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ARTICLE

The Protective Effect of Glycitin on UV-induced Skin Photoaging in Human Primary Dermal Fibroblast

Ga Young Seo · Sanggyu Park · Jung-Sik Huh · Moonjae Cho

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Abstract Exposure of strong and repeated UV on the skin leads to skin aging, characterized with wrinkling, sagging, dyspigmentation, and laxity. Numerous studies revealed that Matrix metalloproteinases are related to skin aging and functions as degrading enzyme of various types of collagen. Here, we attempted to evaluate the effectiveness of glycitin (4'-hydroxy-6-methoxyisoflavone-7-D-glucoside) on skin aging and mechanisms of action in UV-irradiated human dermal fibroblasts. Especially we focused on the expression of Matrix metalloproteinase-1 (MMP-1), which degrades procollagen type-I in dermis, by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, Western blot, and reverse transcription polymerase chain reaction in cell lysates or media. Our results showed that glycitin increased the viability of human dermal fibroblast and alleviated MMP-1 expression caused by UV irradiation. In addition, synthesis of type-I collagen was increased and UV-induced phosphorylation of ERK/JNK/p38 was decreased in dose-dependent manners. Taken together, we demonstrated that treatment with glycitein have a protective effect on skin aging by inhibiting of MMP-1 and increasing of collagen through ERK/JNK/P38 down-regulation, which may be mediated by the inhibition of ERK, JNK, and p38 mitogen-activated protein kinases. We suggest that glycitin is a potential agent for the treatment of skin ageing.

Keywords 4'-hydroxy-6-methoxyisoflavone-7-D-glucoside · collagen type-I · human dermal fibroblast · matrix metalloproteinase-1 · skin ageing · UV

G. Y. Seo
Department of Biochemistry School of Medicine, Jeju National University, Jeju 690-756, Republic of Korea

S. Park
Division of Life & Environmental Science, Daegu University, Daegu 712-714, Republic of Korea

J.-S.Huh (✉)
Department of Urology, School of Medicine, Jeju National University, Jeju 690-756, Republic of Korea
E-mail: urohjs@jejunu.ac.kr

M. Cho (✉)
Department of Biochemistry School of Medicine, Jeju National University, Jeju 690-756, Republic of Korea
Institute of Medical Science, Jeju National University, Jeju 690-756, Republic of Korea
E-mail: moonjcho@jejunu.ac.kr

Introduction

Skin functions as a protective barrier between internal organs and the environment (Kalinin et al., 2002). Skin is divided into three regions: epidermis, dermis, and hypodermis. Dermis is the layer of skin beneath the epidermis that consists primarily of extracellular matrix proteins (Epstein and Munderloh, 1978). UV radiation is one of the causative factor that can affect the structure and functions of human skin (Bae et al., 2010). Chronic exposure to UV irradiation leads to skin aging which is characterized by wrinkling (Lee et al., 2008), sagging, dyspigmentation, and laxity (Kim et al., 2005). Matrix metalloproteinases (MMPs) are a family of structurally related matrix-degrading enzymes that play important roles in photoageing as well as cell proliferation, migration, differentiation (Overall and Lopez-Otin, 2002), angiogenesis, and apoptosis (Werb, 1997). Among the MMP family, collagenolytic MMP (MMP-1) degrades fibrillar collagen and elastin in human skin (Chung et al., 2001).

Activity of MMPs and their regulation mechanisms have been investigated in UV-irradiated human dermal fibroblasts. UV irradiation induces the activation of protein kinase cascades, such as the mitogen-activated protein kinase (MAPK) cascade. A major effecter of the MAPK pathways is the transcription factor AP-1 (Whitmarsh and Davis, 1996). AP-1, which is composed of Jun and Fos family proteins (Janulis et al., 1999), induces MMP synthesis and inhibits collagen synthesis (Yu et al., 2013).

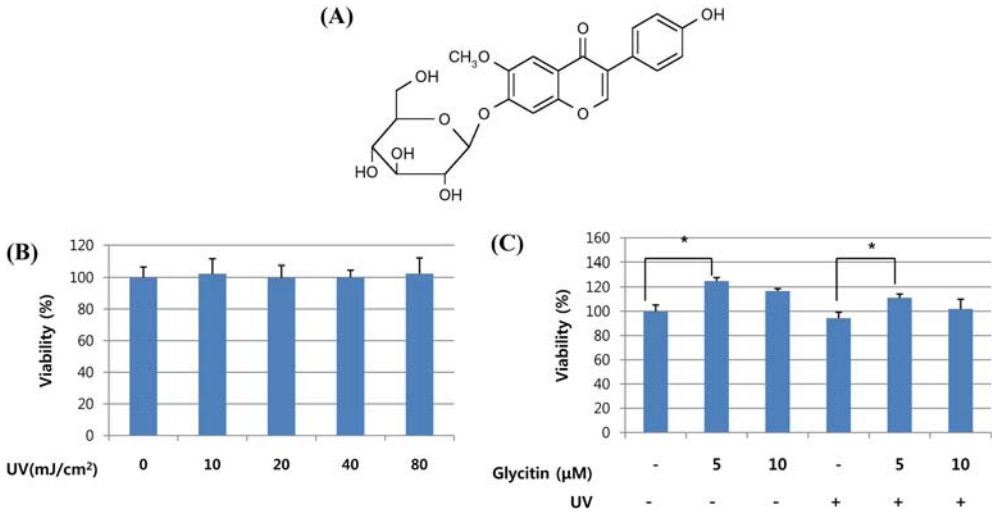

Fig. 1 Glycitin induces proliferation in UV-irradiated fibroblasts. (A) The chemical structure of Glycitin (4'-hydroxy-6-methoxyisoflavone-7-D-glucoside). (B) PHDFs were irradiated with a different intensity (0–80 mJ/cm^2), and proliferation was measured at 48 h by MTT assay as described in Method. (C) UV-irradiated PHDFs were treated with 5 or 10 μM glycitin for 48 h, and proliferation was measured by MTT assay. Data presented as mean of relative to control ± SD from 6 replications, (*) p <0.05.

Environmental stimuli, including UV irradiation, induces the transcription of Jun and Fos and subsequently they are activated by MAPKs; JNK, ERK, and P38 (Hibi et al., 1993).

Glycitin (4'-hydroxy-6-methoxyisoflavone-7-D-glucoside) is an isoform of glycitein, which is an *O*-methylated isoflavone, isolated from soy food products (Fig. 1A). It has been reported to have an anti-oxidant and anti-carcinogenic ability (Chung Hung Lee et al., 2005). However, the effect of glycitin on UV-induced skin ageing has not yet been investigated.

Thus, in the present study, the effects of glycitin isolated from soybean on MMP-1 and collagens in UV-irradiated human primary dermal fibroblasts were investigated. Moreover the molecular mechanism underlying the anti-photoageing effects of glycitin was examined.

Materials and Methods

Cell culture and UV irradiation. Human primary dermal fibroblasts were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FCS and 1% penicillin/streptomycin in a humidified incubator with 5% CO_2 at 37°C. For all experiments, cells were cultured to 60% confluence and the medium was removed. The cells were washed with phosphate-buffered saline (PBS) and added fresh PBS. After washing, cells were irradiated with UV in PBS.

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Cells were seeded on 96-well plates at a density of 3×10^4 cells/mL. Cells were irradiated with UV in PBS and then treated with glycitin or DMSO for 48 h. MTT solutions (10 μL of 5 mg/mL solution; Amresco) was added to each well and incubated at 37°C for 4 h. Subsequently, the medium was gently removed and replaced with 150 μL of DMSO and incubated for 30 min with shaking to dissolve the precipitate. Absorbances of the samples were measured at 570 nm with a spectrophotometer (Lim et al., 2010).

Western blotting. Fibroblast-conditioned medium and cell lysates were collected, and the protein concentrations were determined with BCA protein assay reagents (Thermo Scientific, USA). Equal amounts of protein (~30 μg/lane) were analyzed for each sample by resolving on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Sodium dodecyl sulfate gels were transferred to membranes. After being blocked in 5% non-fat dry milk with Tris-buffered saline Tween 20 (TBST) buffer, membranes were incubated with the indicated antibodies.

Reverse transcription polymerase chain reaction (RT-PCR). Total cellular RNA was isolated using Trizol reagent (Invitrogen, USA). Subsequently, 2 μg of total RNA were used to synthesize cDNA using a Reverse Transcriptase Kit (Promega). The resulting cDNA was used for RT-PCR using the G-Taq kit (Cosmo Genetech, Korea) according to the manufacturer's instructions. The primer sequences were: forward 5'-GTCAGTTCCTTGAGC CG-3' and reverse 5'-GAAGGTAGAGCTTGGGCAGG-3' for *MMP-1*; forward 5'-GAGTCAACGGATTTGGTCGT-3' and reverse 5'-GACAAGCTTCCCGTTCTCAG-3' for β-actin; forward 5'-TGACGAGACCAAGAACTG-3' and reverse 5'-CCATCCAAA CCACTGAAACC-3' for human collagen type I. The results were analyzed using the ImageJ software.

Statistical analysis. Data are expressed as the mean ± SD. Differences with p <0.05 were considered statistically significant.

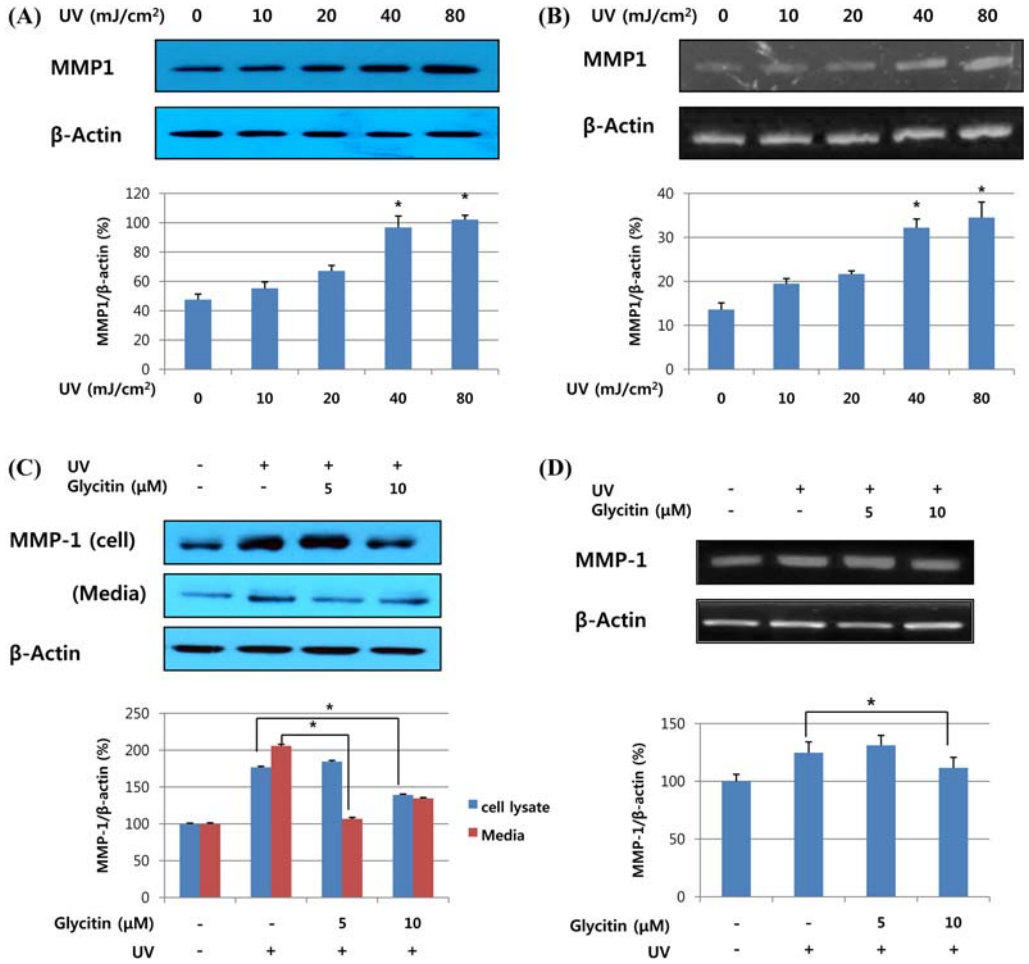

Fig. 2 Glycitin inhibits increased expression and secretion of MMP-1 induced by UV irradiation in fibroblasts. (A) PHDFs were irradiated with a different intensity 0f UV (0–80 mJ/cm^2) for 48 h, and cell lysates were immunoblotted with anti-MMP-1 antibodies. (B) MMP-1 mRNA expression of UV-irradiated PHDF was evaluated by RT-PCR using specific primers. (C) PHDFs were treated with 5 or 10 μM glycitin after UV irradiation. Cell lysates and media were collected after 48 h and measured MMP-1 expression and secretion by Western blot. (D) MMP-1 mRNA expression of PHDF treated with UV and glycitin was evaluated by RT-PCR. Data presented as mean of relative to control ± SD from 3 replications, *p <0.05).

Results

Inhibitory effects of glycitin on viability of UV-irradiated fibroblasts. To examine cell viability in UV-irradiated Primary Human Dermal Fibroblasts (PHDFs), the MTT analysis was performed. PHDFs were cultured with various intensity of UV irradiation (0–80 mJ/cm^2) for 48 h, proliferation of irradiated cells were measured by MTT and proliferation was not affected (Fig. 1B). According to MTT assay result, we selected intensity of UV at 80 mJ/cm^2 as the experiment condition. Furthermore, the effect of glycitin on proliferation was also determined. Treatments of glycitin on irradiated cells with 80 mJ/cm^2 at the concentrations of 5 and 10 μM for 48 h significantly increased cell viability by 10–20% as well as in non-irradiated cells (Fig. 1C). These results suggest that UV irradiation does not have toxicity upto 80 mJ/cm^2, and glycitin increased cell proliferation in human dermal fibroblasts.

Suppression of MMP-1 expression and secretion in UV-irradiated and glycitin treated fibroblast. UV irradiation is known to affect skin especially in dermis, which is the layer of skin beneath the epidermis that consists primarily of extracellular matrix proteins (ECM). UV irradiation degrades ECM caused by increased MMPs expression in UV-treated PHDF. For this reason, the MMP-1 expression was observed by the Western blot. Human dermal fibroblasts were irradiated with UV at 0–80 mJ/cm^2, and then, cell lysates were analyzed by Western blot at 48 h after UV irradiation. The protein level of MMP-1 is increased in a dose-dependent manner at 48 h after UV irradiation (Fig. 2A). The expression of MMP-1 mRNA was measured using RT-RCR and also elevated by about 30% at 80 mJ/cm^2 compared to non-irradiated fibroblasts (Fig. 2B). PHDFs treated with 5 or 10 μM of glycitin for 48 h after UV irradiation at 80 mJ/cm^2. Fibroblast-conditioned media and cell lysate were collected and measured

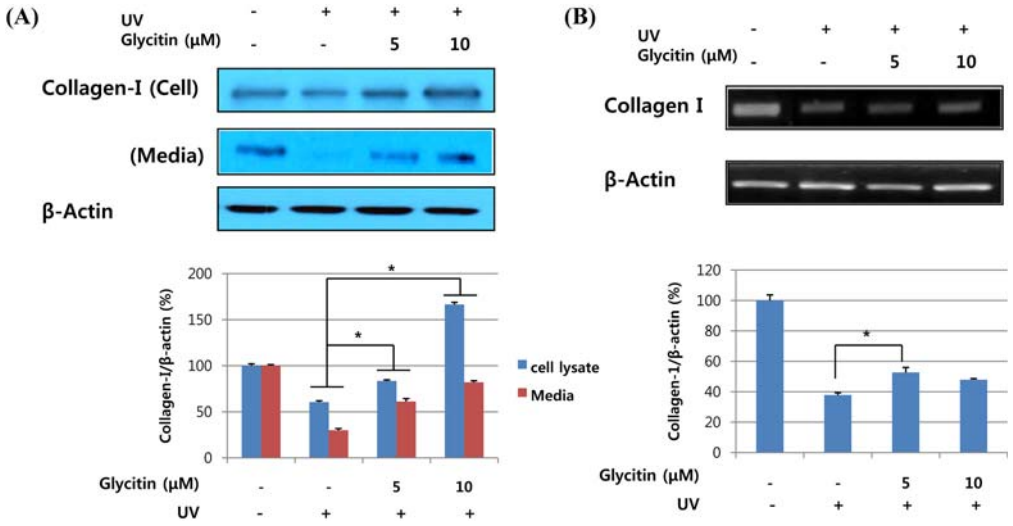

Fig. 3 Glycitin suppresses the degradation of collagen type I in UV-irradiated fibroblasts. (A) PHDFs were treated with 5 or 10 μM glycitin after UV irradiation. Cell lysates and media were collected at 48 h and measured collagen type-I expression and secretion by Western blot. (B) Collagen type-I mRNA expression of PHDF treated with UV and glycitin was evaluated by RT-PCR. Data presented as mean of relative to control ± SD from 3 replications, *p <0.05.

the secretion of MMP-1 using Western blot. The secretion of MMP-1 was suppressed at least 70% in glycitin-treated cell lysates compared to control, but inhibitory effect of glycitin in media was distinguished from cell lysates by its concentration (Fig. 2C and D). And the expression of MMP-1 mRNA was also suppressed out of glycitin at 10 μM and this result took on a similar aspect with cell lysates (Fig. 2D).

These results suggest that glycitin inhibits the expression of MMP-1 protein and mRNA as well as secretion induced by UV-irradiation in human dermal fibroblasts.

Inhibition of collagen degradation in UV-irradiated fibroblasts by glycitin. MMP-1, a matrix-degrading enzyme, is well known to play important roles in photoageing and UV-induced MMP-1 in fibroblasts leading to the degradation of collagen I, III, and other extracellular matrix proteins. In particular, collagen type I is the most abundant protein in skin and activated by collagen type III, which also exists in dermis. However, aged skin features with reduction and impairment of the organization of collagen fibrils and elastin fibers. Therefore, in the present study whether UV irradiation degrades collagen was evaluated and whether glycitin attenuates the collagen degradation of dermal fibroblasts was induced by UV-irradiation. Cells were irradiated with UV (80 mJ/cm^2) and then treated with 5 or 10 μM glycitin. Fibroblast cell lysates and media were collected at 48 h, and Western blot was initiated.The results showed that glycitin increased collagen I expression and secretion by upto 66% in cell lysate and 81% in media (Fig. 3A); the expression of collagen I mRNA was also increased in dose-dependent manner (Fig. 3B).

Inhibitory effect of glycitin on activation of MAPKs signaling in UV-irradiated fibroblasts. To examine the signaling of attenuation of glycitin related to UV irradiation, we focused on the MAPKs pathway that induces MMPs synthesis and inhibits collagen synthesis. Human dermal fibroblasts were irradiated with UV (80 mJ/cm^2), and treated with 5 or 10 μM glycitin. When cells were irradiated, phosphorylation of ERK, JNK, and p38 increased by at least two-fold compared to negative control (Fig. 4). This result suggests that UV irradiation induces MMP expression partly by activation of ERK/JNK/p38 pathway. However, treatment of glycitin on irradiated cells attenuated phosphorylation of ERK, JNK, and p38 in dose-dependent manner. Among them, p38 kinase was shown to be the most affected by glycitin (Fig. 4A and B).

Discussion

The present study demonstrates that glycitin may give inhibitory effects on UV-induced skin ageing through down-regulation of MMP-1. Glycitin is a flavonoid extracted from soy bean and is a glucoside form of glycitein (4',7-dihycroxy-6-methoxyisoflovone). Besides glycitin, soy bean extracts contain two more major isoflavonoids; daidzein (4',7-dihydroxyisoflavone) and genistein (4',5,7-trihydroxyisoflavone) and their respective beta-glycosides, genistin and daidzin (Hsieh et al., 2009). Glycitein, daidzein, and genistein function as ROS scavengers. These functions, which have been widely investigated, showed that they have different estrogenic activity by binding to estrogen receptor protein competitively (Song et al., 1999) and inhibit hydrogen peroxide-induced cell damage by scavenging reactive oxygen species through inhibition of JNK pathway (Kang et al., 2007). However, the effect of glycitin on UV-induced skin ageing has not been investigated.

Treatment of glycitin attenuates UV-induced MMP-1 expression

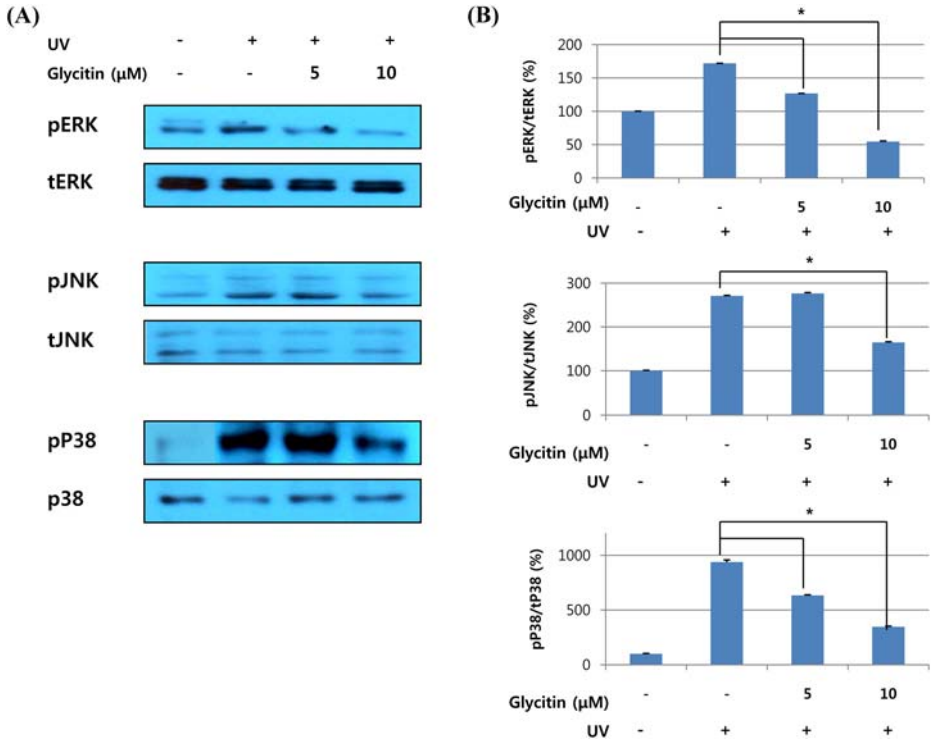

Fig. 4 Glycitin inhibits UV-induced MMP-1 expression by inhibiting MAPKs signaling. (A) UV-irradiated PHDFs were treated with glycitin and cell lysates were collected. Expression and phosphorylation of ERK, JNK, and p38 were measured by Western blot using specific antibodies. (B) Western blot results were quantified by ImageJ. Data presented as mean of relative to control ± SD from 3 replications, *p <0.05.

and secretion in human dermal fibroblasts (Fig. 3). MMP family plays a crucial role in the physiological mechanisms of skin ageing (Kim et al., 2013). In dermis, where the ECM is mainly located, activated MMP-1, which is activated by MMP-3, initiates degradation of type I and III collagens. Several approaches have been used to study new compounds and related pathways that inhibit the MMP expression resulting in ageing. For example, Carnosic acid (0–10 μM) attenuates UV-induced MMP-1, 3, and 9 expressions at 20 mJ/cm^2 in dose-dependent manner in human dermal fibroblasts (Park et al., 2013) and fucosterol also inhibits MMP-1, 2, and 9 expressions in UVB-induced HaCaT cells (Kim et al., 2013). In the present study, we found that glycitin inhibited UV-induced MMP-1 expressions in protein and mRNA level (Fig. 2). We assumed that a reduction of MMP-1 expression and secretion by glycitin are related to MAPKs signaling, leading to activation of AP-1. AP-1, which is composed of c-Jun and c-Fos family proteins, is known as an important factor in synthesis of the MMP-1 and inhibits the collagen synthesis. Among the MAPKs, JNK binds to c-Jun and phosphorylates its N-terminal sites in response to UV irradiation (Minden et al., 1994) or binds to TCF (ternary complex factor) composed of c-fos along with p38 and ERK. Moreover, p38 phosphorylates ATF-2 (activation transcription factor 2) of c-Jun (Muthusamy and Piva, 2010), and further activated and stabilized c-fos and c-Jun form hetero-dimerization of AP-1, resulting in MMP-1 expression (Karin et al., 1997). Our results suggest that glycitin distinctly reduced MMP-1 expression through down-regulation of MAPK, which, in turn, led to deactivation of AP-1 complex at 10 μM of glycitin (Fig. 4).

As a result, reduction of MMP-1 leads to accumulation of collagen (Fig. 3). Collagens confer elasticity and strength to skin. Therefore, their degradation due to UV irradiation results in an aged features to the skin (Kim et al., 2013). The present study shows that UV irradiation augmented collagen degradation most likely via activated MMP-1 (collagenase-1) and disrupted collagen type-1 synthesis. However, glycitin significantly compounded the amount of collagen type-1 in media and also increased synthesis of collagen in a dose-dependent manner.

In conclusion, the present results demonstrate that UV irradiation might be a cause of skin photoageing through the decomposition of cellular matrix proteins such as collagen. However, treatment with glycitin showed a protective effect on skin ageing by inhibiting of MMP-1 and increasing of collagen through AP-1 down-regulation, which may be mediated by the inhibition of ERK, JNK, and p38 MAPKs. Therefore, we conclude that glycitin is a potential agent for the prevention and treatment of skin ageing.

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