Protective Effects of Aucubin Isolated from *Eucommia ulmoides* against UVB-Induced Oxidative Stress in Human Skin Fibroblasts

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Ultraviolet-B (UVB) irradiation has been demonstrated to produce reactive oxygen species (ROS) in the cells and skin, which induces the synthesis of matrix metalloproteinases (MMPs), causing skin photoaging. Using the human skin fibroblast HS68 cell line in the present study, we investigated the photoprotective effects of aucubin from *Eucommia ulmoides*. Pretreatment with aucubin significantly inhibited the production of MMP-1 by 57% when compared to the UVB-irradiated cells. Additionally, the senescence-associated β-galactosidase (SA β-gal) activity was markedly decreased in the presence of aucubin, which indicates it as an antiphoto-induced aging compound. As the effect of aucubin was determined against ROS, the inhibited ROS formation and malondialdehyde (MDA) levels, and the increased cell viability and glutathione (GSH) level were observed with aucubin under UVB irradiation. Based upon these results, it was suggested that aucubin might play an important role in the cellular defense mechanism against UV radiation-induced photoaging. An understanding of the antioxidant properties of aucubin could, in part, act to elucidate its protective mechanism on the human skin photoaging.

Key words photoaging; ultraviolet B (UVB); *Eucommia ulmoides*; aucubin; oxidative stress

Human skin is constantly exposed to potentially harmful compounds and radiation because it serves as a protective barrier between environment and internal organs.[1,2] Ultraviolet (UV) irradiation has deleterious effects on human skin, including sunburn, immune suppression, cancer, and photoaging.[3] UV light is composed of UVA (320—400 nm), UVB (280—320 nm), and UVC (200—280 nm). Though UVC is absorbed by the atmospheric ozone layer, both UVA and UVB reach the surface of the earth and have physiological significance.[4] UVB, in particular, is the most hazardous environmental carcinogen known regards to human health.[5] UVB irradiation is known to provoke oxidative stress through the generation of reactive oxygen species (ROS), such as superoxide anion radical, hydroxy radical, hydrogen peroxide, and singlet oxygen in cells.[2,6—8] These ROS could result in the subsequent activation of complex signaling pathways, followed by MMP induction in skin cells.[9,10] Human skin expresses a number of MMPs, including MMP-1 (interstitial collagenase), MMP-8 (neutrophil collagenase), and MMP-13 (collagenase 3), all of which are capable of attacking native fibrillar collagen.[11] Of those, MMP-1 is the most important MMP in the degradation of the extracellular matrix by photoaging.[12]

Eucommia ulmoides Oliv. has been widely used in Korea, Japan, and China to treat hypertention.[13] Investigations have shown that *E. ulmoides* has pharmacological effects on coronary blood flow, pain relief, diuresis, and lipid metabolism.[14] Recently, a water extract from *E. ulmoides* leaves was reported to possess a potent antioxidant effect and to prevent oxidative DNA damage and lipid-peroxidation.[13,15] In our preliminary study, we isolated a phytochemical from the cortex of *E. ulmoides*, aucubin (1,4a,5,7a-tetra-5-hydroxy-7-(hydroxymethyl)cyclopenta(c)pyran-1-yl-β-D-glucopyranoside), a common iridoid glucoside. Aucubin is known to have a variety of biological activities such as antimicrobial, hepatoprotective, antitumoral, hemodynamic, choleretic, collagen synthesis, and anti-inflammatory effects.[16,17]

In the present study, we investigated the protective effects of aucubin isolated from *E. ulmoides* against the photoaging of human skin fibroblasts irradiated with UVB. We specifically focused on the role of aucubin in UVB-induced MMP-1 production, β-galactosidase activation, ROS production, MDA level, and GSH content.

MATERIALS AND METHODS

Reagents and Samples The normal human newborn foreskin fibroblast cell line, HS68 cell (ATCC CRL 1635), was obtained from American Type Culture Collection (Rockville, MD, U.S.A.). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and antibiotic–antimycotic were purchased from GIBCO (Grand Island, NY, U.S.A.). MMP-1 monoclonal antibody was obtained from Oncogene (Boston, MA, U.S.A.). Secondary antibody, 3,3′, 5,5′-tetramethylbenzidine (TMB), and 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) were purchased from Sigma (St. Louis, MO). Aucubin was previously isolated from *E. ulmoides* in our lab (data not shown). Isolated aucubin (Fig. 1) was identified by the comparison of NMR spectra and MS with commercial aucubin (Wako, Japan). All other chemicals were of analytical reagent-grade.

Cell Culture HS68 cells were plated in 100 mm tissue culture dishes and cultured in DMEM supplemented with 10% FBS and 1% antibiotic–antimycotic. The cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2–95% air. When cells reached above 80% confluency, subculture was conducted at a split ratio of 1 : 5.

UV Irradiation As a UVB source, a UVB lamp (312 nm, Spectroline Model EB-160C, New York, NY, U.S.A.) was used. In brief, serum-starved confluent cells were rinsed

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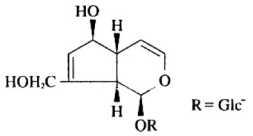

Fig. 1. Chemical Structure of Aucubin Isolated from *Eucommia ulmoides*

Aucubin was purified using the Waters 2690 HPLC analysis system (Symmetry C_{18} reverse column: 3.9×150 mm; mobile phase; water–acetonitrile (97 : 3, v/v); flow rate, 1.0 ml/min; UV detector; absorbance monitor operating at 210 nm; injection volume, 20 μl). To identify the structure of this compound, $^{1}H/^{13}C$-NMR and EI-MS were performed.

twice with phosphate-buffered saline (PBS), and all irradiations were performed under a thin layer of PBS. Immediately after irradiation, fresh serum-free medium was added to the cells. Responses were measured after an incubation period of 24 h. Mock-irradiated controls followed the same schedule of medium changes without UVB irradiation.

Assay for MMP-1 Production The production of MMP-1 was determined by enzyme-linked immunosolvent assay (ELISA).[18] Aliquots of media were transfer into immunowell plates and were incubated at 37 °C for 2 h. The well was blocked with casein, and subsequently incubated with MMP-1 antibody at 37 °C for 1 h. The plate was then washed with wash buffer, incubated with secondary antibody linked to peroxidase at 37 °C for 1 h, thoroughly re-washed with wash buffer, and subsequently re-incubated with tetramethylbenzidine (TMB) until color development. The absorbance was measured with a microplate reader at 450 nm.

SA β-Gal Staining SA β-gal staining was conducted according to the method of Yang and Hu.[19] In brief, the cells were washed twice with PBS and fixed for 5 min in 3% formaldehyde. They were then washed and incubated at 37 °C with fresh SA β-gal stain solution (1 mg/ml of X-Gal, 5 mM of potassium ferricianide, 5 mM of potassium ferrocyanide, 150 mM of NaCl, 2 mM of $MgCl_2$ in PBS at pH 6.0). Staining was evident in 12—16 h. Cells were counted at ×200 magnification.

Cytotoxicity Cell viability was assessed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.[4] Cells were pretreated with aucubin at a concentration of 0.01, 0.1, or 1 μg/ml prior to UVB irradiation, hydrogen peroxide, and *S*-nitroso-*N*-acetyl-DL-penicillamine (SNAP). After incubation for 24 h, MTT solution (final concentration: 0.5 μg/ml) was added and cells were incubated at 37 °C for 3 h. The supernatant was then removed, and 100 μl of dimethyl sulfoxide (DMSO) was added. The absorbance was read on a microplate reader at 570 nm to obtain the percentage of viable cells.

Assay for ROS Production DCFH-DA was used to detect ROS production in cells.[8] DCFH-DA, which had entered the cell was cleaved to form DCFH. Trapped DCFH was oxidized by oxygen free radicals to produce fluorescent DCF. Cells which had been treated with aucubin were incubated with 20 μM of DCF-DA for 30 min. Cells were harvested at the indicated time points after UV irradiation. Immediately after two washes with PBS, the ROS formation was analyzed by a fluorometer (TECAN SER-NR 94572, Salzburg, Austria) using 485 nm of exitation and 530 nm of emission filters.

Malondialdehyde Determination The amount of malondialdehyde (MDA) was measured using thiobarbituric acid (TBA).[20] Lipid peroxidation was evaluated at 24 h after UVB exposure. Cells were mixed with TBA reagent consisting of 0.375% TBA and 15% trichloroacetic acid in 0.25 N of hydrochloric acid. The reaction mixtures were placed in a boiling water bath for 30 min and centrifuged at 2500 rpm for 5 min. The absorbance of the supernatant was read at 535 nm. The MDA concentrations of cells were calculated using an extinction coefficient of $1.56\times10^{5}\ M^{-1}\ cm^{-1}$.

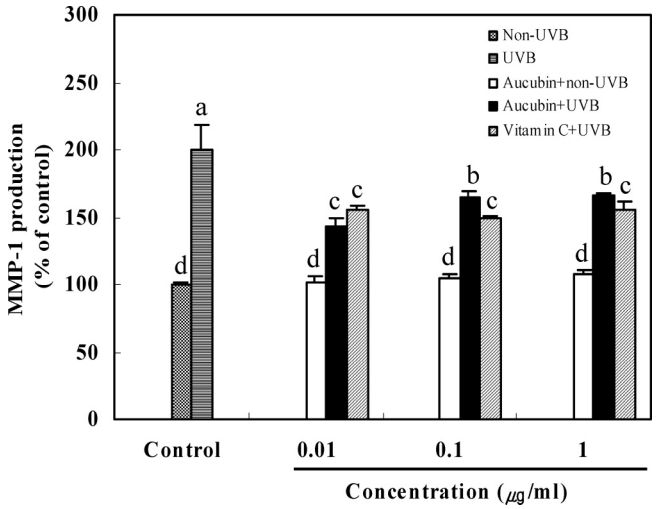

Fig. 2. Inhibition of UVB-Induced MMP-1 Production by Aucubin in Human Skin Fibroblasts

The cells were pretreated with aucubin (0.01, 0.1, 1 μg/ml) prior to UVB irradiation (100 mJ/cm^2) and harvested 24 h later. MMP-1 production was determined by ELISA, as described in Materials and Methods. Vitamin C was used as a positive control. Each bar represents the mean±S.D. (n=3). $p<0.05$.

Glutathione Levels The GSH content was measured according to the method of Moron *et al*.[21] Cells were washed twice with PBS and lysed with passive lysis buffer (Promega Co., Madison, WI, U.S.A.). Proteins were precipitated by 2% sulfosalicylic acid and were subsequently centrifuged. The supernatant was mixed with 0.1 M of sodium phosphate buffer (pH 8.0) and 0.5 mM of 5,5-dithiolbis (2-nitrobenzoic acid) (DTNB), and were incubated at room temperature for 10 min. The absorbance of the product was measured at 412 nm. The GSH concentration was calculated using an extinction coefficient of $13.6\ mM^{-1}\ cm^{-1}$.

Statistical Analysis All experiments were repeated a minimum of three times. Data was analyzed by the difference between means, and statistical significance was calculated using Fisher's least significant difference (LSD) or Student's *t*-test.

RESULTS

Inhibitory Effect of Aucubin on MMP-1 Production and β-Galactosidase Activity Pretreatment of cells with aucubin resulted in suppression of the MMP-1 elevation caused by UVB irradiation (Fig. 2). As a positive control, vitamin C was also examined for inhibitory effect of MMP-1 production. The highest inhibitory effect on MMP-1 was found after treatment with aucubin at a concentration of 0.01 μg/ml. Its inhibitory effect was 57.3%, which was significantly higher than that of vitamin C. However, the inhibitory effects were decreased to 36% and 34%, respectively, at aucubin concentrations of 0.1 and 1 μg/ml. The per-

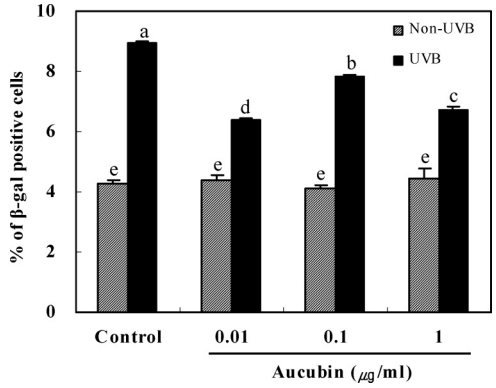

Fig. 3. Effect of Aucubin on β-Galactosidase Activity in Human Skin Fibroblasts

The cells were pretreated with aucubin (0.01, 0.1, 1 μg/ml) prior to UVB irradiation (100 mJ/cm^2) and were then stained for β-gal activity. In all cases, a minimum of 400 cells were counted for each data point. Each bar represents the mean±S.D. (n=3). p<0.05.

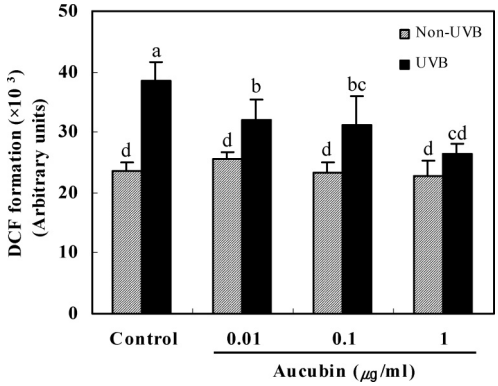

Fig. 4. Effect of Aucubin on ROS Production in Human Skin Fibroblasts after UVB Irradiation

The cells were pretreated with aucubin (0.01, 0.1, 1 μg/ml) prior to UVB irradiation (100 mJ/cm^2). Followed by incubation for 1 h, the production of ROS was determined by DCFH-DA method, as described in Materials and Methods. Each bar represents mean±S.D. (n=4). p<0.05.

Table 1. Effects of Aucubin on Cell Viabilities under UVB Irradiation, Hydrogen Peroxide, and *S*-Nitroso-*N*-acetyl-DL-penicillamine (SNAP)

ROS source[a)]	Dose	Aucubin Treated (%)[b)]			
		0	0.01	0.1	1
None	0	100.0±4.1^a	107.5±8.1^a	104.3±7.1^a	104.6±7.3^a
UVB (mJ/cm^2)	50	71.6±12.0^a	87.9±1.5^a	82.7±9.9^a	79.6±2.1^a
	100	67.8±4.1^b	83.2±1.7^a	85.9±4.1^a	74.7±7.1^b
	150	40.6±0.3^c	60.5±4.3^a	52.2±5.1^b	52.6±0.7^b
Hydrogen peroxide (mM)	0.05	88.8±2.6^c	89.9±1.1^{bc}	97.4±4.8^{ab}	99.8±2.2^a
	0.1	42.3±0.7^b	53.6±2.5^{ab}	64.6±7.4^a	65.9±4.8^a
	0.25	9.3±0.5^b	12.0±0.4^b	22.7±2.6^a	21.0±4.3^a
SNAP (mM)	0.5	60.4±1.2^c	64.3±0.7^{bc}	68.1±1.4^b	73.0±2.3^a
	1	58.2±2.2^b	64.4±0.3^a	67.3±1.3^a	66.6±2.3^a
	2	40.4±1.1^c	55.9±0.9^b	60.1±1.2^a	57.7±0.1^{ab}

a) The cells were treated with UVB (50, 100, 150 mJ/cm^2), hydrogen peroxide (0.05, 0.1, 0.25 mM), and SNAP (0.5, 1, 2 mM). *b*) The cells were pretreated with aucubin (0.01, 0.1, 1 μg/ml) prior to UVB irradiation, hydrogen peroxide, and SNAP. Cell viability was measured by MTT assay, as described in Materials and Methods, and was calculated in terms of relative values. Each value represents mean±S.D. (n=4). Values in a row indicated with different letters are significantly different (p<0.05).

centage of SA β-gal positive cells was increased in a dose-dependent manner upon UVB irradiation (data not shown). The SA β-gal positive cells were 2.1-fold higher in 100 mJ/cm^2 of UVB-irradiated cells than that in non-irradiated cells (Fig. 3). Pretreatment with aucubin significantly inhibited β-galactosidase activity.

Changes of Cell Viability by Aucubin under UVB Irradiation, Hydrogen Peroxide, and SNAP To examine the effect of UVB irradiation, the cultured human skin fibroblasts were exposed to various doses of UVB at 0, 50, 100, and 150 mJ/cm^2, and viabilities of cells were determined 24 h later by MTT assay. The UVB decreased the cell viability of human skin fibroblasts in a dose-dependent manner (Table 1). To further clarify the effect of aucubin on skin fibroblasts under UVB irradiation, aucubin was added at concentrations of 0.01—1 μg/ml. Pretreatment with 0.1 μg/ml of aucubin significantly increased cell viability by 20% compared to only UVB-irradiated (100 mJ/cm^2) cells, with a p-value <0.05. Additionally, treatment with hydrogen peroxide or SNAP as different free radical generation sources decreased cell viability in a concentration-dependent manner. At most of the concentrations, pretreatment with aucubin also increased cell viability (p<0.05).

Effects of Aucubin on Free Radical Generation and GSH Level The ROS formation was evaluated after cells were irradiated at 100 mJ/cm^2 of UVB irradiation. As shown in Fig. 4, pretreatment with aucubin significantly attenuated an increase (p<0.05) in intracellular ROS content, which was triggered by UVB irradiation. At a high concentration of aucubin (1 μg/ml), the intracellular ROS levels were noticeably reduced by *ca*. 34% compared to the control.

Lipid peroxidation was evaluated at 24 h after UVB irradiation. As expected, the MDA content, measured as an index of lipid peroxidation, was increased with an elevated dose of UVB (Fig. 5A). The MDA level at 150 mJ/cm^2 of UVB irradiation was approximately 2.2-fold greater than that of non-irradiated cells (p<0.05). However, MDA levels were significantly decreased with the treatment of aucubin (Fig. 5B). The highest antioxidative effect against lipid peroxidation was observed at an aucubin concentration of 1 μg/ml.

Finally, to examine the relation between GSH and UVB-mediated oxidative stress, changes in intracellular GSH were determined 24 h after UVB irradiation (100 mJ/cm^2). As shown in Fig. 6, UVB caused a significant depletion (p<0.05) in intracellular GSH levels, compared to that of non-irradiated cells. Pretreatment with aucubin at concentrations of 0.1 and 1 μg/ml elevated the GSH levels by 1.3-fold and 1.4-fold, respectively.

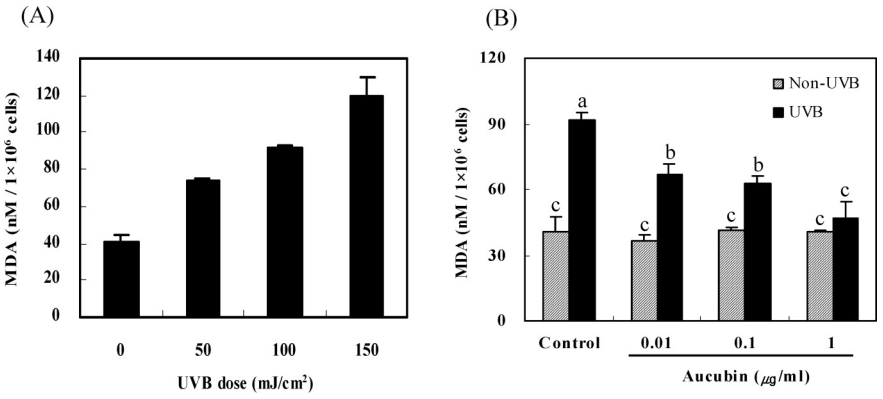

Fig. 5. Protective Effect of Aucubin on UVB-Induced Lipid Peroxidation in Human Skin Fibroblasts

(A) The cells were treated with UVB (50, 100, 150 mJ/cm^2). (B) The cells were pretreated with aucubin (0.01, 0.1, 1 μg/ml) prior to UVB irradiation (100 mJ/cm^2) and harvested 24 h later. MDA levels were measured using the thiobarbituric acid method, as described in Materials and Methods. Each bar represents the mean±S.D. (n=3). p<0.05.

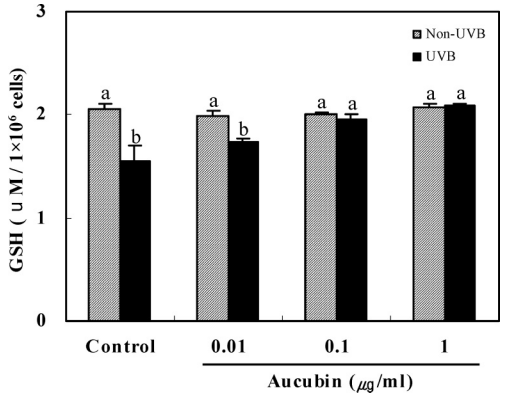

Fig. 6. Intracellular GSH Contents in Human Skin Fibroblasts

The cells were pretreated with aucubin (0.01, 0.1, 1 μg/ml) prior to UVB irradiation (100 mJ/cm^2) and harvested 24 h later. The GSH contents were measured using the DTNB, as described in Materials and Methods. Each bar represents the mean±S.D. (n=3). p<0.05.

DISCUSSION

In recent years, the development of novel compounds with anti-photoaging activities from natural plants has received a great deal of attention. Since the inhibition of the production of MMPs appears to be a useful intervention for preventing collagen damage,[22] we investigated the inhibitory effect of MMP-1 production among 50 traditional herbal plants in the preliminary study, and identified aucubin from *E. ulmoides* as an anti-photoaging compound.

MMP-mediated collagen damage is a major contributor to the phenotype of photoaged human skin.[6] The UVB irradiation which induces expression of various MMPs transiently suppresses the transcription of type I collagen genes, which, in turn, impairs the structural integrity of the dermis.[6,11,23] Therefore, inhibition of the induction of MMPs has been reported to alleviate UV-induced photoaging in terms of the protection from collagen destruction.[22] Of the various types of MMP, MMP-1 acted more efficiently than the other collagenases (MMP-8 and MMP-13) in inducing collagen damage.[11] In the present study, a significant increase in the production of MMP-1 was observed at 24 h after UVB irradiation. In addition, the levels of MMP-1 mRNAs were noticeably elevated (data not shown). However, pretreatment with aucubin led to a significantly high inhibition of the production of MMP-1. The protective effect of aucubin against UVB-induced MMP-1 was comparable or better than that of vitamin C. It has been reported that vitamin C possesses the ability to protect fibroblasts from UV-induced oxidative stress.[3,4] Since photoaging takes place due to the loss of collagen components by enhanced enzymatic degradation through the induction of MMPs,[22] the inhibitory effects of aucubin on the induction of MMP-1 strongly suggest that aucubin might be a valuable compound against UVB-mediated photoaging.

The biomarkers of cellular senescence triggered by UVB or H_2O_2 examine permanent cell cycle arrest, enlarged and flattened cell morphology, and SA β-gal activity.[24] In culture and *in vivo* biopsy, SA β-gal is measured in a variety of cells and tissues to demonstrate the onset of cell senescence.[19] Aucubin reduced the SA β-gal positive cells, which confirms that it plays an important role in the retardation of the accumulation of senescent fibroblasts in human skin.

UV radiation penetrates readily into dermal tissue and is highly implicated in the aging of skin. Therefore, human dermal skin fibroblasts are a useful model for investigating the effect of antioxidants on UV-induced skin photoaging.[3] Regarding the mammalian UV response, two signaling pathways have been identified. The primary pathway consists of UV irradiation-dependent generation of ROS near or within the cell membrane.[23] In our studies, we focused on the involvement of UVB-generated ROS leading to the induction of MMPs. UVB interacts with cellular chromotophores and photosensitizers, which results in the generation of ROS, damage to DNA, and activation of cytoplasmic signal transduction pathways, which are related to growth, differentiation, replicative senescence, and connective tissue degradation.[25] According to many researchers, UVB causes a dramatic dose-dependent decrease in intracellular GSH, intramembrane vitamin E, and membrane fluidity, as well as an increase in MDA levels.[26,27] The DCF fluorescence represents intracellular activity, so an increase in fluorescence is suggestive of the intracellular oxidative stress. Pretreatment with aucubin significantly attenuated DCF fluorescence, with reduction of approximately 34% at the highest concentration (1 μg/ml) of aucubin. Brenneisen *et al*.[23] reported that the diminished generation of ROS by preincubatin of cells with the free radical scavenger, *N*-acetylcysteine, prior to UVB irradiation resulted in significant reductions of c-jun mRNA,

MMP-1 mRNA, and MMP-3 mRNA levels compared to those of UVB-irradiated cells. The use of free radical scavengers to reduce the harmful effects of UVB radiation is a novel approach to photoprotection and skin cancer prevention.[28)]

In this study, we examined the effect of aucubin on cellular antioxidant status in skin fibroblasts under UVB irradiation. The GSH content was significantly reduced in UVB irradiated cells, while aucubin inhibited UVB-induced decline in GSH in skin fibroblasts. The present results suggest that the initial depletion of GSH by UVB induces an increase in ROS production. Because GSH is the main antioxidant system in cells, the depletion of GSH may predominantly affect the cell death due to the ROS accumulation in UVB-irradiated human skin fibroblasts.[29)] Vitamin C and caffeine have been reported to protect the UV irradiation-induced GSH decline.[4,30)]

Lipid peroxidation has been used as an indirect indicator of oxidative stress.[31)] In this study, MDA content of cells, measured as a marker of lipid peroxidation,[4)] was elevated as the dose of UVB increased. Our finding is in agreement with previous studies, which revealed that MDA is increased by UVB irradiation in humans.[20)] With the inclusion of aucubin, lipid peroxidation was inhibited during UVB irradiation.

In conclusion, we confirm that aucubin from *E. ulmoides* possesses photoprotective effects against oxidative stress. The presence of aucubin scavenges UVB-induced free radicals in human skin fibroblasts, which, in turn, decreases MMP-1 production, and the activation of β-galactosidase. Considering these results, aucubin is capable of reducing the oxidative stress induced by UVB irradiation in a human skin cell system and may be of therapeutic value in the prevention of photoaging. Further study is underway to evaluate pharmacological and *in vivo* efficacy in the presence of this compound.

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