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Biflavonoids isolated from *Selaginella tamariscina* regulate the expression of matrix metalloproteinase in human skin fibroblasts

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Abstract—The methanol extract from *Selaginella tamariscina* significantly inhibited UV irradiation induced activity of matrix metalloproteinase-1 (MMP-1) in primary fibroblasts from human skin. Using the technique of bioassay-directed chromatographic separation, five biflavonoids were isolated from the ethyl acetate soluble fraction of *S. tamariscina*. Here, we investigated the effect of these five biflavonoids on the regulation of MMP-1 and -2 in UV irradiated cultured dermal fibroblasts from human neonatal foreskins. Among these biflavonoids, sumaflavone and amentoflavone showed significant MMP-1 inhibitory activity in primary human dermal fibroblasts after UV irradiation. The IC_{50} values of sumaflavone, amentoflavone and retinoic acid, which was used as a positive control, were 0.78, 1.8, and 10 μM, respectively.

1. Introduction

Ultraviolet (UV) irradiation from the sun damages human skin, which causes the skin to age. This skin aging can be attributed to extrinsic and intrinsic aging and is commonly related to increased wrinkling, sagging, and laxity.[1] Extrinsic aging is generally referred to as photo-aging and is caused by repeated exposure to UV light. Whereas naturally aged skin is smooth, pale, and finely wrinkled, photo-aged skin is coarsely wrinkled, and associated with dyspigmentation and talangiectasia.[2,3] Alterations in collagen, the major structural component of skin, in the dermis layer have been suggested to be the cause of the clinical changes observed in naturally aged and photo-aged skin.[4,5] Collagen deficiency may arise from its reduced synthesis as well as increased degradation with a concomitant elevation of matrix metalloproteinase (MMP) expression. UV irradiation induces the synthesis of MMP in human skin in vivo, and MMP-mediated collagen destruction accounts, in a large part, for the connective tissue damage that occurs in aging.[6–8] Therefore, the regulation of MMP activity might be a potential strategy for prevention and/or treatment of UV induced skin damage.

MMPs are a family of zinc-dependent endoproteinases that play pivotal roles in the dynamic remodeling of the extracellular matrix. Based on substrate preference and structural homology, MMPs are sub-classified into functional groups: collagenases, gelatinases, stromelysins, matrilysins, membrane type-MMPs, and other non-classified MMPs.[9] Among the human MMPs that have been described previously, MMP-1, an interstitial collagenase, is mainly responsible for the degradation of dermal collagen in the aging process of human skin.[10,11] Therefore, inhibition of MMP-1 production might contribute to an anti-aging effect. Recently, Leu et al. reported that some flavonoids, such as phloretin, 3-hydroxyphloretin, and quercetin showed inhibition of MMP-1 production in fibroblast cells.[12]

Selaginella tamariscina belongs to the family Selaginellaceae, and has been used in oriental medicine to treat bloody feces, hematuria, prolapse of the anus, and for stanching.[13] In addition, *S. tamariscina* has been reported to lower blood glucose levels and to facilitate the repair of pancreatic islet B cells injured by alloxan.[14]

Keywords: *Selaginella tamariscina*; Biflavonoid; Matrix metalloproteinase (MMP)-1; (MMP)-2; UV; Anti-aging.
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Moreover, crude extracts of *S. tamariscina* were found to reduce the production of proinflammatory cytokines, interleukin-1β and tumor necrosis factor-α in human mesangial cells.[15] Investigation of the phytochemical constituents of *S. tamariscina* revealed it to be a rich source of biflavonoids. Biflavonoids are flavonoid dimers connected with a C–C or a C–O–C bond. Although biflavonoids are known to display a variety of biological activities, such as anti-inflammatory activity,[16–19] mast cell histamine release inhibitory activity,[20,21] anti-tumor activity,[22,23] and phospholipase A2 inhibitory activity,[24] their ability to inhibit MMP-1 and -2 has not been previously studied. Concerning the MMP-9 inhibitory activity of biflavonoids, Yoon et al. reported that isoginkgetin decreased MMP-9 production profoundly, but up-regulated the level of tissue inhibitor of metalloproteinase (TIMP)-1, an inhibitor of MMP-9, in HT 1080 human fibrosarcoma cells.[25] In addition, Suh et al. reported that ochnaflavone inhibited TNF-α induced MMP-9 expression in human aortic smooth muscle cells via inhibition of transcription factors NF-κB and AP-1 binding activities.[26]

In an ongoing investigation into anti-aging compounds from natural products, a methanol extract of *S. tamariscina* was found to significantly inhibit MMP-1 expression in vitro. In the present study, we isolated five biflavonoids and examined their ability to inhibit the expression of MMP-1 and -2 induced by UV irradiation. We found that sumaflavone and amentoflavone have potent anti-aging effects and these activities were ascribed to the blocking of MMP-1 expression. This is the first report on the MMP-1 inhibitory activity of naturally occurring biflavonoids.

2. Results and discussion

2.1. Extraction and isolation

Dried whole plants of *S. tamariscina* Spring were obtained from a herbal drug store in Gwangju (September 2003). The plants were identified by Professor Eun-Rhan Woo at the College of Pharmacy, Chosun University. Voucher specimens (853-16) were deposited in the Herbarium of the College of Pharmacy, Chosun University. The whole plant of *S. tamariscina* (1.8 kg) was extracted three times with MeOH at room temperature and evaporated to dryness under reduced pressure to obtain 78.54 g of residue. The methanol extract was suspended in water and then partitioned by dichloromethane, ethyl acetate, and *n*-butanol in

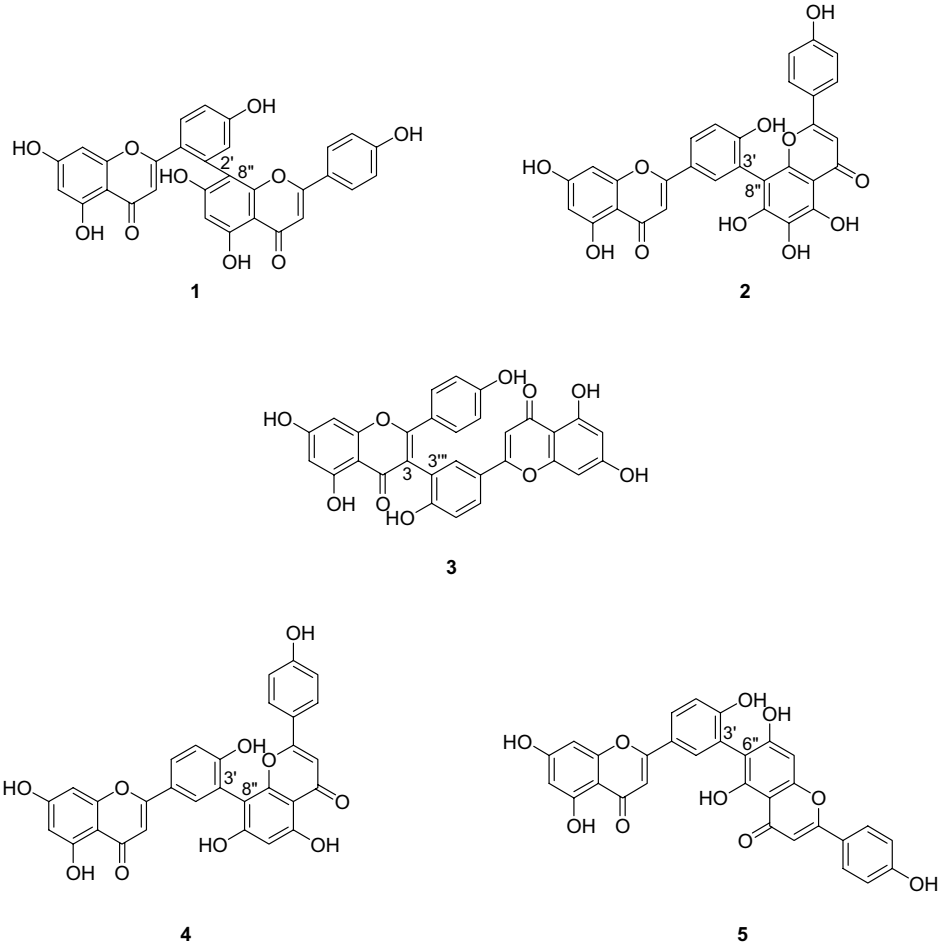

Figure 1. Chemical structures of the five biflavonoids isolated from *Selaginella tamariscina*. 1, 2′,8″-biapigenin; 2, sumaflavone; 3, taiwaniaflavone; 4, amentoflavone; 5, robustaflavone.

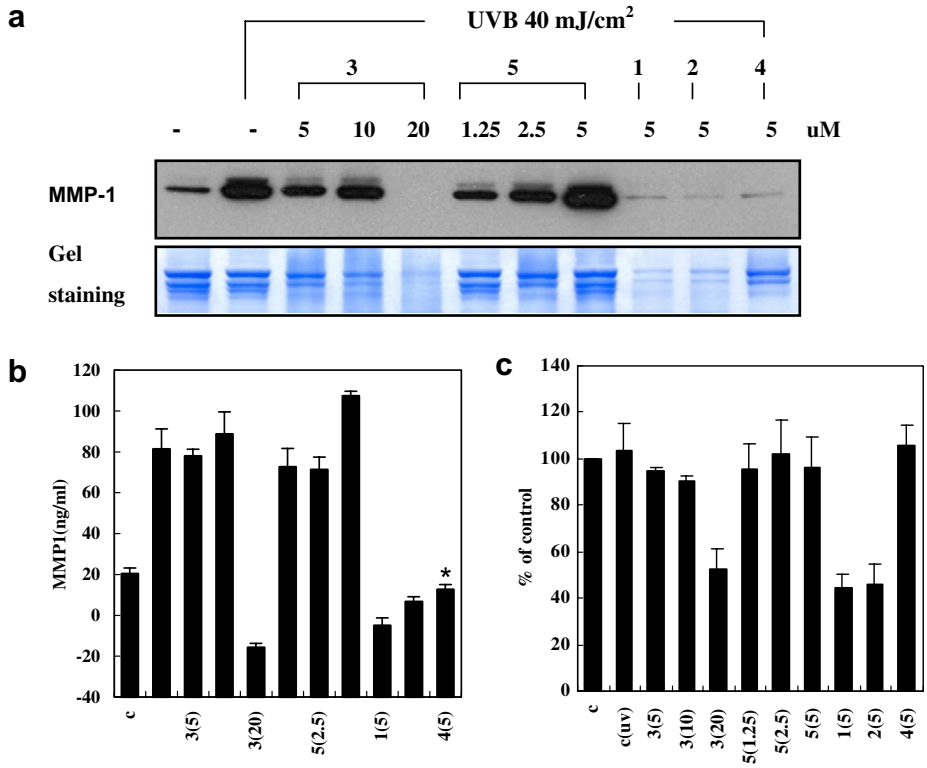

Figure 2. The effect of the five biflavonoids on the production of MMP-1 by HDFs. After UVB exposure, HDFs were cultured with or without biflavonoids for 48 h. MMP-1 content in the culture medium was measured by Western blotting (a) and ELISA (b), as described in Section 3 details. Gel staining (lower panel of a) represents a polyacrylamide gel with protein loading control of each sample. Similar results were obtained from two independent experiments. Cell viability was determined by measuring protein concentration. The protein content of the cells without UVB exposure (control: C) was established as the reference value of 100%. Values are expressed as means ± SD of two independent experiments, each using triple culture flasks. The student's *t*-test was employed for statistical analysis of data. ($^{*}p < 0.01$, vs C (UV)). C, control; C (UV), exposed UVB; 1, 2′,8″-biapigenin; 2, sumaflavone; 3, taiwaniaflavone; 4, amentoflavone; 5, robustaflavone.

turn. The EtOAc fraction (6.8 g) was subjected to column chromatography over a silica gel (Merck 43–60 and 63–200 μm, Germany; 300 g) eluting with a $CHCl_3$–MeOH–H_2O (12:1:0.1 → 8:1:0.1 → 5:1:0.1 → 2:1:0.1 → 1:1:0.1 → MeOH only) gradient system. Fractions were combined based on their TLC pattern to yield subfractions designated as E1–E10. Subfraction E4 (995.0 mg) was purified by column chromatography over a Sephadex LH 20 eluting with a MeOH/H_2O 2:1 to give four subfractions (E41–E44). Subfraction E44 (393.3 mg) was finally purified by column chromatography over a MCI gel to afford 2′,8″-biapigenin (247.8 mg). Subfraction E3 (586.0 mg) was purified by repeated column chromatography over a silica gel, RP-18, and Sephadex LH 20 to afford amentoflavone (340.0 mg). Subfraction E5 (4.3 g) was purified by column chromatography over a MCI gel eluting with a MeOH/H_2O 2:1 to give four subfractions (E51–E54). Subfraction E52 (949.2 mg) was finally purified by column chromatography over a silica gel, MCI gel, and Sephadex LH 20 to afford taiwaniaflavone (30.9 mg). Subfraction E6 (101.9 mg) was finally purified by repeated column chromatography over Sephadex LH-20 and MCI gel to obtain sumaflavone (26.6 mg). In addition, subfraction E10 (30.5 mg) was finally purified by repeated column chromatography to obtain robustaflavone (14.1 mg). The physical and chemical data, including MS, ^{1}H NMR, ^{13}C NMR, and HSQC, of five biflavonoids were identical with those reported previously[27–30] (Fig. 1).

2.2. Biological activity

2.2.1. Inhibitory effect of five biflavonoids on the production of MMP-1 by human dermal fibroblasts (HDFs). We determined the effect of five biflavonoids on the production of MMP-1 by HDFs using Western blotting and ELISA. Exposure of HDFs to UVB irradiation (40 mJ/cm^2) increased the level of MMP-1 in the culture medium after 48 h by three- to fourfold (Figs. 2b and 3b). However, the enhancement of MMP-1 production induced by UVB irradiation was completely inhibited by 5 μM 2′,8″-biapigenin (**1**), 5 μM sumaflavone (**2**), 5 μM amentoflavone (**4**), and 20 μM taiwaniaflavone (**3**) (Fig. 2a and b). In contrast, robustaflavone (**5**) (1.25, 2.5 and 5 μM) and taiwaniaflavone (**3**) (5 and 10 μM) had no effect on MMP-1 expression induced by UVB irradiation (Fig. 2a and b). However, 5 μM 2′,8″-biapigenin (**1**), 5 μM sumaflavone (**2**) and 20 μM taiwaniaflavone (**3**) also inhibited the growth of HDFs (Fig. 2c). The protein content of the cells treated with 5 μM 2′,8″-biapigenin (**1**), 5 μM sumaflavone (**2**), and 20 μM taiwaniaflavone (**3**) decreased by approximately 50%. Therefore, the inhibitory effects of MMP-1 production of these biflavonoids might be due to poor cell viability. Accordingly, we tested the effect of 2′,8″-biap-

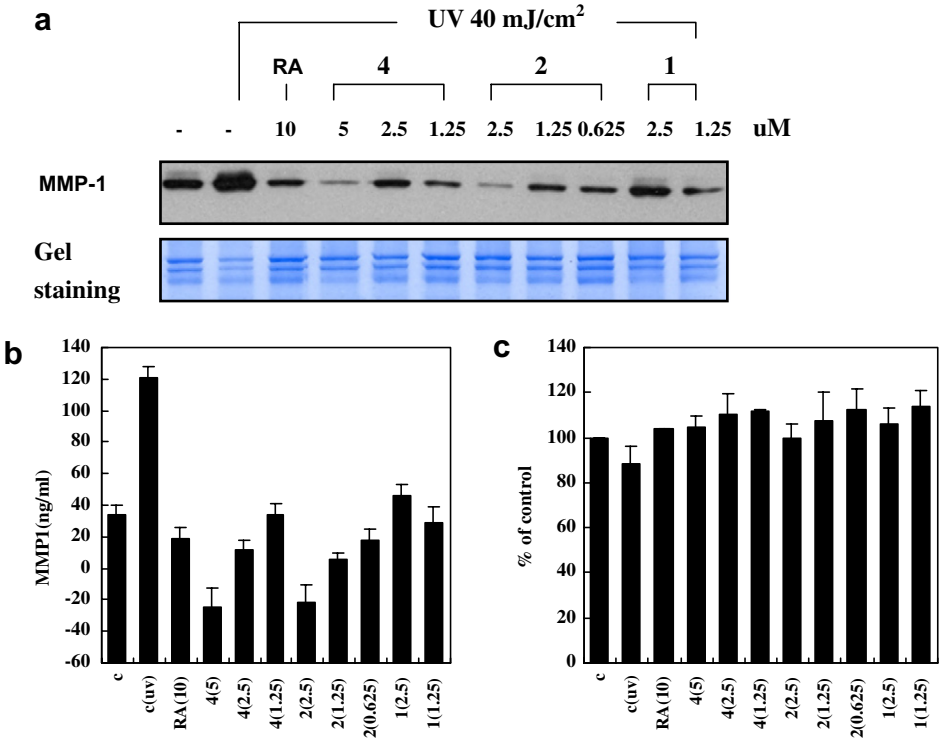

Figure 3. The effect of 2′,8″-biapigenin, sumaflavone and amentoflavone on the production of MMP-1 by HDFs. After UVB exposure, HDFs were cultured with or without biflavonoids for 48 h. MMP-1 content in the culture medium was measured by Western blotting (a) and ELISA (b), as described in Section 3 details. Gel staining (lower panel of a) represents a polyacrylamide gel with protein loading control of each sample. Similar results were obtained from two independent experiments. Cell viability was determined by measuring protein concentration. The protein content of the cells without UVB exposure (control: C) was established as the reference value of 100%. Values are expressed as means ± SD of two independent experiments, each using triple culture flasks. The student's *t*-test was employed for statistical analysis of data. *p* value of all data is lower than 0.01, versus C (UV). C, control; C (UV), exposed UVB; RA, retinoic acid (positive control); 1, 2′,8″-biapigenin; 2, sumaflavone; 4, amentoflavone.

igenin (**1**), sumaflavone (**2**), and amentoflavone (**4**) on the production of MMP-1 by HDFs at concentrations below 5 μM (Fig. 3a and b). As shown in Figure 3a and b, sumaflavone (**2**) and amentoflavone (**4**) suppressed MMP-1 production induced by UVB irradiation in a dose-dependent manner. However, 2′,8″-biapigenin (**1**) did not inhibit MMP-1 expression at concentrations of 1.25–2.5 μM. Therefore, we conclude that sumaflavone (**2**) and amentoflavone (**4**) suppress MMP-1 expression induced by UVB irradiation in HDFs. IC_{50} values of sumaflavone (**2**) and amentoflavone (**4**) were 0.78 and 1.8 μM, respectively. In our assay system, sumaflavone (**2**) and amentoflavone (**4**) were more effective inhibitors of MMP-1 expression than the commercially available retinoic acid (IC_{50} value 10 μM).

As described above, 2′,8″-biapigenin, taiwaniaflavone, and robustaflavone showed negligible MMP-1 inhibitory effect, whereas sumaflavone and amentoflavone were potent MMP-1 inhibitors. This difference in ability of these biflavonoids to inhibit MMP-1 is probably due to structural differences, in particular with respect to the location of C–C bond (i.e. the 3′–8″ bond of sumaflavone and amentoflavone, the 2′–8″ bond of 2′,8″-biapigenin, the 3–3‴ bond of taiwaniaflavone, and the 3′–6″ bond of robustaflavone). Moreover, sumaflavone is more active than amentoflavone. This could be explained by the number of hydroxyl groups in the B rings of the flavonoid skeleton. Sumaflavone, which carries a 5″, 6″, 7″ trihydroxy group in the B ring increased MMP-1 inhibition compared to amentoflavone, which carries a 5″, 7″ dihydroxy group in the B ring of flavonoid.

2.2.2. Effect of five biflavonoids on the enzymatic activity of MMP-2 produced by HDFs. After UVB exposure, HDFs were cultured with or without the five biflavonoids for 48 h. Enzymatic activity of MMP-2 in the culture medium was measured by zymography. Gelatin in polyacrylamide gel is degraded by proteinase activity of MMP-2. Degraded gelatin does not stain with Commassie Blue. Enzymatic activity of MMP-2 is determined by measuring the area not stained in the polyacrylamide gel. The results are shown in Figures 4 and 5. After UVB exposure, the cell viability of HDFs cultured with 5 μM 2′,8″-biapigenin (**1**), 5 μM sumaflavone (**2**), and 20 μM taiwaniaflavone (**3**) decreased by approximately 50% (Fig. 4b). Therefore, the disappearance and decrease of proteinase bands in a blue background by 5 μM 2′,8″-biapigenin (**1**), 5 μM sumaflavone (**2**), and 20 μM taiwaniaflavone (**3**) were caused by decreased cell viability (Fig. 4a and b). When HDFs were cultured with 2′,8″-biapigenin (**1**) and sumaflavone (**2**) at concentrations below 5 μM, enzymatic activity of MMP-2 in the medium did not decrease, compared to that of control (Fig. 5a and b). These results showed that the five biflavonoids did not inhibit MMP-2 enzymatic activity.

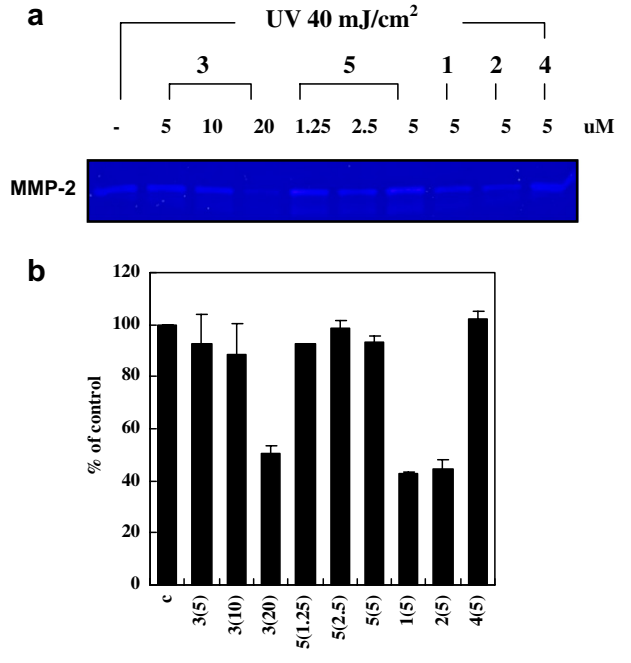

Figure 4. The effect of five biflavonoids on the enzymatic activity of MMP-2 produced by HDFs. After UVB exposure, HDFs were cultured with or without biflavonoids for 48 h. Enzymatic activity of MMP-2 in the culture medium was measured by zymography (a), as described in Section 3 details. Cell viability was determined by measuring protein concentration. The protein content of the cells treated without biflavonoids (control: C) was established as the reference value of 100%. Similar results were obtained from two independent experiments. C, control; 1, 2′,8″-biapigenin; 2, sumaflavone; 3, taiwaniaflavone; 4, amentoflavone; 5, robustaflavone.

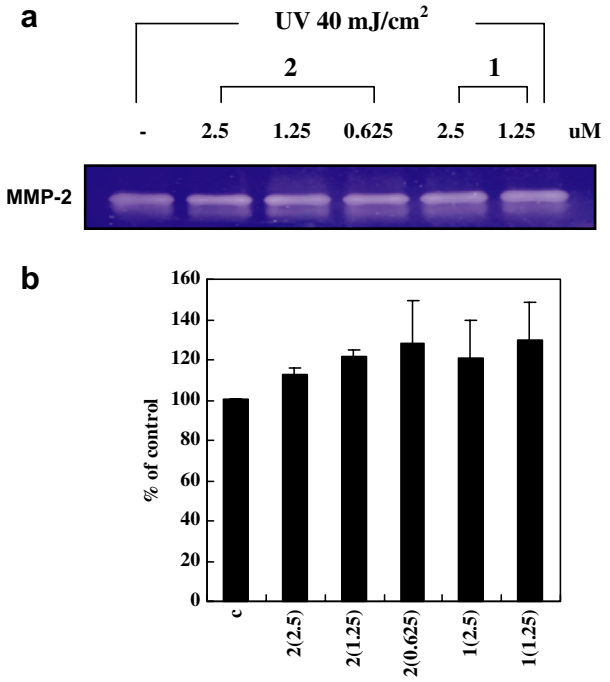

Figure 5. The effect of 2′,8″-biapigenin and sumaflavone on the enzymatic activity of MMP-2 produced by HDFs. After UVB exposure, HDFs were cultured with or without biflavonoids for 48 h. Enzymatic activity of MMP-2 in the culture medium was measured by zymography (a), as described in Section 3 details. Cell viability was determined by measuring protein concentration. The protein content of the cells treated without biflavonoids (control: C) was established as the reference value of 100%. Similar results were obtained from two independent experiments. C, control; 1, 2′,8″-biapigenin; 2, sumaflavone.

Extracellular matrix (ECM) degradation by UV irradiation is associated with wrinkle formation in skin.[31,1,8] MMPs are enzymes that are responsible for the degradation of ECM components such as collagen and elastin. MMP-1, interstitial collagenase, is one of a subfamily of MMPs that can specifically degrade the collagen triple helix. MMP-2 is a gelatinase that degrades denatured collagens and elastin. It has been determined that UV irradiation induces the degradation of ECM components by increasing the production of MMPs in skin.

In this study, we examined the effects of five biflavonoids on MMP-1 production and MMP-2 enzymatic activity in HDFs exposed to UV irradiation. We observed that sumaflavone and amentoflavone inhibited MMP-1 expression in a dose-dependent manner, but had no effect on MMP-2 enzymatic activity. Recently, we reported that amentoflavone potently suppresses nitric oxide (NO) production in macrophages treated with LPS, and we determined that this effect is associated with the transcriptional inhibition of the *iNOS* gene via nuclear factor-κb (NF-κB) inactivation.[32] In addition, we found that sumaflavone inhibits NO production in a concentration-dependent manner and blocks the LPS-induced expression of *iNOS* via AP-1 inhibition in macrophages.[33] NO is a multifunctional messenger molecule generated from L-arginine by inducible *iNOS*. NO production in some cells, including keratinocytes and fibroblasts, is induced by UV irradiation.[34] MMP expression is mediated by NO and inhibited by *iNOS* inhibitors.[35,36] Therefore, we suggest that the inhibitory effect of sumaflavone and amentoflavone on MMP-1 expression in HDFs might be associated with their potent NO blocking effect.

In conclusion, five biflavonoids were isolated from the whole plant of *S. tamariscina*. Various pharmacological studies have been performed previously on these five biflavonoids. Inhibition of MMP-1 and -2, however, has not been previously studied. In the present study, we found that sumaflavone and amentoflavone could inhibit enzyme expression of MMP-1 in human dermal fibroblasts. These results suggest that sumaflavone and amentoflavone could be developed as potential preventive or therapeutic agents of skin aging.

3. Experimental

3.1. Chemistry

The NMR spectra were recorded on a Varian Unity Inova 500 spectrometer. The FAB-MS was recorded on a JEOL JMS 700 mass spectrometer. TLC and preparative TLC were carried out on precoated Kieselgel 60 F_{254} (art. 5715, Merck) and RP-18 F_{254}s (art. 15389,

Merck) plates. Column chromatography was performed on silica gel 60 (40–63 and 63–200 μm, Merck), TSK gel TOYOPEARL HW-40© (cat. No. 07149, TOSOH), MCI gel (CHP 20P 75–150 μL, Lot number: 9E504; Mitsubishi Chemical Corp., Tokyo, Japan), and Sephadex LH-20 (25–100 μm, Sigma).

3.1.1. 2′,8″-Biapigenin (1). FAB-MS (*m*/*z*) 539 $[M+H]^+$, 419, 307, 154.

3.1.2. Sumaflavone (2). FAB-MS *m*/*z* 555 $[M+H]^+$, 539, 460, 381, 154.

3.1.3. Taiwaniaflavone (3). FAB-MS *m*/*z* 561 $[M+Na]^+$, 413, 381, 329, 176.

3.1.4. Amentoflavone (4). FAB-MS *m*/*z* 539 $[M+H]^+$, 460, 359, 307, 154.

3.1.5. Robustaflavone (5). FAB-MS *m*/*z* 539 $[M+H]^+$, 413, 391, 279, 149.

3.2. Biology

3.2.1. Chemicals. All chemicals used were of analytical grade and were purchased from Sigma–Aldrich (St. Louis, MO, USA) and TCI (Tokyo, Japan).

3.2.2. Cell culture. HDFs were obtained from neonatal foreskins and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 IU/mL penicillin, 50 μg/mL streptomycin, and 10% fetal bovine serum at 37 °C in a humidified atmosphere of 5% CO_2. HDFs were cultured until they were 90% confluent before being passaged. HDFs from passages 4 to 6 were used for the experiments.

3.2.3. UV irradiation. HDFs were cultured until 80% confluent. After 6 h starvation, the cells were washed twice with phosphate-buffered saline (PBS) and exposed to UVB irradiation (UVB lamp: G15TBE, SANKYO DENKI, Japan). The total energy dose of UVB irradiation was 40 mJ/cm^2. After UVB exposure, serum free DMEM containing the compounds under investigation was added to the cells. Culture medium was harvested 48 h later. The concentration of MMP-1 and enzyme activity of MMP-2 in the culture medium were determined as described below.

3.2.4. ELISA and Western blotting for MMP-1. MMP-1 expression in the culture medium was assessed by ELISA (Amersham, UK) and Western blotting. The ELISA was performed according to the manufacturer's instructions. All reactions were performed in triplicate. Protein concentration of the culture medium was measured using a Pierce Protein Assay Kit (Pierce Biotechnology, Inc., Rockford, IL, USA) with bovine serum albumin as the standard. Total protein of the culture medium was separated by electrophoresis on a 10% polyacrylamide gel. Following transblotting onto nitrocellulose, membranes were incubated with a monoclonal antibody specific for human MMP-1 (Calbiochem, 1:100 dilution). Anti-mouse IgG-HRP conjugate was used as the secondary antibody (Amersham, 1:1000 dilution). Immunoreactive bands were detected by chemiluminescence using ECL Western blotting detection reagents (Amersham).

3.2.5. Zymography for MMP-2. Zymography in 10% polyacrylamide gel containing 0.1% gelatin (Invitrogen, USA) was performed according to the method of Demeule et al.[37] Gelatin was used to detect MMP-2 activity in the culture medium. Fifteen microliters of cell-free medium was mixed with Tris–glycine SDS sample buffer (2×) without reducing agent and electrophoresed. After electrophoresis, SDS gel was incubated with 1× zymogram renaturing buffer (Invitrogen, USA) for 30 min at room temperature. 1× zymogram developing buffer (Invitrogen, USA) was then added to the gel. After incubation of the gel at 37 °C for 24 h, the gels were stained with 0.05% Commassie Blue and destained in 10% acetic acid and 40% methanol until proteinase bands were clearly visible in a blue background.

Acknowledgments

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