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Effects of Aucubin Isolated from *Eucommia ulmoides* on UVB-induced Oxidative Stress in Human Keratinocytes HaCaT

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Abstract Ultraviolet B (UVB) radiation provokes the generation of reactive oxygen species (ROS) in the cells and skin, which induce oxidative stress in the exposed cells, leading to photoaging and cancer. Using the human keratinocytes HaCaT cell line, we investigated the photoprotective effects of aucubin isolated from *Eucommia ulmoides*. Pretreatment with aucubin markedly suppressed UVB-induced oxidative stress, which manifests as a decrease in intracellular lipid peroxidation, elevation of catalase activity, and reduced glutathione content. In addition, aucubin significantly reduced expression of matrix metalloproteinase-1 (MMP-1) protein (54%) and mRNA. Taken together, these results suggest that aucubin may offer protection against UVB-induced oxidative stress and may be used as a potential agent in prevention of UVB-induced photoaging.

Keywords: photoaging, ultraviolet B (UVB), aucubin, oxidative stress, matrix metalloproteinase-1 (MMP-1)

Introduction

Human skin, as a protective barrier between internal organs and environment, constantly exposed to potentially harmful effects of various exogenous physical and chemical agents including ultraviolet (UV) radiation (1). UV radiation is responsible for the cutaneous damage after both acute and chronic exposure, and is believed to be an important etiology in human skin cancer and premature skin aging (2). UVB has a low level of skin penetration, but it can readily affects macromolecules in the epidermal layer, thus altering cellular functions via DNA damage, generation of reactive oxygen species (ROS), decreased in skin content of antioxidant compounds (3). Recent studies have shown that ROS such as superoxide anion, hydroxyl radical, and hydrogen peroxide are responsible for UV-induced oxidative damage (4,5). These ROS could result in the subsequent activation of complex signaling pathways, followed by matrix metalloproteinase (MMP) induction in skin cells (6,7).

The MMPs form a family of structurally and functionally related zinc endopeptidases that exhibit different substrate specificities (8). MMPs expression is low in unstimulated skin cells or normal skin tissues, but some MMPs are induced by various extracellular stimuli, e.g., UV or infrared radiation, growth factors, cytokines, and tumor promoters (9). The enzyme mainly responsible for collagen breakdown in the skin is MMP-1, which is produced by both dermal fibroblasts and epidermal keratinocytes, cleaves type 1 collagen into specific fragments (8,10). Once collagen is initially cleaved by MMP-1, MMP-3, and other MMP promote further collagen breakdown (8).

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In recent years, various natural compounds from both nutritive and non-nutritive sources were reported to protect against UV-induced skin damage (11,12). *Eucommia ulmoides* Oliv. is a traditional medicine, widely used in Korea, Japan, and China for the treatment of hypertention (13). Recently, Yen and Hsieh (13,14) has reported that water extract from *E. ulmoides* leaves possess antioxidant effect in oxidative DNA damage and lipid-peroxidation model. In our previous study, we isolated aucubin (1,4a,5,7a-tetra-5-hydroxy-7-(hydroxymethyl)cyclopenta(c) pyran-1-yl-β-D-glucopyranoside) from the cortex of *E. ulmoides* as a photoprotective phytochemical in skin fibroblasts (15). Aucubin has been shown various activities such as antimicrobial, hepatoprotective, antitumoral, hemodynamic, choleretic, collagen synthesis, and anti-inflammatory effects (16).

Thus, in the present study, we investigated the protective effects of aucubin against the UVB-induced photodamage in human skin keratinocytes HaCaT. We focused on their effects on ROS induced cellular oxidative stress, antioxidant activities, and MMP-1 production.

Materials and Methods

Reagents and samples Human skin epidermal keratinocytes HaCaT were kindly provided from Dr. Fusening (German Cancer Research Center, Heidelberg, Germany). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and antibiotic-antimycotic were purchased from Gibco (Grand Island, NY, USA). MMP-1 monoclonal antibody was obtained from Oncogene (Boston, MA, USA). Secondary antibody, 3,3',5,5'-tetramethylbenzidine (TMB), and 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Aucubin was previously isolated from *Eucommia ulmoides* in our laboratory (15). Isolated aucubin was identified by the comparison of nuclear

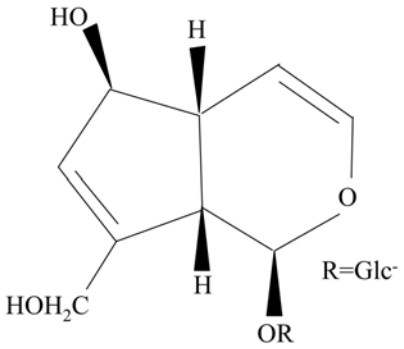

Fig. 1. Chemical structure of aucubin isolated from *Eucommia ulmoides*. Aucubin was purified using the Waters 2690 HPLC analysis system (μ-bondapack C_{18} reverse column, 3.9×150 mm; mobile phase, water-acetonitrile (97:3, v/v); flow rate, 1.0 mL/min; UV detector, absorbance monitor operating at 210 nm; injection volume, 20 μL). $^{1}H/^{13}C$-NMR and EI-MS were performed to identify the structure of this compound.

magnetic resonance (NMR) spectra and mass spectrometry (MS) with commercial aucubin (Wako, Osaka, Japan, Fig. 1). (-)-Epigallocatechin-3-gallate (EGCG) was used as a positive control (17). All other chemicals were of analytical reagent-grade.

Cell culture The HaCaT cells (immortalized human keratinocyte cell line) were cultured in DMEM supplemented with 10% FBS and 1% antibiotic-antimycotic. The cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2. When cells reached above confluency, subculture was conducted at a split ratio 1:4.

UVB irradiation A UVB lamp (312 nm, Model EB-160C; Spectroline, New York, NY, USA) was used as a UVB source. In brief, serum-starved confluent cells were rinsed 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 hr. Mock-irradiated controls followed the same schedule of medium changes without UVB irradiation.

Measurement of cell viability Cell viability was measured using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (18). Cells were pretreated with aucubin at a concentration of 0.1, 1, or 10 μM prior to UVB irradiation. After incubation for 24 hr, MTT solution (final concentration: 0.5 mg/mL) was added and cells were incubated at 37°C for 3 hr. 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 (19). 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 2 washes with PBS, the ROS formation was analyzed by a fluorometer (SER-NR 94572; TECAN, Salzburg, Austria) using 485 nm of exitation and 530 nm of emission filters.

Glutathione levels The glutathione (GSH) content was measured according to the method of Moron *et al*. (20). Cells were washed twice with PBS and lysed with passive lysis buffer (Promega Corp., Madison, WI, USA). 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-dithiol-bis (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 13.6×10^{4}/M·cm.

Malondialdehyde determination The amount of malondialdehyde (MDA) was measured using thiobarbituric acid (TBA) (21). Lipid peroxidation was evaluated at 24 hr 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 500×g 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 1.56×10^{5}/M·cm.

Antioxidant enzyme activities The catalase activity was measured according to the method of Aebi (22). One unit of catalase was defined as the amount of enzyme required to decompose 1 μM of H_2O_2 in 1 min. The assay mixture consisted of 50 mM phosphate buffer (pH 7.0), 20 mM H_2O_2 and cell lysate as an enzyme source in a final volume of 1.0 mL. The rate of decomposition of hydrogen peroxide was measured spectrophotometrically at 240 nm for 1 min. Superoxide dismutase (SOD) activity was based on the method described by McCord and Fridovich (23). For the determination of SOD activity, xanthine and xanthine oxidase are used to generate superoxide radicals reacting with 2-(4-iodophenyl)3-(4-nitrophenol)-5-phenyl tetrazolium chloride to form a red formazan dye. SOD activity was then measured at 505 nm.

Assay for MMP-1 production The production of MMP-1 was determined by enzyme-linked immunosorbent assay (ELISA) (24). Aliquots of media were transferred into immunowell plates and were incubated at 37°C for 2 hr. The well was blocked with casein, and subsequently incubated with MMP-1 antibody at 37°C for 1 hr. The plate was then washed with wash buffer, incubated with secondary antibody linked to peroxidase at 37°C for 1 hr, 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.

Reverse transcription-polymerase chain reaction (RT-PCR) RT-PCR (25) was performed to determine aucubin

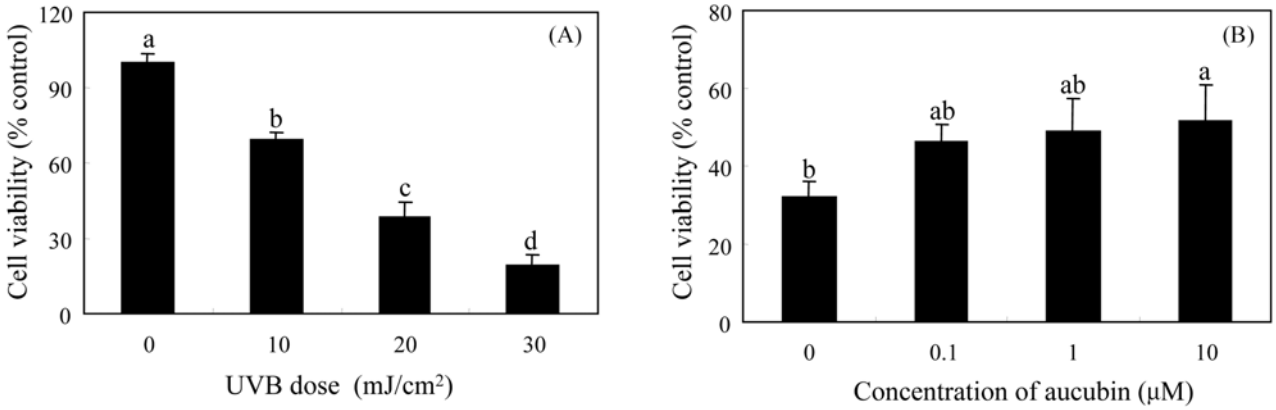

Fig. 2. Effect of aucubin on cell viability of human skin keratinocytes. (A) Cell viability of human Keratinocytes following UVB irradiation (0, 10, 20, 30 mJ/cm^2). (B) Effect of aucubin on cell viabilities under UVB irradiation (20 mJ/cm^2). Each bar represents the mean±SD (n=5). The bars with a different mark represent differences with statistical significance (p<0.05).

affecting MMP-1 gene expression. Total RNA was prepared from 2×10^6 cells, cultured with or without aucubin using easy-Blue Total RNA Extraction kit (iNtRON Biotech., Seoul, Korea). The primers for MMP-1 were 5'-AGGTTAT CCCAAAATGATAG-3' and 5'-TGCAGTTGAACCAGCT ATTA-3', those for β-actin were 5'-TCCTTCTGCATCCT GTCGGCA-3' and 5'-CAAGAGATGGCCACGGCTGCT-3'. Approximately 0.5 μg of total RNA was used for cDNA amplification. Sequential cycles included 25 cycles at 94°C for denaturation (30 sec), 60°C for annealing (30 sec), and 72°C for extension (45 sec). The expected lengths of PCR products for MMP-1 and β-actin were 173 and 275 bp, respectively. Amplified products were separated by 2% agarose gel electrophoresis and were identified by ethidium bromide staining (Gel Doc 2000 and the Quantity One program; Bio-Rad, Sydney, Australia).

Statistical analysis All experiments were repeated a minimum of 3 times. Data were analyzed by the difference between means, and statistical significance was calculated using analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) test or Student's t-test.

Results and Discussion

Changes of cell viability by aucubin under UVB irradiation Mammalian skin keratinocytes are important cells in the skin and connective tissues, and are continuously exposed to UV irradiation (26). Keratinocytes exposed to solar UV irradiation exhibit a complex alteration of gene expression, which results in cutaneous inflammation, premature aging, tumor promotion, and cell death (27).

MTT assay was performed to examine the toxicity of aucubin on keratinocytes. Pretreatment with aucubin at 0.1, 1, 10, and 20 μM did not significantly affect cell viability (data not shown). To confirm the cytotoxic effect of UVB irradiation, cultured human skin keratinocytes were exposed to various doses of UVB at 0, 10, 20 and 30 mJ/cm^2, and viabilities of cells were determined 24 hr later by MTT assay. As shown in Fig. 2A, UVB irradiation of keratinocytes led to a dose-dependent suppression of cell viability (69%, 10 mJ/cm^2; 39%, 20 mJ/cm^2; 20%, 30 mJ/cm^2), compared with non-irradiated cells. A 20 mJ/cm^2 irradiation dose was used in the following studies if not specified otherwise. Pretreatment with 10 μM aucubin significantly increased cell viability by 15% (Fig. 2B) compared to UVB-irradiated control cells (p<0.05).

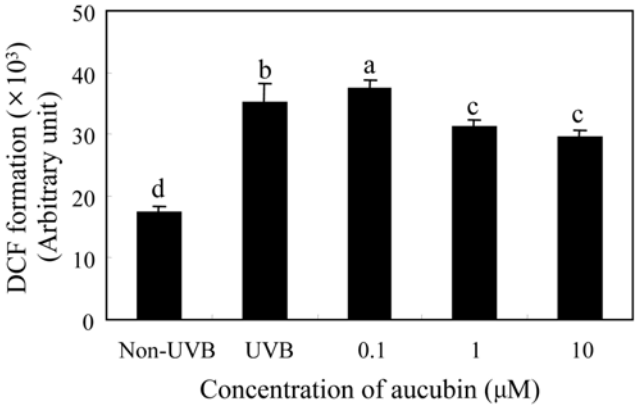

Fig. 3. Effects of aucubin on the generation of cellular reactive oxygen species in human skin keratinocytes induced by UVB irradiation. The cells were pretreated with aucubin (0.1, 1, 10 μM) prior to UVB irradiation (20 mJ/cm^2) and harvested 24 hr later. Each bar represents the mean±SD (n=5). The bars with a different mark represent differences with statistical significance (p<0.05).

Effects of aucubin on free radical generation UV irradiation is associated with formation of ROS leading to skin aging and photocarcinogenesis (28). Increased ROS generation can overwhelm antioxidant defense mechanisms, resulting in oxidative stress and oxidative photodamage of proteins and other macromolecules in the skin (29). Therefore, it is likely that ROS generated by UV irradiation plays a critical role in UV-induced skin damage (29). We measured the intracellular oxidative stress levels using the redox sensitive dye DCFH-DA (2). UV irradiation significantly increased ROS generation by 2-fold versus the non-irradiated control cells. The increase in ROS was markedly reduced (p<0.05) in the presence of aucubin in a concentration dependent manner (Fig. 3). Several reports have suggested that botanical antioxidants, including polyphenols, have been shown to be associated with reduced incidence of

Table 1. Activities of catalase and levels of glutathione (GSH) and malondialdehyde (MDA) in human skin keratinocytes[1)]

Aucubin (μM)	Catalase (U/mg protein)	SOD (U/mg protein)	GSH (μM/mg protein)	MDA (nM/mg protein)
Non-UVB	2.80±0.11[a]	3.36±0.05[a]	11.07±0.16[a]	0.65±0.13[d]
UVB	1.02±0.00[d]	1.35±0.18[d]	6.81±1.12[c]	2.72±0.18[a]
0.1	1.34±0.16[b]	2.34±0.10[b]	7.29±0.00[c]	2.21±0.25[bc]
1	1.28±0.00[bc]	2.04±0.03[c]	8.95±0.06[b]	1.88±0.32[c]
10	1.04±0.10[cd]	2.13±0.05[bc]	9.90±0.62[ab]	2.31±0.17[b]

[1)]The cells were pretreated with aucubin (0, 0.1, 1, and 10 μM) prior to UVB irradiation (20 mJ/cm^2). Each value represents mean±SD (n=5). Values in the same column indicated with different letters are significantly different (p<0.05).

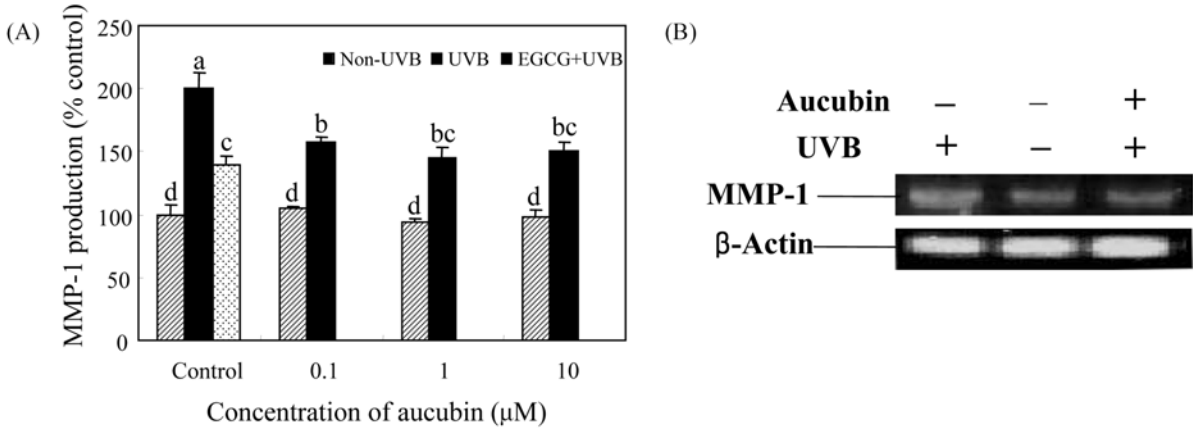

Fig. 4. Inhibition of UVB-induced MMP-1 production by aucubin in human skin keratinocytes. The cells were pretreated with aucubin (0.1, 1, 10 μM) or EGCG (1 μM) prior to UVB irradiation (20 mJ/cm^2) and harvested 24 hr later. (A) MMP-1 production was determined by ELISA, as described in the text. Each bar represents the mean±SD (n=5). The bars with a different mark represent differences with statistical significance (p<0.05). (B) Total RNA was isolated and analyzed by RT-PCR to determine MMP-1. The molecular weight of the products was estimated by comparison to a 100-bp ladder.

ROS-mediated photocarconogenesis and photoaging (30, 31). Also, our results demonstrated the protective potential of aucubin in human keratinocytes.

Antioxidant activities UVB irradiation induces the formation of ROS and depletes cellular antioxidant stores (2). Recently Na *et al.* (2) demonstrated that antioxidant may reduce the UVB-induced skin damage. Excessive exposure to UV irradiation is thought to overwhelm and deplete this antioxidant supply, thereby leading to a state of oxidative stress (32). Keratinocytes contain high levels of antioxidants such as GSH and various enzymes involved in antioxidant defense, including SOD and catalase (33).

Antioxidant activity of aucubin was measured by levels of GSH and MDA and activities of catalase and SOD. The antioxidant activities in UVB irradiated keratinocytes are shown in Table 1. SOD is a nuclear-encoded antioxidant enzyme belonging to a complex interdependent antioxidant enzymatic network that protects cells from oxidative damage by rapidly converting superoxide anions to H_2O_2 and molecular oxygen (34). Catalase, an antioxidant enzyme responsible for catalyzing the conversion of UV-induced H_2O_2 to water and oxygen, is diminished after UV irradiation (35). The activities of catalase and SOD at 20 mJ/cm^2 of UVB irradiation were decreased by 36.4 and 40.2% compared to that of non-irradiated cells. However, activities of catalase and SOD significantly increased with aucubin treatment (p<0.05). As shown in Table 1, the level of intracellular GSH as a strong non-enzymatic antioxidant in UVB irradiated keratinocytes at 24 hr was also significantly reduced by 38% compared with that of non-irradiated cells (p<0.05). Pretreatment with the aucubin (0.1, 1, and 10 μM) significantly elevated the intracellular GSH level by 1.1, 1.3, and 1.5-fold, respectively (p<0.05).

In this study, MDA, an end product of lipid peroxidation, has been used as an indicator of oxidative stress (36). As expected, the MDA content was increased with an elevated dose of UVB (data not shown). UVB-induced oxidative stress was effectively prevented by pretreatment with aucubin. The MDA level at 20 mJ/cm^2 of UVB irradiation was approximately 4.2-fold greater than that of non-irradiated cells. The increase in MDA level induced by UVB was markedly suppressed by pretreatment of 1 μM aucubin to 31% (p<0.05). The diminution in cellular GSH and increased level of lipid peroxidation products were due to ROS production in keratinocytes. Pretretment of aucubin preserved the cellular antioxidant capacity such as GSH, catalase, and SOD which were significantly reduced after UVB irradiation. These results indicated that pretreatment of aucubin might play an important role in protection against UVB-induced oxidative damage.

Inhibitory effect of aucubin on MMP-1 production Exposure of human skin to UV irradiation results in the

induction of a series of MMPs, which have been implicated in photoaging (29). UV irradiation upregulates synthesis of several MMPs by keratinocytes, including MMP-1, -3, and -9 (37). Especially, MMP-1 is involved in UV-induced premature skin aging by degrading various components of the dermal extracellular matrix (8).

Because aucubin was shown to have strong antioxidant activity, we tested whether it affected UVB-induced MMP-1 expression in human keratinocytes HaCaT. The MMP-1 inhibitory effects of aucubin measured by ELISA and RT-PCR are shown in Fig. 4. Pretreatment of cells with aucubin resulted in suppression of the MMP-1 elevation caused by UVB irradiation (Fig. 4A). Aucubin significantly inhibited the expression of MMP-1 protein by 43% at 0.1 μM, 54% at 1 μM, and 49% at 10 μM, respectively ($p<0.05$). The MMP-1 inhibitory effects of aucubin were similar to EGCG at the concentration of 1 μM. An increasing concentration of aucubin exhibited a non-linear dose response in the inhibition of UVB-induced MMP-1 production. Higher doses (1 and 10 μM) of aucubin had less inhibitory effect on MMP-1 production than the lower dose (0.1 μM), the reason for this being unclear at present. Similar results, showing a biphasic effect, have been reported in UV-induced ROS with a melatonin treatment (38). As shown in Fig. 4B, the level of MMP-1 mRNA was also decreased by pretreatment with 1 μM aucubin when compared to UVB-irradiated cells. Previously, we have reported that aucubin possessed inhibitory activities on UVB-induced MMP-1 production in human skin fibroblasts (15). Recently, it was suggested that excessive matrix degradation by UV-induced MMPs secreted by various cells (e.g., keratinocytes, fibroblasts, and inflammatory cells) contributes substantially to the connective tissue damage that occurs during photoaging (39). Inhibition of MMPs induction has been reported to alleviate UV-induced photoaging by preventing from collagen destruction (40). Several studies reported that the protective effects of asiatic acid, ursolic acid, and melanocin A against UV-induced MMPs production in kertinocytes HaCaT (41,42). Our results showed that the inhibition of UVB-induced MMP-1 expression by aucubin in both fibroblasts and keratinocytes may be of therapeutic value in the prevention of photoaging.

In conclusion, our results suggested that aucubin may provide protection against oxidative stress induced by UVB irradiation in human keratinocytes and these protective effects may be mediated via its antioxidant properties. Further studies are needed to unravel exactly under the molecular mechanisms or proper *in vivo* models for underlie the various pharmacological actions of aucubin.

Acknowledgments

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