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Inhibitory effect of *Astragalus membranaceus* root on matrix metalloproteinase-1 collagenase expression and procollagen destruction in ultraviolet B-irradiated human dermal fibroblasts by suppressing nuclear factor kappa-B activity

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Keywords
Astragalus membranaceus; Hs68 fibroblast; nuclear factor kappa-B P65; procollagen; ultraviolet

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Abstract

Objectives The root of *Astragalus membranaceus*, regarded as a tonic in traditional Korean medicine, has been prescribed for long periods to treat chronic illness by boosting the immune system. Ultraviolet (UV) irradiation causes damage to skin connective tissue by degrading collagen, which is a major structural component of the extracellular matrix. Such damage is considered to be a cause of the wrinkling observed in premature ageing of the skin. This study has investigated the photoprotective effect of *A. membranaceus* on UVB radiation-induced activation of nuclear factor kappa-B (NF-κB) activity in human dermal fibroblasts.

Methods Hs68 fibroblast cells cultured with various concentrations of *A. membranaceus* were exposed to UVB (40 mJ/cm^2). Activation of NF-κB P65 and expression of matrix metalloproteinase-1 (MMP-1) and type 1 procollagen were measured by Western blotting. Translocation of NF-κB P65 and MMP-1 regulation were also examined by immunocytochemistry.

Key findings Western blotting and immunocytochemistry results showed that *A. membranaceus* inhibited UVB-induced translocation of NF-κB P65 and MMP-1 expression. The data suggested that *A. membranaceus* restored type 1 procollagen synthesis by inhibiting NF-κB P65 activity and MMP-1 expression in UVB-exposed human dermal fibroblasts.

Conclusion *A. membranaceus* is a candidate for use in skin protection from UVB-induced skin inflammation and photoageing.

Introduction

Skin ageing occurs from not only internal ageing but also photoageing caused by repeated exposure to ultraviolet (UV) light.[1] UVB irradiation (290–320 nm) is a main factor in biological reactions in the skin, inducing inflammatory responses and apoptosis, and subsequently skin damage.[2] Intense exposure to UVB results in inflammatory changes, production of inflammatory mediators, alteration of vascular responses, and infiltration of inflammatory cells.[3]

The functional properties of skin rely on the integrity of collagens, which are the most plentiful structural proteins in the dermis.[4] Type 1 collagen, which constitutes 70–90% of all collagen, is synthesized by fibroblasts as a soluble precursor type 1 procollagen, and secreted and proteolytically modified to form insoluble collagen fibres in the dermis.[5,6] The production and fibrillar organization of collagen in the skin are decreased by UV irradiation, whereas protein expression of matrix metalloproteinases (MMPs) increases.[7] Among MMPs, MMP-1 is the one most responsible for the degradation of type 1 collagen in the skin.[8–10] Collagen degradation weakens the structural integrity of fibres during photoageing. Therefore, control of collagen metabolism is important in a variety of therapeutic and cosmetic applications.

Nuclear factor kappa-B (NF-κB), a critical factor in the immuno-inflammatory response, is implicated in several skin diseases including allergic dermatitis, psoriasis vulgaris, and skin cancer.[11–13] Although NF-κB is involved in maintaining skin homeostasis, excessive expression is thought to be pathogenic.[14] NF-κB is an inducible dimeric P65 and p50 transcription factor that belongs to the Rel/NF-κB family of transcription factors. NF-κB is initially localized in the cytoplasm and in its inactive form is united with IκB, which is a repressive factor of NF-κB. Various stimulants, such as

interleukin-1 (IL-1), tumour necrosis factor α (TNF-α), and UV, can give rise to the dissociation of this complex by phosphorylation of IκB, which leads to NF-κB release from the complex. NF-κB then translocates to the nucleus, where it combines with specific DNA recognition sites to induce gene transcription.[15,16]

Astragalus membranaceus Bunge (family Fabaceae) is known as a tonic in traditional Korean medicine. Root of *A. membranaceus* is cultivated in the north-eastern province of Heilongjiang and the south-western province of Sichuan of China.[17] *A. membranaceus* has been prescribed over long periods to treat general weakness and chronic illness by boosting overall vitality. Also the efficacy of *A. membranaceus* in immunopotentiation has been demonstrated.[18] The main constituents of *A. membranaceus* root includes polysaccharides, saponins, flavonoids, and amino acids.[16] These components are useful for treating inflammation related immune deficiencies.[19] However, little evidence exists to show that *A. membranaceus* treatment improves defects in inflammation and decreased collagen levels in fibroblasts. Also, *A. membranaceus* is a medicinal herb that is broadly used for the treatment of inflammatory diseases, tumours, and various cardiovascular diseases, and it exhibits neuroprotective and radical scavenging activity.[20–25] However, no study has investigated the anti-skin-ageing effects of *A. membranaceus* treatment mediated by its effects on human dermal fibroblasts, especially in terms of molecular mechanisms by which *A. membranaceus* protects skin damage. Here, we have examined the anti-skin-ageing effect of *A. membranaceus* on the UVB-induced NF-κB pathway in human dermal fibroblasts.

Materials and Methods

Materials

Fetal bovine serum (FBS), Dulbecco's Modified Eagle Medium (DMEM) and trypsin-EDTA were purchased from Gibco (Grand Island, NY, USA). Polyclonal goat anti-human NF-κB P65, monoclonal mouse anti-human pro-collagen type 1 and monoclonal mouse anti-human MMP-1 were purchased from Santa Cruz and Calbiochem company (Santa Cruz, CA, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazoliumbromide (MTT), and dimethyl sulfoxide (DMSO) were purchased from Sigma (St Louis, MO, USA). Rotary evaporatory (Eyela, Tokyo, Japan), freeze dryer (Eyela, Tokyo, Japan), deep freezer Themo Scientific (Asheville, NC, USA), microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA), UVB irradiator (UVP Inc, Upland, CA, USA) and CO_2 incubator (Sanyo, Tokyo, Japan) were used. Individual astragaloside standards (I, II, III, and IV) were purchased from Chromadex Inc. (Irvine, CA, USA). The solvents used for extraction and quantification of astragalosides were of HPLC grade from Fisher Scientific Company (Pittsburgh, PA, USA)

Preparation of *A. membranaceus* extract

Root of *A. membranaceus* Bunge was purchased from Wonkwang Herbal Drug Co. Ltd (Seoul, Korea). Dried *A. membranaceus* root (100 g) was boiled in 2 l water for 2 h. The suspension was then filtered and concentrated under reduced pressure. The filtrate was lyophilized and yielded 29.8 g (29.8%) of powder. Dried extract was dissolved in phosphate buffered saline (PBS), sonicated for 2 min at room temperature, and filtered before being used in experiments.

Standard preparation and extraction procedure for *Astragalus* saponins

Astragaloside standards were individually dissolved in a 25 ml mixture of acetonitrile and H_2O (50 : 50) and analysed by HPLC. Crude *A. membranaceus* (5.0 g) was finely grounded and freeze dried (4.2 g). Then, *Astragalus* was prepared using the same extraction solvent mixture for further HPLC analysis.

High performance liquid chromatography analysis of astragalosides

A high performance liquid chromatography (HPLC) system comprising a vacuum degasser, two pumps, autosampler, column temperature control compartment and diode array detector (Shimadzu, MD, USA) was used for the quantification of astragalosides. The column, Ultra II C18 5 μm, 150 × 4.6 mm (Restek, Bellefonte, PA, USA), was maintained at 40°C. Low pressure gradient elutions of mobile phase A (acetonitrile) and mobile phase B (H_2O) was used (35 (A) : 65 (B) for 0–1.5 min, 65 : 35 for 1.5–18 min, 35 : 65 for 20–30 min). The flow rate was 0.5 ml/min, sample injection volume was 40 μl and chromatogram was monitored at 200 nm. Quantification was made on the comparison basis of the peak area from astragaloside standards.

Cell culture and UVB irradiation

Human dermal fibroblasts were cultured in DMEM containing 10% FBS, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C humidified atmosphere of 5% CO_2 in air. Fibroblasts were plated at 90–95% confluence in all experiments. The nontoxic concentration of *A. membranaceus* used in the 24-h culture experiments was 100 μg/ml, as determined by the MTT assay. Dermal fibroblasts at a density of 2.0×10^6 cells on a 100-mm dish were treated with *A. membranaceus* (μg/ml) and exposed to 40 mJ/cm^2 UVB in serum-free DMEM (312 nm UVB light source, UVP). Subsequently cells were incubated for 24 h in DMEM containing equivalent doses of *A. membranaceus* extract.

Cell viability

At the end of the incubation time the MTT assay was performed to quantitate cellular viability.[26] Human dermal fibroblasts were incubated in fresh medium containing MTT (1 mg/ml) for 4 h at 37°C. After removal of unconverted MTT, the purple formazan product was dissolved in 0.5 ml isopropanol through gentle shaking. Absorbance of formazan dye was measured colorimetrically at k = 570 nm.

$$\text{Cell viability (\%)} = 100 \times A_T / A_C$$

Where A_C is the absorbance of control, and A_T is the absorbance of tested extract solution.

Immunocytochemistry

After the human dermal fibroblasts were grown on the 24-well dish with cover slides, the cover slides were thoroughly washed with PBS, the cells were fixed with 90% EtOH for 30 min and washed for 5 min, three times with PBS containing 0.1% gelatin and 0.1% saponin. For blocking any nonspecific binding, cells were incubated for 1 h with 10% normal donkey serum. For the in-situ detection of NF-κB P65 and MMP-1 expression, fixed cells were washed with PBS, and polyclonal goat anti-human NF-κB P65 (1 : 100) and MMP-1 (1 : 100) were sufficiently added to cells, respectively, and incubated overnight at 4°C. After washing three times for 5 min, cells were incubated with Fluorescein (FITC) conjugated anti-rabbit IgG (1 : 50) as a secondary antibody and washed with PBS three times for 5 min. All samples were counterstained with propidium iodide (PI). Fluorescent images were obtained by a fluorescence microscopy with an Olympus BX51 fluorescent microscope with differential interference contrast and reflected light fluorescence with 400 × magnification.

Western blot assay

Western blot analysis was performed using whole cell lysates prepared from human dermal fibroblasts at a density of 2.0×10^6 cells. Whole fibroblast lysates were prepared in a lysis buffer containing 1% β-mercaproethanol, 1 M β-glycerophosphate, 0.1 M Na_3VO_4, 0.5 M NaF and protease inhibitor cocktail. Cell lysates containing equal amounts of total proteins or equal volumes of culture supernatants were electrophoresed on 10% SDS-PAGE gels and transferred onto a polyvinylidene fluoride membrane. Nuclear extracts were collected according to the instructions of the nuclear extract kit (Sigma, USA). Nonspecific binding was blocked by soaking the membrane in a TBS-T buffer (50 mM Tris–HCl (pH 7.5), 150 mM NaCl, and 0.1% Tween 20) containing 5% skim milk for 1 h. The membrane was incubated with monoclonal mouse anti-human MMP-1 (1 : 1000), polyclonal rabbit anti-human NF-κB P65 (1 : 1000), and polyclonal goat anti-human type 1 procollagen (1 : 1000). The membrane was then incubated with a secondary antibody, a goat anti-rabbit IgG (1 : 2000), goat anti-mouse IgG (1 : 2000) conjugated to horseradish peroxidase. The protein levels were analysed by using Supersignal West Pico Chemiluminescence detection reagents (Pierce Biotech. Inc., Rockford, IL, USA) and Konica X-ray film (Konica Co., Tokyo, Japan). Incubation with polyclonal mouse anti-human β-actin antibody (1 : 1000) was performed for comparative control.

Data analysis

The results are presented as means ± SD for each treatment group. Statistical analyses were conducted using Statistical Analysis Systems (SAS Institute Inc., Cary, NC, USA). Significance was determined by Kruskal-Wallis test followed by Dunn's test for multiple comparisons and were considered significant at $P < 0.05$.

Results

Detection of astragaloside using high performance liquid chromatography analysis

To identify and confirm the compounds of *A. membranaceus*, we analysed the extract of *A. membranaceus* with astragaloside standards I, II, III and IV, which are representative compounds for *A. membranaceus*, using a HPLC method (Figure 1a). The *A. membranaceus* extract contained 0.36% astragaloside II (Figure 1b). The other astragaloside standards were not detected.

Cell viability

To examine the effect of *A. membranaceus* on cell viability, 10, 100, and 300 μg/ml *A. membranaceus* extract was used to treat cultured cells for 24 h; cell viability was then determined by MTT analysis. *A. membranaceus* treatment (300 μg/ml) caused a significant increase in cell viability by 12.9% compared with the untreated group. When *A. membranaceus* treatment of dermal fibroblasts was performed before UVB exposure (40 mJ/cm^2), viability was substantially enhanced to 142.8% at 100 μg/ml compared with the UVB-exposed control group. Thus, *A. membranaceus* exhibited a protective effect against UVB-induced dermal damage (see Figure 2).

Inhibition of UVB induced NF-κB P65 translocation by the *A. membranaceus* extract

To examine the effect of *A. membranaceus* on UVB-induced NF-κB P65 activation, cytoplasmic and nuclear extracts were isolated from human fibroblast cells irradiated with UVB (40 mJ/cm^2) in the presence of 100 μg/ml *A. membranaceus*. Western blot analysis showed that *A. membranaceus*

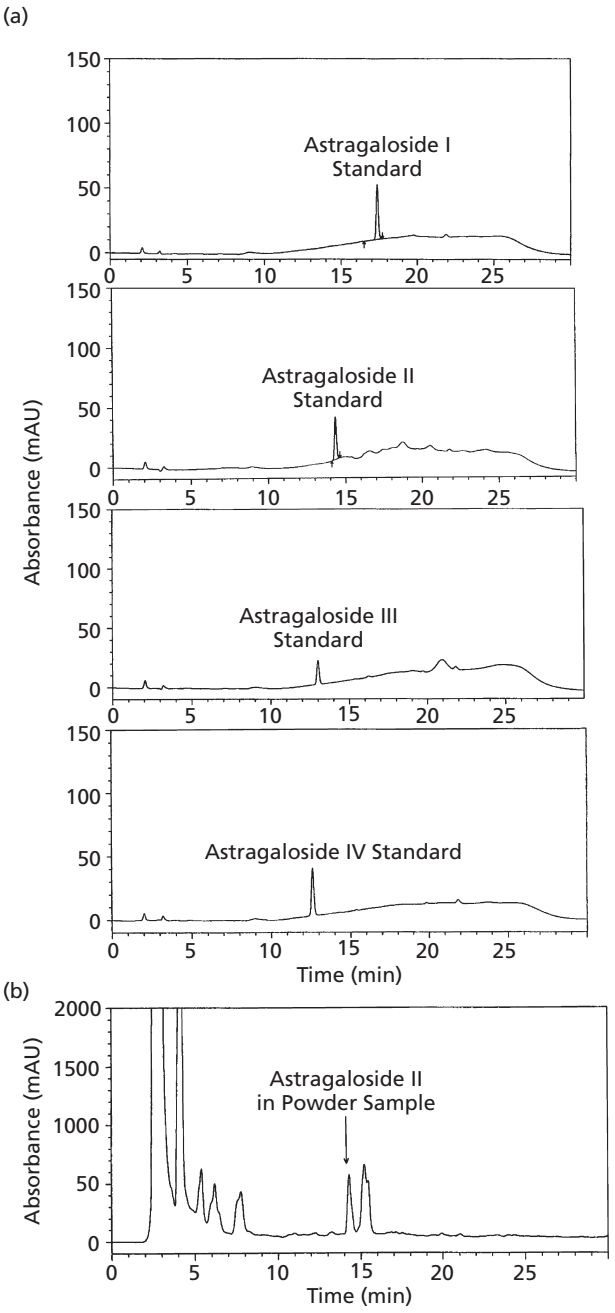

Figure 1 Detection of astragaloside from *Astragalus membranaceus* extract using HPLC analysis. (a) Astragaloside standards I, II, III and IV were detected at corresponding retention times. (b) HPLC analysis showed that *A. membranaceus* extract contained astragaloside II.

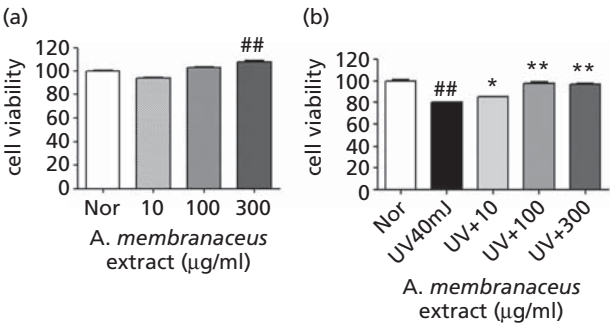

Figure 2 Proliferative effect of the water extract of *Astragalus membranaceus* on Hs68 fibroblast cells. Hs68 fibroblast cells (2×10^6 cells/ml) were incubated with various concentrations of *A. membranaceus* water extract (AM) for 24 h with or without UVB (40 mJ/cm^2) treatment. An MTT assay was performed. Data are expressed as a percentage of the control and represent the mean of three independent determinations. $^*P < 0.05$, $^{**}P < 0.01$, $^{\#\#}P < 0.01$ compared with control.

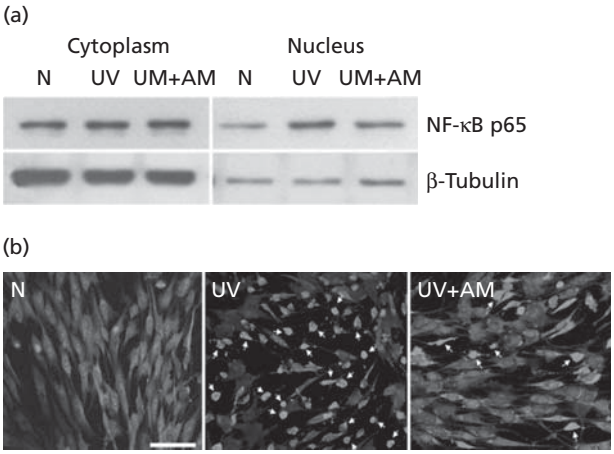

Figure 3 Effect of *Astragalus membranaceus* on UVB-induced activation of nuclear factor kappa-B P65 in Hs68 fibroblast cells. (a) Cultured cells were exposed to UVB (40 mJ/cm^2) with or without *A. membranaceus* (AM) pretreatment (100 μg/ml) for 24 h. Cells were harvested at 5-h time points after UVB exposure and cell lysates were prepared to determine the level of nuclear factor kappa-B (NF-κB) activation using a Western blot assay. (b) Immunocytochemical analysis showing inhibition of translocation of NF-κB P65 to nucleus in *A. membranaceus*-treated (100 μg/ml) and 40 mJ/cm^2 UVB-exposed human dermal fibroblasts. NF-κB P65 localization from the cytoplasm to nucleus is visualized in the UVB control. The white arrow indicates translocation of NF-κB P65 to the nucleus. Cultured cells were incubated with anti-P65 antibody overnight at 4°C. Representative fluorescent images taken at 400 × magnification are shown. Scale bar: 75 μm.

treatment resulted in inhibition of NF-κB P65 translocation to the nucleus (see Figure 3a). NF-κB P65 levels determined by an immunocytochemical analysis, showed that translocation of NF-κB P65 was significantly decreased in human fibroblast cells by *A. membranaceus* treatment compared with that of nontreated UVB-exposed cells (see Figure 3b).

Regulation of MMP-1 expression by *A. membranaceus* extract

To investigate whether the inhibition of NF-κB P65 translocation by *A. membranaceus* treatment affected MMP-1

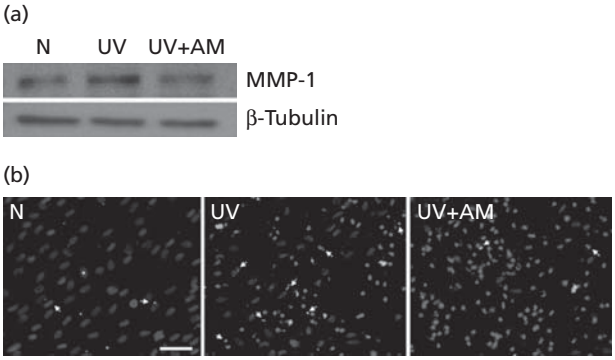

Figure 4 Effect of *Astragalus membranaceus* on UVB-induced activation of matrix metalloproteinase-1 in Hs68 fibroblast cells. (a) Cultured cells were exposed to UVB (40 mJ/cm^2) with or without *A. membranaceus* (AM) pretreatment (100 μg/ml) for 24 h. Cells were harvested at 5-h time points after UVB exposure and cell lysates were prepared to determine the level of matrix metalloproteinase-1 (MMP-1) activation using a Western blot assay. (b) Immunocytochemical analysis showing inhibition of MMP-1 by *A. membranaceus* treatment (100 μg/ml) in UVB (40 mJ/cm^2)-exposed human dermal fibroblasts. MMP-1 was visualized in UVB-exposed control cells. Cultured cells were incubated with MMP-1 antibody overnight at 4°C. Representative fluorescent images taken at 400 × magnification are shown. Scale bar: 75 μm.

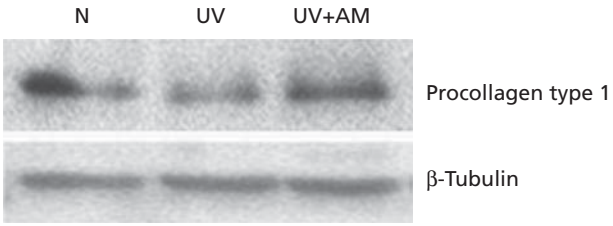

Figure 5 Inhibitory effect of *Astragalus membranaceus* on UVB-induced activation of type 1 procollagen in fibroblast cells. Cultured cells were exposed to UVB (40 mJ/cm^2) with or without *A. membranaceus* (AM) pretreatment (100 μg/ml) for 24 h. Cells were harvested at 5-h time points after UVB exposure and cell lysates were prepared. Total protein levels of type 1 procollagen were detected using a specific antibody and Western blot assay. N, normal cells.

expression, MMP-1 protein levels were measured in human fibroblast cells irradiated with UVB in the presence of *A. membranaceus* extract (100 μg/ml). Western blot analysis showed that treatment of cells with *A. membranaceus* resulted in inhibition of the elevated MMP-1 gene expression in UVB-stimulated fibroblast cells (see Figure 4a). In addition, an immunocytochemical analysis showed that MMP-1 levels significantly decreased in human fibroblast cells treated with *A. membranaceus* compared with those of UVB-irradiated cells (see Figure 4b).

Effect of *A. membranaceus* on type 1 procollagen expression

To confirm the inhibitory effect of *A. membranaceus* extract on the synthesis of MMP-1, we investigated type 1 procollagen levels in normal and UVB-irradiated cells in the presence or absence of *A. membranaceus*. Type 1 procollagen levels were increased in UVB-irradiated human fibroblast cells treated with 100 μg/ml *A. membranaceus* (see Figure 5).

Discussion

Photoageing is considered to begin through the production of reactive oxygen species, activation of activator protein 1, NF-κB, and consequent induction of MMPs and inflammation.[27] Transcription factor NF-κB induces gelatinase expression and stimulates transcription of pro-inflammatory cytokine genes, for example TNF-α, IL-1β, IL-6, and IL-8, and gathers neutrophils, which guide preformed neutrophil collagenase into UVB-irradiated fibroblast skin.[1]

UV irradiation is reported to bring about NF-κB translocation from the cytoplasm to the nucleus and so induce the production of MMPs. Matrix degradation by UV-induced MMPs accounts for connective tissue damage during photoageing.[28,29] Among the MMPs, MMP-1 (fibroblast collagenase) is primarily responsible for collagen fragmentation in the skin. Type 1 procollagen consists of two polypeptide chains, proα 1 (I) and proα 2 (I), at a ratio of two to one, respectively.[30] Diverse biological factors can either irritate or suppress collagen biosynthesis at various levels of gene expression. Tumour growth factor-β (TGF-β) is also another regulator of type 1 procollagen production.[31,32] Thus, development of NF-κB inhibitors is regarded as a promising method of enhancing the synthesis of type 1 procollagen for photoageing and skin inflammation therapies.

We investigated the NF-κB pathway involved in matrix degradation and found that UVB-induced MMP-1 protein expression was repressed by *A. membranaceus* treatment. *A. membranaceus* extract increased cell viability in both normal and irradiated cells, implying that *A. membranaceus* may be effective in cell proliferation as well as photoageing. *A. membranaceus* extract pretreatment (100 μg/ml) increased cell viability by up to 142.8% over the UVB-irradiated control group. Moreover, *A. membranaceus* was shown to decrease NF-κB P65 translocation and MMP-1 expression in human dermal fibroblast cells. Although the precise mechanism underlying cutaneous ageing remains unknown, internal biological cutaneous ageing results in a lack of collagen and an increase in MMP-1 expression induced by NF-κB.[28] The ability of *A. membranaceus* to control UVB-induced collagen degradation was implicated in fluctuations of collagenolytic MMP-1 production through the regulation of NF-κB activity.

Many plant extracts have been shown to trigger collagen production and inhibit MMPs during photoageing, such as

the ascorbic acid in amla extract.[33,34] The main components of *A. membranaceus* roots are polysaccharides, saponins, flavonoids, amino acids, and trace elements.[35] In our study, astragaloside II was detected as a traceable compound of *A. membranaceus*. Recent reports have shown that astragaolsides induced osteogenic activity of osteoblasts, prevented MPP-induced SH-SY5Y cell death, exhibited healing and antiscar effects, and protected against focal cerebral ischaemia/reperfusion injury.[36–39] Though we could not exclude the involvement of other compounds of *A. membranaceus* in its effects on MMP expression, astragaloside is expected to decrease UV-induced NF-κB production and retard photoageing by downregulating MMP-1 to enhance collagen expression.

Conclusions

We have demonstrated that *A. membranaceus* increased procollagen synthesis through inhibition of NF-κB activation and subsequent MMP-1 expression in UVB-irradiated fibroblasts (see Figure 6). These data suggest that *A. membranaceus* could be of pharmaceutical value for skin protection from UVB-induced irradiation. Further research on the mechanisms underlying the beneficial effects of *A. membranaceus* on skin ageing, for example signalling pathways and clinical methods, is necessary to verify the benefit of *A. membranaceus* in protecting human skin.

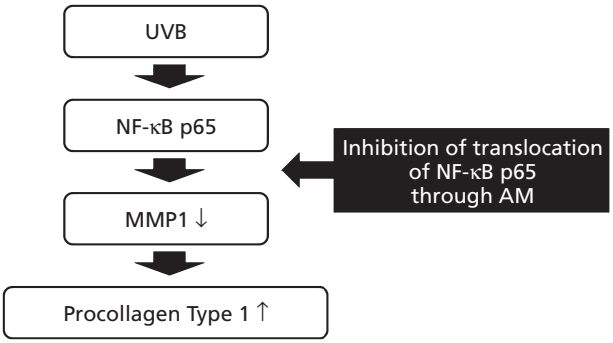

Figure 6 Schematic diagram showing the inhibitory effects of *Astragalus membranaceus* in UVB irradiation-induced dermis photoageing. *A. membranaceus* (AM) blocked UVB irradiation-induced matrix metalloproteinase-1 (MMP-1) production and procollagen type 1 degradation by interfering with nuclear factor kappa-B (NF-κB) activation.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

Funding

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