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Original Article

Asparagus racemosus root extract induced procollagen I expression and inhibited matrix metalloproteinase-1 (MMP-1) in UVB treated human dermal fibroblasts

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Abstract

Asparagus racemosus Willd., known as Shatavari (Rak-Sam-Sib in the Thai language), has been used in traditional medicine and as a food supplement for females with hormone related health conditions due to its estrogenic effects. In cosmetic use, a clinical study in adult volunteers showed an efficacy in delayed wrinkle formation with topical application. In this study, we focused on the effect of *A. racemosus* root extract on the expression of type I procollagen and matrix metalloproteinase-1 (MMP-1) in ultraviolet B (UVB) treated human dermal fibroblasts. The saponin enriched extract of *A. racemosus* root was shown to promote type I procollagen synthesis in non-UVB-exposed cells and prevent a decrease in type I procollagen secretion in UVB exposed cells. Moreover, the enhanced MMP-1 expression upon UVB irradiation was suppressed by *A. racemosus* root extract. These results suggested *A. racemosus* root extract could be a potential agent as an anti-photoaging ingredient in cosmeceuticals.

Keywords: *Asparagus racemosus*, photoaging, procollagen, MMP-1, UVB

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1. Introduction

Collagen is the major protein component in the extracellular matrix of skin and connective tissue that provides structural support and plays mechanical functions. The synthesis of type I procollagen, a precursor of the fundamental unit of different proteins contained in the dermis, begins within dermal fibroblasts. Newly-synthesized type I procollagen is further processed to mature collagen I which is a larger protein of more complex structure and organization that provides tensile strength and elasticity to the skin dermis. A decrease in type I procollagen synthesis becomes evident due to both chronological and photo-induced aging of the skin (Canty & Kadler, 2005; Farage, Miller, Elsner, & Maibach, 2008).

Exposure to ultraviolet (UV) radiation accounts for the premature aging of skin or photo-induced aging, which is characterized by the presence of wrinkles, roughness and dyspigmentation. Ultraviolet A (UVA) (320-400 nm), which accounts for 90–99% of irradiance to reach the earth's surface, is known to be a major contributor in the mechanism of photo-induced aging. Ultraviolet B (UVB) (290–320 nm), although absorbed by the ozone layer, can also reach the earth's surface and is responsible for sunburn and skin erythema. The UV of both wavelength bands once absorbed into cells can result in the formation of reactive oxygen species (ROS) within the cells of the epidermis and dermis (Rittié & Fisher, 2002; Trautinger, 2001). Following UVB irradiation, keratinocytes are activated to secrete proinflammatory cytokines such as IL-1, IL-6, and TNF-α which signal fibroblasts to up-regulate the level of mRNA of MMP-1, 2, and 3 (Kammeyer & Luiten, 20 15; Quan *et al.*, 2009).

Matrix metalloproteinases (MMPs) are matrix-degrading enzymes involved in the pathogenesis of photo-induced aging of the skin. MMP-1, so called collagenase-1, is responsible for degradation of fibrillar collagen such as type I and III collagen. Following UV irradiation, the upregulation of MMP-1 is observed within 6 h whereas collagen breakdown occurs within 24 h. The activation of MMP-1 also inhibits the expression of type I procollagen mRNA causing a decrease in type I procollagen synthesis (Peter, Helmut, & Karin, 2002; Quan *et al.*, 2009)

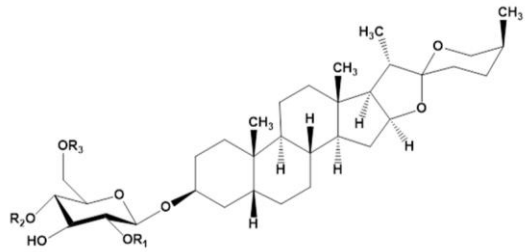
Asparagus racemosus, known in the Thai language as Rak-Sam-Sib, has been reported with many uses in ayurvedic medicine. The tuberous root of the plant has been used to treat dysentery, dyspepsia, fever, infectious diseases, and rheumatism. Moreover, its phytoestrogenic properties were evident as a tonic for females with sexual and gynecological disorders and as a galactagogue during lactation (Alok *et al.*, 2013; Bopana & Saxena, 2007). In addition, *A. racemosus* root extract was reported to have various pharmacological activities, such as anti-oxidant, anti-inflammatory, immunomodulation, anti-lipid peroxidation, and anti-fungal properties (Onlom, Khanthawong, Waranuch, & Ingkaninan, 2014; Onlom, Phrompittayarat, Putalun, Waranuch, & Ingkaninan, 20 17). The principal chemical constituents of *A. racemosus* are shatavarins which is a group of steroidal saponins present primarily in the roots of plants. Shatavarin IV is the major saponin among the other shatavarins (I, V, IX, and X). Other constituents are asparagamine, quercetin, racemosol, and sitosterol (Hayes *et al.*, 2008). The anti-aging properties of *A. racemosus* were studied previously in human fibroblasts undergoing UVA irradiation. The expression of MMP-1 was suppressed with pretreatment with *A. racemosus* root extract (Rungsang, Waranuch, Ingkaninan, & Viyoch, 2012). The clinical use of *A. racemosus* root extract was demonstrated to delay progression of wrinkles among human volunteers (Rungsang, Tuntijarukorn, Ingkaninan, & Viyoch, 2015). However, the effects of the plant extract have not been elucidated with fibroblast cells undergoing UVB irradiation.

In this study, we aimed to investigate the cellular response of human dermal fibroblasts under exposure to UVB irradiation with the focus on the expressions of the major extracellular matrix proteins which are type I procollagen and MMP-1. We also addressed the impact of *A. racemosus* root extract on the same parameters when incubated with fibroblasts prior to UVB exposure.

2. Materials and Methods

2.1 Reagents and plant material

Shatavarin IV was purchased from ChromaDex Inc., CA, USA. Human serum albumin was a product of Sigma-Aldrich Chemie GmbH and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) was purchased from Wako, Osaka, Japan. Monoclonal antibody to shatavarin IV was produced as described by Onlom *et al.* (2017). Peroxidase conjugated goat IgG fraction to mouse IgG Fc was purchased from MP Biomedicals, Illkirch-Graffenstaden, France. Acetonitrile and methanol were analytical grade and purchased from Merck KG. Fresh roots of *A. racemosus* were collected from Rayong Province, Thailand. The collected roots were sliced and dried in an air oven at 50 °C and then ground into powder. The extraction method for the enriched saponin content was as follows. One hundred grams of dried root powder were macerated with 500 mL of 90% acetonitrile in water under constant stirring. The resultant solution was filtered and evaporated using vacuum at 45 °C. Finally, the obtained extract was stored in airtight amber containers at –20 °C until use.



COMPOUNDS	R1	R2	R3
Shatavarin IX	β-D-glucose	β-D-glucose	H
Shatavarin V	α-L-rhamnose	β-D-glucose	H
Shatavarin IV	β-D-glucose	α-L-rhamnose	H

Figure 1. Chemical structures of shatavarins found in the root of *Asparagus racemosus* (Onlom *et al.*, 2017)..

2.2 Quantitation of saponin content by ELISA

Enzyme-linked immunosorbent assay (ELISA) was used for the quantitation of shatavarin IV as described by Onlom *et al.* (2017). Since the monoclonal antibody to shatavarin IV is specific to sarsasapogenin, which the shared aglycone structure found in other types of shatavarins, all other

shatavarins found in *A. racemosus* root extract were collectively quantified. A calibration curve was plotted against logarithmic concentration of shatavarin IV (78–2500 ng/mL) from which shatavarin IV-equivalent could be calculated.

2.3 Cell cultures, treatment, and UVB irradiation

Human dermal fibroblasts were extracted from the foreskins of anonymous donors aged 6 months to 3 years. The protocol was approved by Naresuan University Institutional Review Board (COA No.577/2017 IRB No. 0547/60). The tissues were soaked in 5% dispase at 4 °C overnight and the epidermis was then removed. The dermis tissues were incised, placed into a 6-well plate (Maxisorb Nunc, Roskilde, Denmark) and maintained with Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (Gibco-BRL, Gaithersburg, ME, USA), 1% glutamine and 1% penicillin/streptomycin in 37 °C humidified atmosphere of 5% CO_2 in air. After dermal fibroblasts presented radial outgrowth of single cells from the attached tissue, the fibroblasts were trypsinized for further experiment. *A. racemosus* root extract was dissolved with culture media at pre-specified concentrations (10 and 25 µg/mL) and used to pre-treat cells for 24 h before UVB irradiation. After pretreatment, the cells were washed twice with phosphate buffer solution (PBS). The cells were exposed to UVB at the dose of 25 and 50 mJ/cm^2 under PBS with a 313 nm UVB light source (BS-03; Dr Grobe GmbH, Ettlingen, Germany). Then, serum-free DMEM was replaced and the cells were incubated a further 24 h before total RNA extraction and viability assay.

2.4 Cell viability assay

Cell viability was determined by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazoliumbromide (MTT) assay. Fibroblast cells (1x10^4 cells/well) were seeded into a 96-well plate. Cell viability was evaluated in each well by the addition of 50 µL of MTT (1 mg/mL). After 4 h of incubation, the supernatants were removed completely from each well, and 100 µL of dimethyl sulfoxide (DMSO) was added. Absorbance was measured at 595 nm using a spectrophotometric multiwell microplate reader (Multiskan MS; Thermo Electron Corp., Waltham, MA, USA).

2.5 Measurement of procollagen I and matrix metalloproteinase-1 (MMP-1) protein by ELISA protein

After exposure to UVB irradiation, the cells were washed twice with PBS before incubation with serum-free DMEM for 24 h. Then, the cell culture supernatants were collected and quantified for the amount of procollagen I protein by ELISA Human Pro-Collagen I alpha 1 Matched Antibody Pair Kit; ab216064 (Abcam, San Francisco, CA, USA) according to the manufacture's instructions. A calibration curve was plotted against the concentration of a standard type-1 procollagen (6.25–200 pg/mL).

A human MMP-1 ELISA kit (Raybiotech, Norcross, GA, USA) was used to determine MMP-1 protein in the culture supernatants. A calibration curve was plotted against the logarithmic concentration of the MMP-1 protein (75–18000 pg/mL). All reactions were performed in triplicate.

2.6 Reverse transcriptase polymerase chain reaction (RT-PCR)

After exposure to UVB, the fibroblasts were trypsinized and collected. Total RNA obtained from 7.5x10^5 cells was extracted using Total RNA Extraction kit (RBC Bioscience, Taipei, Taiwan). The concentration and purity of R NA were determined by measuring the absorbance at both 260 and 280 nm with NanoDrop ND-1000 UV-vis spectrophotometer (NanoDrop Technologies, Waltham, MA, USA). The cDNA were then synthesized using MyTaq^{TM} One-step RT-PCR kit (Bioline, Taunton, MA, USA) by reverse transcription of 30 ng total RNA at 48 °C for 20 min. The sets of specific primers (Macrogen Inc, Seoul, Korea) used in the reactions are shown in Table 1 as described in the reference study protocol (Han *et al.*, 2010; Huh *et al.*, 2015; Moon *et al.*, 2008). The housekeeping gene β-actin was used for internal normalization. The program included an initial denaturation at 94 °C for 5 min, followed by 30 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s. Final extension was at 72 °C for 10 s. PCR products were then mixed with Novel Juice (GeneDireX, Taiwan) containing tracking dyes bromophenol blue, xylene cyanol FF, and orange G, separated by electrophoresis through a 1.5% agarose in Tris-boric acid-EDTA buffer. The bands were visualized upon UV illumination under ChemiDoc XRS (Biorad Inc, Philadelphia, PA, USA)

Table 1. Primer sequences used in the study.

Primers	Sequences	Size (bp)	Tm (°C)
β-actin	Forward: 5'- GGA CCT GAC AGA CTA CCT CA-3' Reverse: 5'- GTT GCC AAT AGT GAT GAC CT-3'	229	56.5
Procollagen-1 (PROCOL-I)	Forward: 5'- CTC GAG GTG GAC ACC ACC CT-3' Reverse: 5'- CAG CTG GAT GGC CAC ATC GG-3'	366	61
Collagenase-1 (MMP-1)	Forward: 5'-ATT CTA CTG ATA TCG GGG CTT TGA-3' Reverse: 5' -ATG TCC TTG GGG TAT CCG TGT AG-3'	409	59

2.7 Statistics

Data from the statistical analysis are presented as mean±SD for three independent experiments. Comparison between the groups were analyzed by one-way ANOVA followed by Fisher's LSD test for multiple comparison and considered significant at $P<0.05$.

3. Results and Discussion

3.1 Quantitation of saponin

The dried root of *A. racemosus* extracted with 90% acetonitrile in water provided a yield of approximately 13.6%

w/w. The total saponin content (equivalent to shatavarin IV was 38.4±4.2% determined by ELISA.

3.2 Effect of *A. racemosus* root extract on cell viability in UVB-exposed human fibroblasts

To examine the cytotoxic effects of UVB irradiation, the fibroblasts were exposed to UVB in the dose range of 25–100 mJ/cm^2. At 24 h after UVB exposure, the cell viability was determined. The dose of UVB irradiation at 100 mJ/cm^2 significantly diminished cell viability, while at 75 mJ/cm^2 cell viability was not reduced; however, severe changes in morphology were observed (data not shown). The cell viability of the human fibroblasts was not significantly affected by irradiation at 25 mJ/cm^2 or 50 mJ/cm^2 (data not shown). Treatments with *A. racemosus* root extract were carried out at concentrations of 10, 25, 50, and 100 µg/mL. There were no significant changes in cell viability at extract concentrations of 10, 25 µg/mL (data not shown). Therefore, the UVB doses of 25 mJ/cm^2 and 50 mJ/cm^2 were used for further investigations. With pretreatment of *A. racemosus* root extract for 24 h prior to UVB exposure, cell viability substantially increased compared to the control (Figures 2A and 2B).

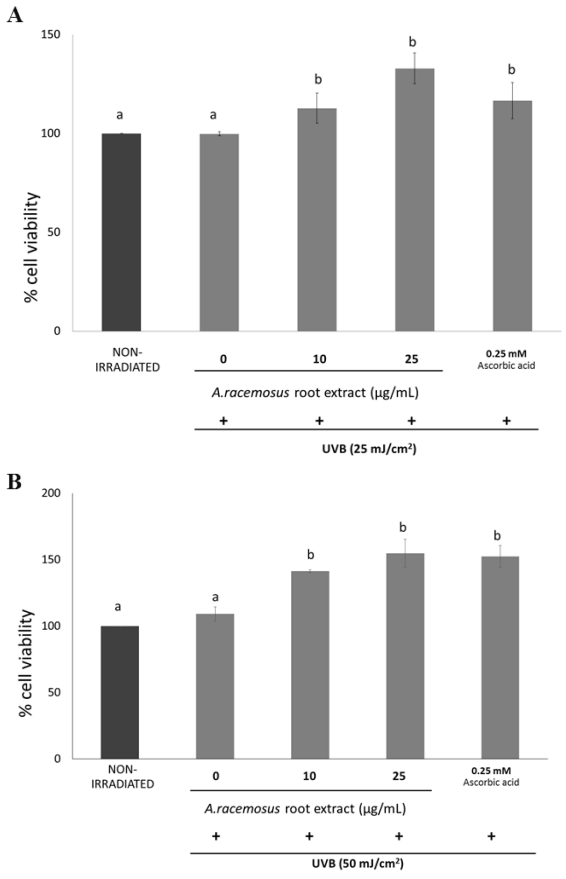


Figure 2. Effects of *A. racemosus* root extract on human fibroblast cell viability. Fibroblasts exposed to UVB (A) 25 mJ/cm^2 and (B) 50 mJ/cm^2. Results are expressed as % control (mean±SD). Means followed by different letters differ statistically (P<0.05). Values are determined by one-way ANOVA; n=3.

3.3 Effect of *A. racemosus* root extract on procollagen I synthesis in human dermal fibroblasts

Under non-UVB exposed conditions, treatment with 25 µg/mL *A. racemosus* root extract and ascorbic acid significantly enhanced the expression of procollagen I mRNA and protein (Figure 3).

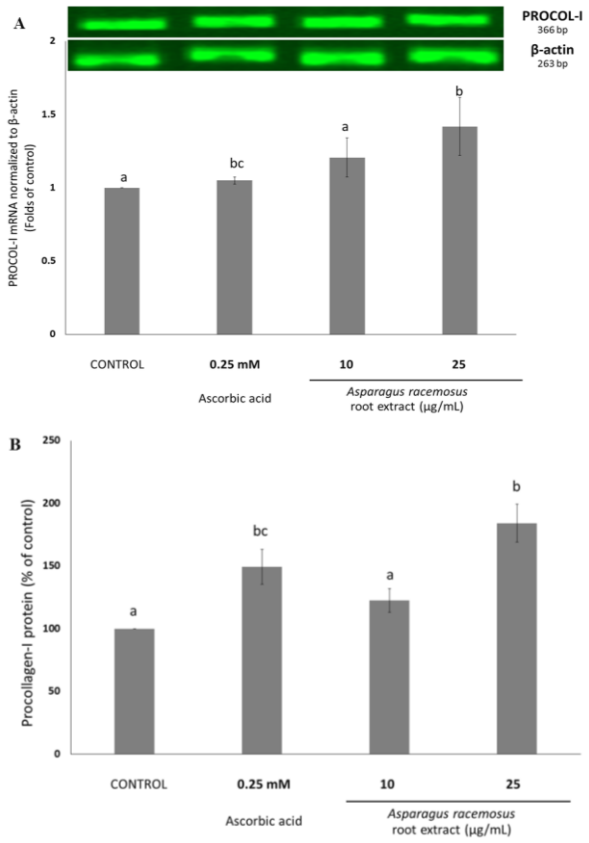


Figure 3. Effects of ascorbic acid and *A. racemosus* root extract on (A) procollagen I mRNA expression and (B) type I procollagen protein in non-irradiated fibroblasts (expressed as the mean±SEM, n=3). Means followed by different letters differ statistically (P<0.05) by one-way ANOVA.

3.4 Effect of *A. racemosus* root extract on procollagen I expression in UVB irradiated human fibroblasts

The expression of procollagen I mRNA in UVB irradiated fibroblasts was not significantly altered at 24 h after exposure compared to the non-irradiated cells. *A. racemosus* root extract did not significantly affect the expression of procollagen I mRNA among the fibroblast cells at both irradiation doses. However, procollagen I protein level significantly decreased by nearly 23% in the cell culture supernatant at 24 h after irradiation under 25 mJ/cm^2 UVB. The level of procollagen I protein in the pre-treatment group with the extract did not differ from the non-treated control (Figure 4). At 50 mJ/cm^2, the level of procollagen I protein was significantly decreased by nearly 56%; however, the level of procollagen I protein was significantly higher when the fibroblasts were pre-

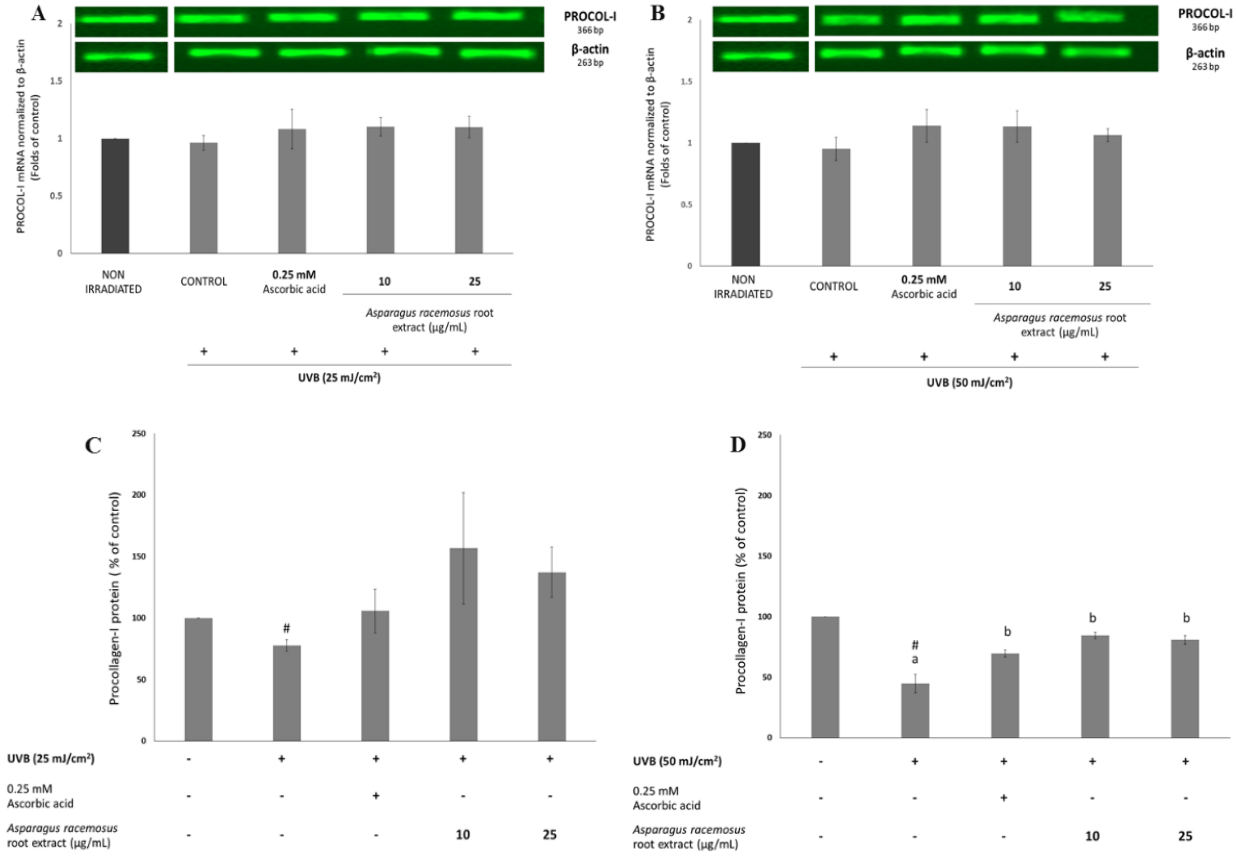


Figure 4. Effect of ascorbic acid and *A. racemosus* root extract on the expression of procollagen-I mRNA in UVB irradiated human fibroblasts, (A) 25 mJ/cm^2 and (B) 50 mJ/cm^2 and type I procollagen secretion in the culture medium of fibroblasts under exposure of UVB irradiation (C) 25 mJ/cm^2 and (D) 50 mJ/cm^2. The data are presented as mean±SEM ($^{\#}$P<0.05 versus non-irradiated control by student's *t*-test; Means followed by different letters differ statistically; P<0.05 versus UVB irradiated group by one-way ANOVA; n=3).

treated with the extract (both 10 and 25 μg/mL). This suggested a protective effect of *A. racemosus* extract on the UVB-induced decrease in procollagen I secretion.

3.5 Effect of *A. racemosus* root extract on MMP-1 expression in UVB irradiated human fibroblasts

After 24-h irradiation at 25 mJ/cm^2 UVB, there was approximately a 4-fold increase in MMP-1 mRNA with a significant increase of nearly 50% in the amount of MMP-1 protein. The extract showed no effect on the prevention of UVB-induced MMP-1 mRNA expression (Figure 5). At 50 mJ/cm^2, the amount of MMP-1 protein was elevated by approximately 2-fold. Only ascorbic acid could significantly inhibit MMP-1 secretion as seen with a significant reduction of approximately 57% in MMP-1 protein level. The extract from the roots of *A. racemosus* showed some trend on the prevention of UVB-induced MMP protein synthesis, although it was not statistically significant. Our results revealed the beneficial effect of *A. racemosus* on procollagen I synthesis in non-UVB irradiated fibroblasts by increasing the expression of both mRNA and protein in a similar manner as ascorbic acid. However, further investigation is needed to describe the exact mechanisms of action as several phytoestrogens were reported to promote collagen synthesis in human dermal fibroblast by either activation of TGF-β/smad2 signaling pathway (Zhao, Shi, Dang, Zhai, & Ye, 2015) or interacting with an estrogen receptor (i.e. ER-β or ER-α) (Kuiper *et al.*, 1998).

Upon exposure to UVB irradiation, matrix metalloproteinase enzymes are activated as a consequence of increasing pro-inflammatory cytokines and activation of transcription factors such as activation protein-1 and nuclear factor-κB (NF-κB). This phenomenon is followed by the degradation of extracellular matrix proteins such as collagen type-I and III. The expressions of TGF-β and procollagen I mRNA are also suppressed, leading to a decrease in matrix protein synthesis (Quan, He, Kang, Voorhees, & Fisher, 2002). Several plant extracts rich in phytoestrogens have demonstrated beneficial effects for the prevention of skin ageing through various mechanisms (Hwang *et al.*, 2012; Lee *et al.*, 2012; Lee *et al.*, 2014). The root extract of *A. racemosus* at doses in the range of 100–200 μg/mL was reported to prevent early procollagen I degradation and inhibit MMP-1 secretion under exposure to UVA irradiation. The level of MMP-1 protein was reduced by approximately 6-fold whereas procollagen-1 protein was increased by nearly 2.7-fold when pretreated with the extract (Rungsang *et al.*, 2012). In this study, the *A. Racemosus* root extract showed a significant protection from UVB

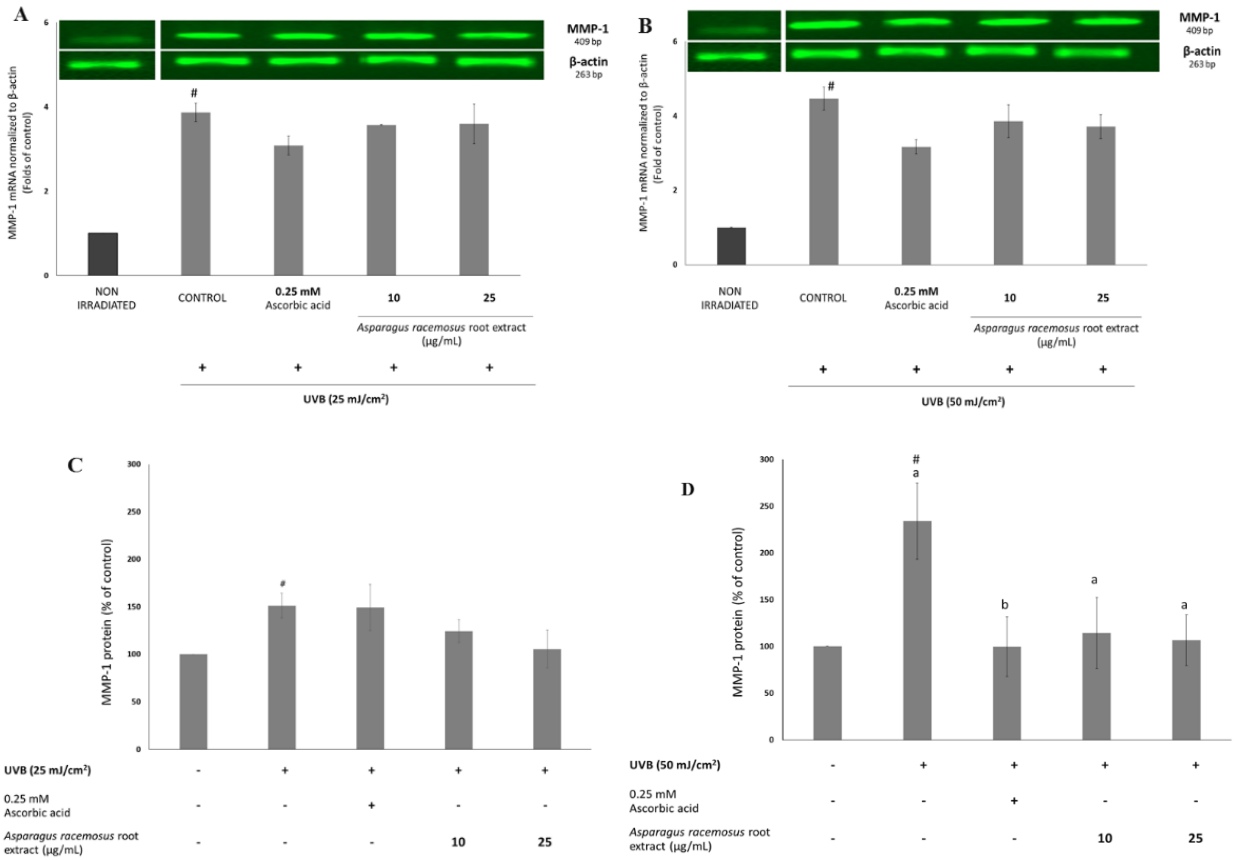


Figure 5. Effect of ascorbic acid and *A. racemosus* root extract on the expression of MMP-1 mRNA in UVB irradiated human fibroblasts, (A) 25 mJ/cm^2 and (B) 50 mJ/cm^2 and MMP-1 secretion in the culture medium of fibroblasts under exposure of UVB irradiation (C) 25 mJ/cm^2 and (D) 50 mJ/cm^2. The data are presented as mean±SEM ($^{\#}$P<0.05 versus non-irradiated control by student's *t*-test; Means followed by different letters differ statistically; P<0.05 versus UVB irradiated group by one-way ANOVA; n=3).

with regard to procollagen I protein secretion. At 24 h post irradiation, there was a noteworthy trend to inhibit UVB induced MMP-1 expression in pre-treated *A. racemosus* root extract fibroblasts. In our experiment, it is not known what could be the mechanism by which shatavarins, a group of saponins present in high abundance of *A. racemosus* root extract, could mediate their pharmacological effects in fibroblast cells, under both non-UVB irradiated or UVB irradiated conditions. Nevertheless, it is important to mention that the concentration of *A. racemosus* root extract treated with fibroblast cells in our study was close to the concentration reported to possess anti-lipid peroxidation activity (Onlom *et al.*, 2017). Thus, it could be one possible mode of action in ameliorating UVB induced activation of MMP-1 expression since it is known that MMP activation could be mediated by NF-κB signaling after the inflammation cascade by lipid peroxidation (Ayala *et al.*, 20 14; Yadav & Ramana, 2013).

4. Conclusions

A. racemosus root extract significantly increased type I procollagen protein in non-irradiated human fibroblasts. UVB irradiation increased the expression of MMP-1 at the gene and protein levels of the cells. The cells that were pre-treated with *A. racemosus* root extract for 24 h showed a trend to attenuate the UVB-induced MMP-1 mRNA expression and MMP-1 protein. Notably, pretreatment with *A. racemosus* root extract could significantly prevent a decrease in procollagen I protein secretion under UVB irradiation to the same extent as ascorbic acid. These findings mark the dual benefits of *A. racemosus* root extract on collagen metabolism involved in skin aging. However, the mechanism of action in promoting procollagen synthesis and the protection against UVB-induced decrease in procollagen I protein secretion was not determined and needs to be investigated.

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