differences were considered statistically significant for $p < 0.05$.

RESULTS

Antioxidant activities of MGME. The total content of the antioxidants polyphenol and flavonoid in MGME was 74.8 mg TAE (tannic acid equivalent)/g and 85.6 mg RE (rutin equivalent)/g, respectively (Fig. 1A). The electron donating ability of MGME at 500 and 1,000 μg/mL was 36.5% and 63.7%, respectively; these values were lower than for the control catechin at the same concentrations, which were 87.9% and 97.8%, respectively (Fig. 1B). The ABTS radical scavenging ability of MGME at 500 and 1,000 μg/mL was 54.7% and 70.6%, respectively; these values were lower than for the control catechin (98.8% and 100.7%, respectively; Fig. 1C). The SOD-like activity of MGME at 500 and 1,000 μg/mL was 14.8% and 20.6%, respectively, which was lower than for catechin (22.8% and 30.0%, respectively; Fig. 1D). Together, these results indicate that MGME has lower antioxidant activities than the control compound catechin.

Human dermal fibroblast viability. The MTT assay was used to determine the maximum permissible level (MPL) of MGME in human dermal fibroblasts. MGME
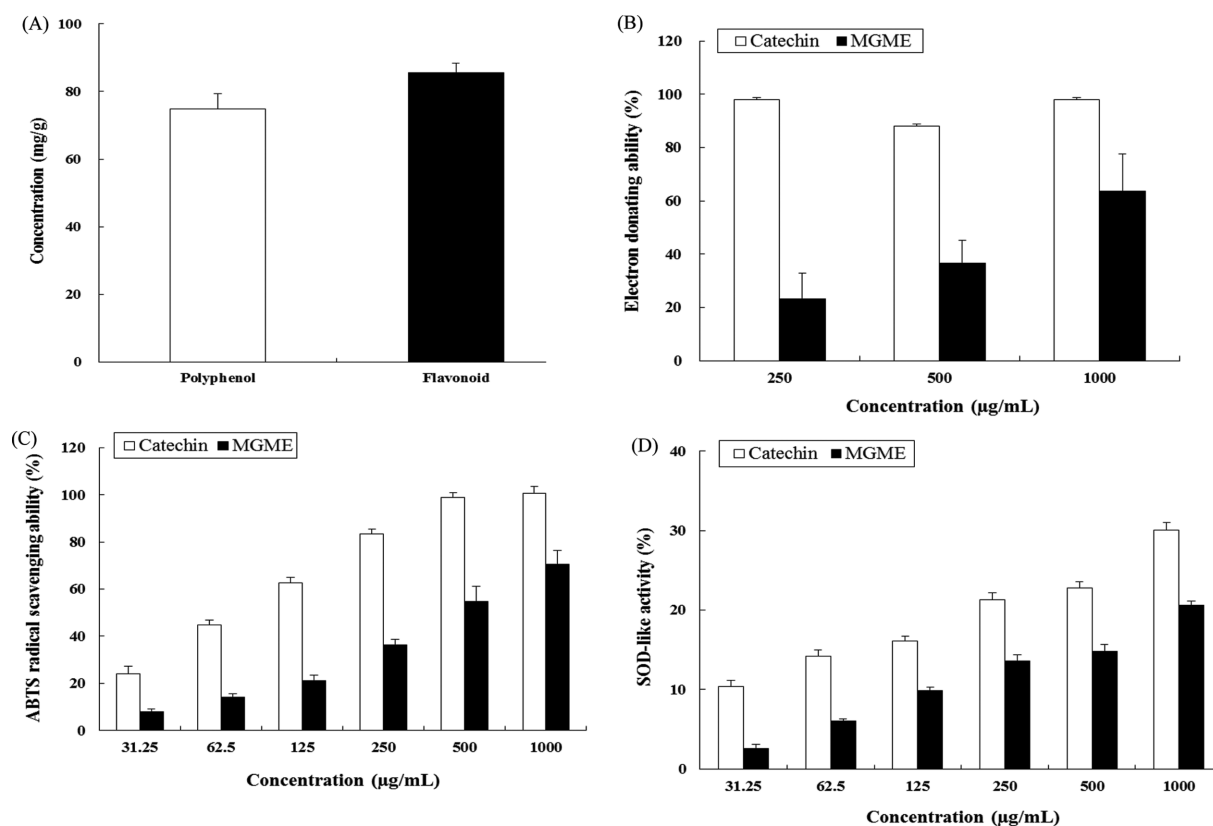
Fig. 1. Antioxidant activity of marigold methanol extract (MGME). (A) Total polyphenol and flavonoid contents, (B) Electron donating ability, (C) ABTS radical scavenging ability, (D) SOD-like activity. Bars represent mean ± SD (n = 3).
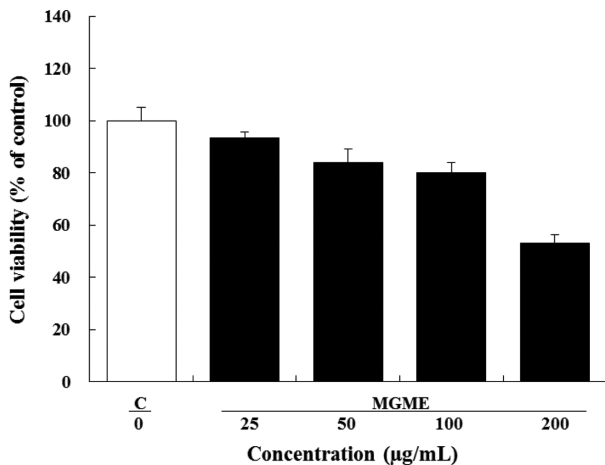
Fig. 2. Effect of MGME on cell viability. Human dermal fibroblasts were treated with indicated concentrations of MGME or vehicle (control, C). Bars represent mean ± SD (n = 3).

treatments at concentrations of 100 and 200 μg/mL reduced cell viability by 19.8% and 46.8%, respectively, compared to vehicle-treated cells, corresponding to an MPL of 100 μg/mL (Fig. 2).

Morphological observation of human dermal fibroblasts. Fibroblasts treated with low or intermediate concentrations of MGME (Fig. 3B-3E) maintained the flat, spindle-like shape observed in vehicle-treated control cells (Fig. 3A); however, at a concentration of 200 μg/mL, cells had irregular or rounded shape, and the cell density was decreased (Fig. 3F).

Type I procollagen synthesis. MGME treatment at concentrations of 25, 50, and 100 μg/mL led to significant ($p < 0.05$) increase in collagen production by 43.5%, 55.8%, and 83.7%, respectively, compared to DMSO-treated control cells (Fig. 4).

MMP activity and expression. MGME treatment resulted in a decreased MMP-2 protein expression, as observed by zymography (Fig. 5A). At concentrations of 12.5, 25, 50, and 100 μg/mL, MMP-2 activity was significantly ($p < 0.05$) decreased by 28.8%, 31.8%, 33.5%, and 36.5%, respectively, compared to UVA-irradiated control cells (Fig. 5B). In addition, at these concentrations, *MMP-1* transcript levels were significantly ($p < 0.05$) reduced in a dose-dependent manner by 50.3%, 51.5%, 60.2%, and
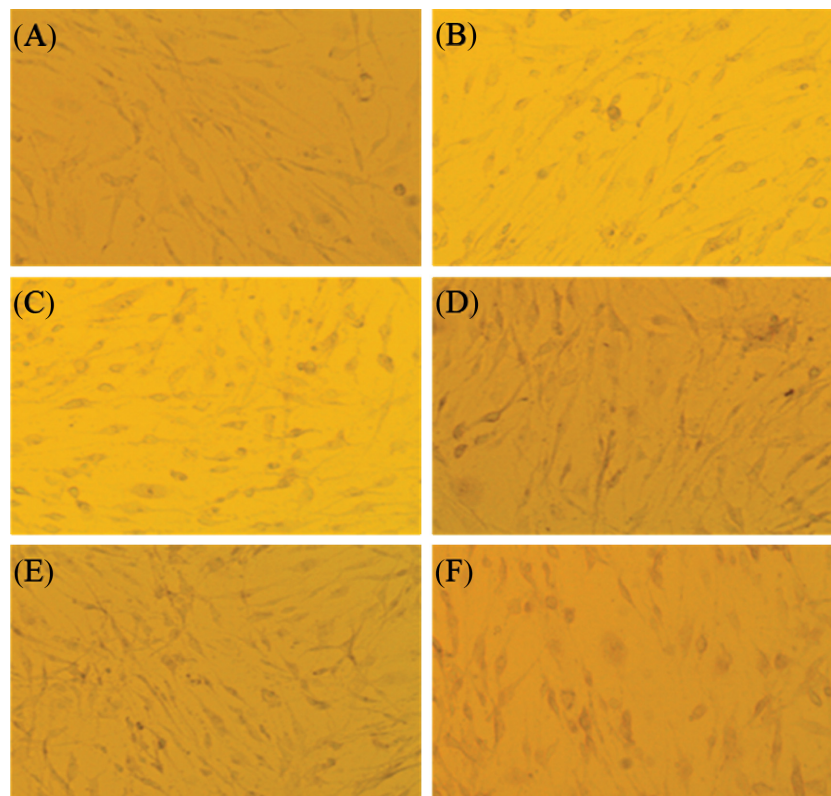

Fig. 3. Morphological changes in human dermal fibroblasts upon treatment with MGME. (A) DMSO-treated control cells were flat, with spindle-like morphology; MGME concentrations of (B) 12.5, (C) 25, (D) 50, or (E) 100 μg/mL had no effect on cell morphology, but at a concentration of (F) 200 μg/mL, cells were frequently observed to have a rounded, irregular shape and were clustered. 200× magnification.
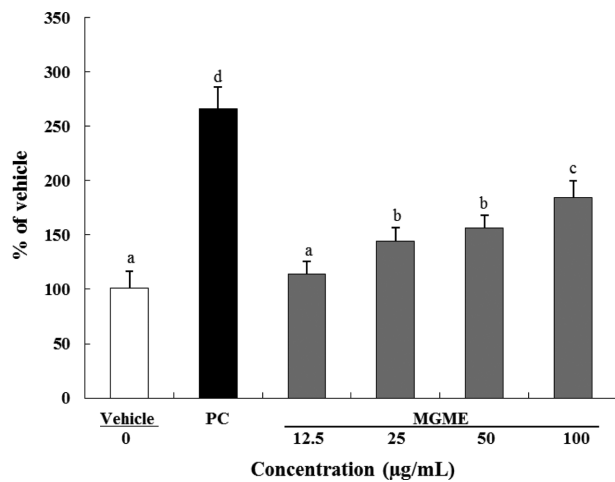
Fig. 4. Effect of MGME on collagen production in human dermal fibroblasts. Cells were treated with the vehicle DMSO, 5 ng/mL TGF-β1 (positive control, PC), or MGME at the indicated concentrations, and the production of procollagen was measured by ELISA. Bars represent mean ± SD (n = 3). Statistically significant differences, as determined by ANOVA and the Duncan multiple range test, are indicated by different letters ($p < 0.05$, a < b < c < d).
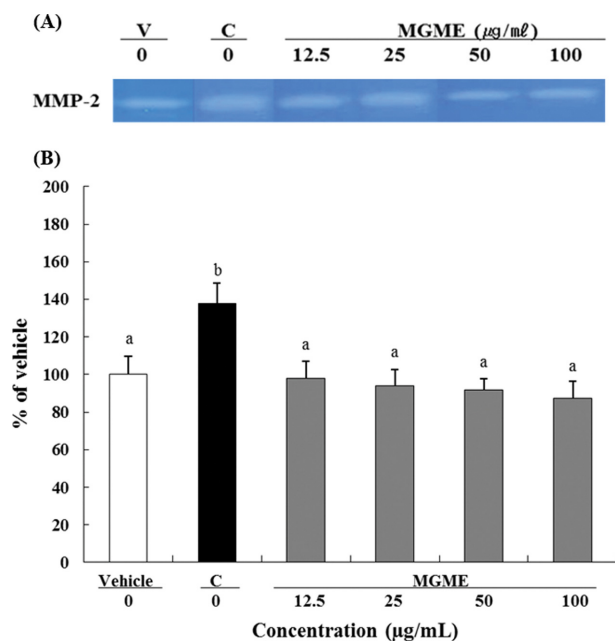
Fig. 5. Effect of MGME on MMP-2 activity in human dermal fibroblasts. (A) MMP-2 protein expression decreased upon treatment with MGME in a dose-dependent manner compared to UVA-irradiated control cells (C). (B) Quantification of MMP-2 activity in cells treated with the vehicle DMSO, 6.3 J/cm² UVA radiation (C), or MGME at the indicated concentrations. Bars represent mean ± SD (n = 3). Statistically significant differences, as determined by ANOVA and the Duncan multiple range test, are indicated by different letters ($p < 0.05$, a < b).
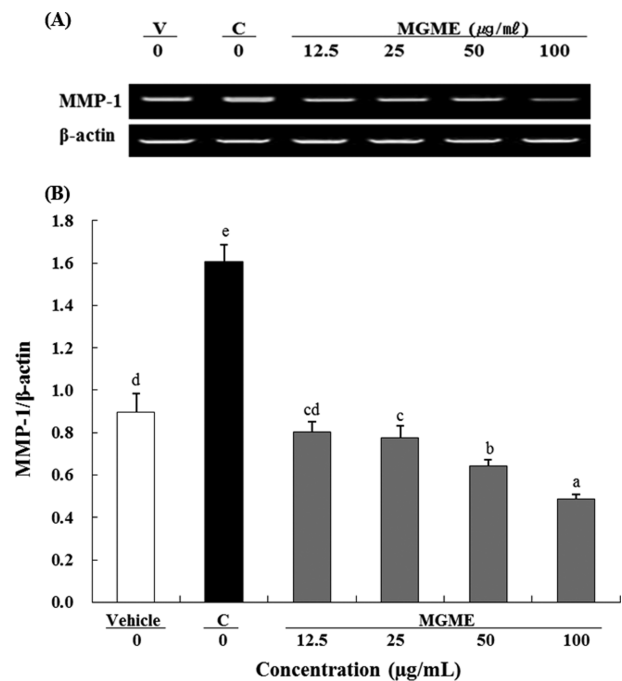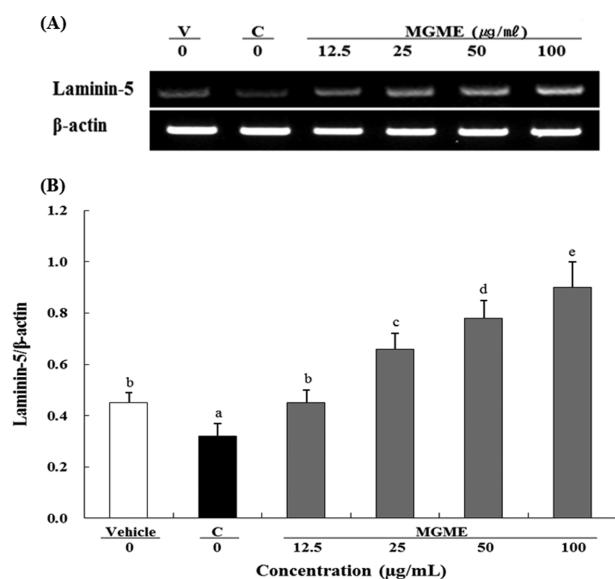

Fig. 6. Effect of MGME on *MMP-1* mRNA expression in human dermal fibroblasts. (A) *MMP-1* transcript levels decreased upon treatment with MGME in a dose-dependent manner compared to UVA-irradiated control cells (C), as determined by RT-PCR. Expression was normalized to *β-actin* levels. (B) Quantification of *MMP-1* transcript expression in cells treated with the vehicle DMSO, 6.3 J/cm² UVA radiation (C), or MGME at the indicated concentrations. Bars represent mean ± SD (n = 3). Statistically significant differences, as determined by ANOVA and the Duncan multiple range test, are indicated by different letters ($p < 0.05$, a < b < c < d < e).

69.5%, respectively, as determined by RT-PCR (Fig. 6).

Laminin-5 mRNA expression. Treatments of cells with MGME at 12.5, 25, 50, and 100 μg/mL significantly ($p < 0.05$) increased *laminin-5* mRNA expression in a dose-dependent manner by 40.6%, 106.2%, 143.7%, and 181.2%, respectively, compared to UVA-irradiated control cells, as determined by RT-PCR (Fig. 7). Taken together, these results indicate that exposure of human dermal fibroblasts to MGME decreases cell viability, reduces MMP expression and its enzyme activity, it increases the expression of matrix components *laminin-5* as well as collagen production.

DISCUSSION

Antioxidant phenolic compounds have anti-aging effects on the skin (24). Polyphenols exhibit excellent anti-inflammatory, anti-bacterial, and UV-protective properties (25). Flavonoids are potent antioxidants comprising naturally occurring plant phenolic compounds, including flavones,

Fig. 7. Effect of MGME on *laminin-5* mRNA expression in human dermal fibroblasts. (A) *Laminin-5* transcript levels increased upon treatment with MGME in a dose-dependent manner compared to UVA-irradiated control cells (C), as determined by RT-PCR. Expression was normalized to *β-actin* levels. (B) Quantification of *Laminin-5* transcript expression in cells treated with the vehicle DMSO, 6.3 J/cm^2 UVA radiation (C), or MGME at the indicated concentrations. Bars represent mean ± SD (n = 3). Statistically significant differences, as determined by ANOVA and the Duncan multiple range test, are indicated by different letters ($p < 0.05$, a < b < c < d < e).

flavonols, isoflavones, flavonones, and chalcones (26). These compounds are known to be the main components of *T. erecta* L (27). In this study, the total polyphenol and flavonoid contents of MGME were 74.8 mg TAE/g and 85.6 mg RE/g, respectively; we observed a higher total flavonoid content in the methanol extract of whole Korean marigold plant than that (68.9 mg RE/g) reported by Kaisoon *et al*. for a methanol extract from Thai marigold flower (28). Li *et al*. reported that total flavonoid contents in the ethanol extracts from eleven different cultivars of Chinese marigold flower varied from 28.6 to 93.3 mg RE/g (29). Therefore, we propose that differences in the cultivar, plant parts, and extraction solvent contribute to the difference in flavonoid content observed in this study.

The DPPH assay is an easy colorimetric method routinely utilized for assessing the free radical-scavenging potential of an antioxidant molecule (30). The ABTS method is also used to determine the antioxidant activity of both lipophilic and hydrophilic antioxidants in various matrices (31). In this study, MGME (1,000 μg/mL) exhibited free radical-scavenging capacities of 63.7% (DPPH assay) and 70.6% (ABTS assay). The DPPH radical-scavenging capacity of the soluble phenolic fraction is reported to be 94.3% in *T. erecta* L. (28); however, it is reported to

vary between 39.4 and 93% in the ethanol extracts from eleven different cultivars of Chinese marigold flower (29). The essential oil derived from the flowers of *T. erecta* L. (100 μg/mL) exhibits DPPH and ABTS radical-scavenging capacities of 71.5 and 57.3%, respectively (32). SOD is an antioxidant enzyme family including metal-containing enzymes that act as a chief ROS scavenger in the cell (33). In this study, MGME (1,000 μg/mL) exhibited an SOD-like activity of 20.6%. From the above results, we found that MGME has a potent free radical-scavenging capacity that contributes to its antioxidant effects.

Cytotoxicity of MGME against human dermal fibroblasts was determined by the MTT assay, and the MPL was determined to be 100 μg/mL. The dermis contains 85~90% type 1 collagen. Procollagen (collagen precursor) is synthesized in dermal fibroblasts in the form of polypeptide chains of type 1 collagen (34). UV irradiation impairs collagen synthesis, primarily through the down-regulation of type 1 procollagen expression (34). Topical treatment with 0.05% all-trans retinoic acid for 10 weeks in non-irradiated hairless mice is reported to increase the amount of type 1 collagen in the skin by 22% compared with vehicle treatment (35). In this study, collagen production significantly increased by 83.7% in MGME (100 μg/mL)-treated fibroblasts compared to that in control fibroblasts, indicating that MGME is a potent inducer of collagen synthesis.

UV light is known to induce the expression of MMP-1, -2, and -9 in normal human epidermis *in vivo* (36), and MMP inhibition may be a promising strategy to prevent photo-aging (37). MMP-1 acts as a primary mediator of UVB-induced skin damage and premature aging (1). In this study, MGME (100 μg/mL) significantly decreased the expression of *MMP-1* mRNA by 69.5% in human dermal fibroblasts compared with control treatment. MGME significantly inhibits hyaluronidase (IC$_{50}$ of 11.70 μg/mL) and elastase (IC$_{50}$ of 4.13 μg/mL) activities and *MMP-1* expression to a greater extent than oleanolic acid (38). Chronic UVB exposure increases skin MMP-2 levels as measured by gelatin zymography (2). In this study, MGME (100 μg/mL) significantly decreased MMP-2 activity by 36.5% in human dermal fibroblasts compared with control treatment. Based on its effect on MMP, MGME acts as a potent collagen degradation inhibitor.

Laminin-5, present in the BM, initiates hemidesmosome formation to facilitate attachment of the epidermis to the dermis, and maintains structural integrity of cells and physiological homeostasis of the skin (39). Consequently, increased laminin-5 expression rejuvenates the aged skin (40). In this study, laminin-5 expression significantly increased by 181.2% in MGME (100 μg/mL)-treated human dermal fibroblasts compared to that in vehicle-treated fibroblasts, indicating that MGME holds remarkable skin anti-aging potential.

Results of this study indicate that MGME, owing to the high levels of polyphenols, induces collagen synthesis and inhibits its degradation in human dermal fibroblasts. These findings suggest that MGME could protect human skin against photo-aging by suppressing MMP expression and/or activity as well as by stimulating collagen synthesis. Therefore, we conclude that MGME has significant skin anti-aging potential.

REFERENCES

1. Brennan, M., Bhatti, H., Nerusu, K.C., Bhagavathula, N., Kang, S., Fisher, G.J., Varani, J. and Voorhees, J.J. (2003) Matrix metalloproteinase-1 is the major collagenolytic enzyme responsible for collagen damage in UV-irradiated human skin. *Photochem. Photobiol.*, **78**, 43-48.

2. Inomata, S., Matsunaga, Y., Amano, S., Takada, K., Kobayashi, K., Tsunenaga, M., Nishiyama, T., Kohno, Y. and Fukuda, M. (2003) Possible involvement of gelatinases in basement membrane damage and wrinkle formation in chronically ultraviolet B-exposed hairless mouse. *J. Invest. Dermatol.*, **120**, 128-134.

3. Buechner, N., Schroeder, P., Jakob, S., Kunze, K., Maresch, T., Calles, C., Krutmann, J. and Haendeler, J. (2008) Changes of MMP-1 and collagen type I alpha1 by UVA, UVB and IRA are differentially regulated by Trx-1. *Exp. Gerontol.*, **43**, 633-637.

4. Dong, K.K., Damaghi, N., Picart, S.D., Markova, N.G., Obayashi, K., Okano, Y., Masaki, H., Grether-Beck, S., Krutmann, J., Smiles, K.A. and Yarosh, D.B. (2008) UV-induced DNA damage initiates release of MMP-1 in human skin. *Exp. Dermatol.*, **17**, 1037-1044.

5. Amano, S., Ogura, Y., Akutsu, N., Matsunaga, Y., Kadoya, K., Adachi, E. and Nishiyama, T. (2005) Protective effect of matrix metalloproteinase inhibitors against epidermal basement membrane damage: skin equivalents partially mimic photoaging process. *Br. J. Dermatol.*, **153**, 37-46.

6. Aumailley, M. and Rousselle, P. (1999) Laminins of the dermo-epidermal junction. *Matrix Biol.*, **18**, 19-28.

7. Miyazaki, K., Kikkawa, Y., Nakamura, A., Yasumitsu, H. and Umeda, M. (1993) A large cell-adhesive scatter factor secreted by human gastric carcinoma cells. *Proc. Natl. Acad. Sci. U.S.A.*, **90**, 11767-11771.

8. Varani, J., Warner, R.L., Gharaee-Kermani, M., Phan, S.H., Kang, S., Chung, J.H., Wang, Z.Q., Datta, S.C., Fisher, G.J. and Voorhees, J.J. (2000) Vitamin A antagonizes decreased cell growth and elevated collagen-degrading matrix metalloproteinases and stimulates collagen accumulation in naturally aged human skin. *J. Invest. Dermatol.*, **114**, 480-486.

9. Brisaert, M. and Plaizier-Vercammen, J. (2000) Investigation on the photostability of a tretinoin lotion and stabilization with additives. *Int. J. Pharm.*, **199**, 49-57.

10. Kim, B.H., Lee, Y.S. and Kang, K.S. (2003) The mechanism of retinol-induced irritation and its application to anti-irritant development. *Toxicol. Lett.*, **146**, 65-73.

11. Hwang, J.Y., Lee, C.M. and Kim, Y.C. (2015) Anti-wrinkle efficacy of *Stachys riederi* var. *japonica* ethanol extract in human dermal fibroblasts. *J. Invest. Cosmetol.*, **11**, 293-301.

12. Kim, S.E., Lee, C.M. and Kim, Y.C. (2016) Anti-wrinkle efficacy of *Oenothera laciniata* methanol extract in human dermal fibroblasts. *J. Invest. Cosmetol.*, **12**, 197-203.

13. Hamburger, M., Adler, S., Baumann, D., Förg, A. and Weinreich, B. (2003) Preparative purification of the major anti-inflammatory triterpenoid esters from Marigold (*Calendula officinalis*). *Fitoterapia*, **74**, 328-338.

14. Bashir, S. and Gilani, A.H. (2008) Studies on the antioxidant and analgesic activities of Aztec marigold (Tagetes erecta) flowers. *Phytother. Res.*, **22**, 1692-1694.

15. Khan, M.T. and Evans, F.J. (1996) Clinical evaluation of *Tagetes erecta* in the treatment of parakeratosis. *Phytother. Res.*, **10**, 186-188.

16. Gutiérrez, R.M.P., Luna, H.H. and Garrido, S.H. (2006) Antioxidant activity of *Tagetes erecta* essential oil. *J. Chil. Chem. Soc.*, **51**, 883-886.

17. Folin, O. and Denis, W. (1912) On phosphotungstic-phosphomolybdic compounds as color reagents. *J. Biol. Chem.*, **12**, 239-249.

18. Davis, R., Massey, R.C. and Mcweeny, D.J. (1980) The catalysis of the *N*-nitrosation of secondary amines by nitrosophenols. *J. Food Chem.*, **6**, 115-122.

19. Pérez, G.R.M., Vargas, S.R., Martinez, M.F.J. and Cordova, R.I. (2004) Antioxidant and free radical scavenging activities of 5,7,3′-trihydroxy-3,6,4′-trimethoxyflavone from *Brickellia veronicaefolia*. *Phytother. Res.*, **18**, 428-430.

20. Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M. and Rice-Evans, C. (1999) Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radic. Biol. Med.*, **26**, 1231-1237.

21. Marklund, S. and Marklund, G. (1974) Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. *Eur. J. Biochem.*, **47**, 469-474.

22. Son, E.D., Choi, G.H., Kim, H., Lee, B., Chang, I.S. and Hwang, J.S. (2007) Alpha-ketoglutarate stimulates procollagen production in cultured human dermal fibroblasts, and decreases UVB-induced wrinkle formation following topical application on the dorsal skin of hairless mice. *Biol. Pharm. Bull.*, **30**, 1395-1399.

23. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, **193**, 265-275.

24. Dudonné, S., Vitrac, X., Coutière, P., Woillez, M. and Mérillo, J.M. (2009) Comparative study of antioxidant properties and total phenolic content of 30 plant extracts of industrial interest using DPPH, ABTS, FRAP, SOD, and ORAC assays. *J. Agric. Food Chem.*, **57**, 1768-1774.

25. Lee, C.J., Chen, L.G., Chang, T.L., Ke, W.M., Lo, Y.F. and Wang, C.C. (2011) The correlation between skin-care effects and phytochemical contents in Lamiaceae plants. *Food Chem.*, **124**, 833-841.

26. Pokorny, J., Yanishlieva, N. and Gordon, M. (2001) Antioxidants in food: practical applications. Woodhead Publishing Limited, Cambridge, pp. 22-70.

27. Xu, L.W., Juan, C., Qi, H.Y. and Shi, Y.P. (2012) Phyto-

chemicals and their biological activities of plants in *Tagetes* L. *Chinese Herbal Medicines*, **4**, 103-117.

28. Kaisoon, O., Siriamornpun, S., Weerapreeyakul, N. and Meeso, N. (2011) Phenolic compounds and antioxidant activities of edible flowers from Thailand. *J. Funct. Foods*, **3**, 88-99.

29. Li, W., Gao, Y., Zhao, J. and Wang, Q. (2007) Phenolic, flavonoid and lutein ester content and antioxidant activity of 11 cultivars of Chinese marigold. *J. Agric. Food Chem.*, **55**, 8478-8484.

30. Mishra, K., Ojha, H. and Chaudhury, N.K. (2012) Estimation of antiradical properties of antioxidants using DPPH assay: a critical review and results. *Food Chem.*, **130**, 1036-1043.

31. Cano, A., Acosta, M. and Arnaro, M.B. (2000) A method to measure antioxidant activity in organic media: application to lipophilic vitamins. *Redox Rep.*, **5**, 365-370.

32. Gutiérrez, R.M.P., Luna, H.H. and Garrido, S.H. (2006) Antioxidant activity of *Tagetes erecta* essential oil. *J. Chil. Chem. Soc.*, **51**, 883-886.

33. Chen, X., Zhang, E., Fang, L., Zhang, J., Zhu, J., He, W. and Luo, X. (2013) Repair effects of exogenous SOD on *Bacillus subtilis* against gamma radiation exposure. *J. Environ. Radioact.*, **126**, 259-263.

34. Fisher, G.J., Kang, S., Varani, J., Bata-Csorgo, Z., Wan, Y., Datta, S. and Voorhees, J.J. (2002) Mechanisms of photoaging and chronological skin aging. *Arch. Dermatol.*, **138**, 1462-1470.

35. Chaqour, B., Seité, S., Coutant, K., Fourtanier, A., Borel, J.P. and Bellon, G. (1995) Chronic UVB- and all-trans retinoic-acid-induced qualitative and quantitative changes in hairless mouse skin. *J. Photochem. Photobiol. B*, **28**, 125-135.

36. Chung, J.H. (2003) Photoaging in Asians. *Photodermatol. Photoimmunol. Photomed.*, **19**, 109-121.

37. Moon, H.J., Lee, S.R., Shim, S.N., Jeong, S.H., Stonik, V.A., Rasskazov, V.A., Zvyagintseva, T. and Lee, Y.H. (2008) Fucoidan inhibits UVB-induced MMP-1 expression in human skin fibroblasts. *Biol. Pharm. Bull.*, **31**, 284-289.

38. Maity, N., Nema, N.K., Abedy, M.K., Sarkar, B.K. and Mukherjee, P.K. (2011) Exploring *Tagetes erecta* Linn flower for the elastase, hyaluronidase and MMP-1 inhibitory activity. *J. Ethnopharmacol.*, **137**, 1300-1305.

39. Schneider, H., Műhle, C. and Pacho, F. (2007) Biological function of laminin-5 and pathogenic impact of its deficiency. *Eur. J. Cell Biol.*, **86**, 701-717.

40. Seo, M.Y., Chung, S.Y., Choi, W.K., Seo, Y.K., Jung, S.H., Park, J.M., Seo, M.J., Park, J.K., Kim, J.W. and Park, C.S. (2009) Anti-aging effect of rice wine in cultured human fibroblasts and keratinocytes. *J. Biosci. Bioeng.*, **107**, 266-271.