



Potential prevention effects of *Rubus occidentalis* seed on UVB-induced MMP-1 production and procollagen degradation in CCD-986sk cells

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Abstract UV exposure induces matrix metalloproteinases (MMPs) and extracellular matrix-degrading enzymes expression. We studied the protective effect of *Rubus occidentalis* seed against UVB-generated skin photoaging using human fibroblast cells (CCD-986sk). We used an ELISA kit to measure the supernatants of procollagen type I and MMP-1 in CCD-986sk cells after they were exposed to UVB irradiation. The CCD-986sk cells that were used with RC-E/E after the UVB irradiation caused higher levels of type I procollagen and lesser levels of MMP-1 compared with the control group. Furthermore, the RC-E/E treated group showed lesser MMP-1 levels and higher procollagen type I levels than the untreated counterpart. Therefore, it can be concluded that *Rubus occidentalis* seed can prevent from skin photoaging.

Keywords Anti-wrinkle · CCD-986sk · MMP-1 · Procollagen · *Rubus occidentalis* seed

Introduction

The skin aging process consists of the intrinsic (chronologic) aging that internal organs and the extrinsic (photoaging) aging that is induced by exposure to environmental factors. Exposure to ultraviolet (UV) irradiation from the sun causes extrinsic aging, which can induce photoaging (Bae et al. 2008); however,

photoaging is mainly due to UVB irradiation exposure and is characterized by an altered pigmentation, inflammation and collagen degradation (Bennett 2008; Ahn et al. 2013). Chronic exposure of skin to UVB irradiation also causes the production of matrix metalloproteinases (MMPs), resulting in the alteration of collagenous skin tissues (Ahn et al. 2013). MMPs are a family of extracellular matrix (ECM)-degenerating enzymes and are generated from different types of cells including fibroblasts and endothelial cells, as well as immune cells. MMPs play a key role in the dynamic remodeling of the ECM and are comprised of functional subclassified groups including membrane-type MMPs, and other nonclassified MMPs (Visse and Nagase 2003); in particular, collagenase-1 (MMP-1) production is significantly enhanced in late passage skin fibroblasts (Tsuji et al. 2001; Tsukahara et al. 2001; Varani et al. 2002; Brennan et al. 2003). Collagenase can cleave the interstitial collagenases that are found in the skin such as type I collagen (Lee et al. 2008). Type I collagen accounts for 70 to 90 % of total collagen (Kerrigan et al. 2000) and the functional characteristic of skin rely on the preservation of dermal collagen. The metabolic control of type I collagen may therefore be useful for a diversity of cosmetic materials.

The fruits of *Rubus occidentalis* Miq. (*R. occidentalis*), a member of the Rosaceae family, have long been used as part of traditional Korean folk medicines to treat asthma and allergic diseases (Shin et al. 2014). Phenolic acid, organic acid, triterpenoids, flavonoids, and ellagitannin are the reported compounds of *R. occidentalis* Miq. fruits (Ju et al. 2009). The fruits are mostly used to make traditional wine through the fermentation and maturation of perfectly matured fruits that are harvested in June and July; therefore, we especially focused on the usage of wine-processing leftovers. The *R. occidentalis* seed, which is rich in unsaturated fatty acids and α -linoleic acids, is used in S. Korea for the prevention of gingivitis, rashes, eczema, and other skin lesions (Pourrat et al. 1973). We determined the effects of *R. occidentalis* seed on UVB-induced MMP-1 production levels and type I procollagen contents in human skin fibroblast (CCD-986sk) cells.

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Materials and Methods

Materials

All of the cell-culture reagents that were used in the present study were obtained from Gibco BRL Co. (Rockville, NE, USA), while 3-[4,5-dimethylthylthiazol]-2-yl]-2,5-diphenyl-tetrazoliumbromide (MTT) was obtained from Sigma Chemical Co. (St. Louis, MO, USA). The productions of MMP-1 (Abcam, Cambridge, MA, USA) and type I procollagen (Takara Co., Otsu, Japan) were measured using commercial ELISA- and EIA-kit antibodies, and the MMP-1 and type I procollagen were purchased from Santa Cruz (Santa Cruz, CA, USA).

Preparation of *R. occidentalis* seeds extract

The *R. occidentalis* seeds were obtained from the “Sam do bong Farm” company that is located in Kimcheon, Gyeongbuk, Korea. A 100 g of the *R. occidentalis* seed was measured and the seeds were soaked in 70 % ethyl alcohol (EtOH) for 24 h at room temperature (RT). The mixture was filtered with Whatman No. 2 (Sigma Chemical Co.) and the procedure was repeated two more times. The total extract was then evaporated and 5.4 g of EtOH extract was obtained. The aqueous phase of EtOH extract was suspended again, mixed with an same volume of n-hexane, and separated by a separating funnel. A hexane extract was evaporated and 0.5 mg was obtained. These procedures were repeated with other solvent ethyl acetate (EtOAc) and butyl alcohol (BuOH) sequentially. In terms of powder samples, 0.8, 1.4, and 2.0 g were obtained from the EtOAc, BuOH, and final aqueous layer, respectively.

Cell culture

After the CCD-986sk human skin fibroblast cell line was got from the American Type Culture Collection ATCC (Manassas, VA, USA), it was commonly cultured in a Dulbecco’s modified Eagle medium (Hyclone Laboratories, Logan, UT, USA) added with 10 % (v/v) FBS and 1 % (v/v) penicillin-streptomycin, while under 37 °C in an incubator with a humidified atmosphere of 5 % CO₂.

Determination of cell viability

The cell toxicity of the *R. occidentalis* seeds extract (RCE) and RCE fractions were determined by measuring the reduction of tetrazolium in accordance with a modified version of Shin et al.’s protocol (Shin et al. 2014). Briefly, the cells were seeded at a consistency of 5×10^3 cells/well in 96-well plates. After 24 h storage, the CCD-986sk cell was exposed to UVB for 20 mJ/cm², followed by indicated concentrations of the RCE; then, 10 µL of the extract was added to the fibroblast cells while the equal volume of dilution solution was added to the control. After 48 h of incubation, MTT solution (5 mg/mL) was added to 4 h incubation period. After the MTT solution was removed, 100 µL of DMSO was added followed by a further 10 min incubation period. The wavelength was set at 540 nm and the cell toxicity was measured

using the Sunrise ELISA plate reader (Tecan Austria GmbH, Grödig/Salzburg, Austria).

MMP-1 inhibition and type I procollagen synthesis assay

The cells were seeded at a consistency of 5×10^4 in 12-well plates and, after 24 h of incubation, they were treated with the RCE and RCE fractions for 48 h. To determine the production levels of the MMP-1 and type I procollagen, the medium was measured using commercially available ELISA kits in accordance with the manufacture’s instructions (Abcam and Takara). The absorbances were measured at 450 nm measuring an ELISA plate reader.

cDNA synthesis and RT-PCR

The cells were incubated in the RCE-E treatment for 48 h. The total RNA was extracted using Trizol (Invitrogen, Carlsbad, CA, USA) following the manufacture’s directions. Two µg of RNA were used for the 1 h cDNA synthesis that involved the use of oligo (dT) primer and reverse transcriptase (Promega, WI, USA) at 42 °C. A PCR was performed with the cDNA to analyze the transcript expressions of MMP-1, MMP-2, and MMP-3, where by a 55 °C annealing temperature was applied for 30 sec and 72 °C was applied for a 1 min extension; the total for the PCR is 30 cycles. The transcripts were separated in a 1.5 % agarose gel and stained with Redsafe (iNtRON Biotechnology Inc., Sungnam, Gyeonggi, Korea), followed by the use of a LAS4000 (Fujifilm, Tokyo, Japan) image analyzer for the confirmation.

Western blot analysis

Shortly, the cells were seeded in 100 mm culture dish and grown for 24 h, followed by RCE-E treatment for 48 h. After the RCE-E treatment, the cells were, then, washed twice with cold PBS and lysed for 5 min on ice in a 1 mL buffer of radio immunoprecipitation assay with protease inhibitor cocktails (100 X). The lysates were clarified by centrifugation at 12,000 rpm for 20 min at 4 °C, and the total protein concentrations were measured using a BSA kit. A western blot analysis was conducted to investigate the protein expressions of MMP-1 and type I collagen. First, 15 µg of each protein was separated using 10 % sodium dodecyl sulfate polyacrylamide gel electrophoresis and they were then transferred onto PVDF membranes; the membranes were incubated for 1 h in 5 % skim milk with TBST followed by their incubation with the designated primary antibodies (1:1000) the membranes were then washed and incubated with secondary HRP-labeled antibodies (1:1000) for 2 h at RT. Chemiluminescent images of the blots were detected using the SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, IL, USA) under an LAS 4000 image analyzer (Fuji Film Life Science, Tokyo, Japan). The membranes were then stripped and reprobed with β-actin antibody serving as the loading control.

Data analysis

This study was conducted in triplicate. An analysis of variance

was run using the raw data, and the mean values and standard deviations were analyzed using the ANOVA system. Differences among the means were determined by a *t*-test, and significance occurs at $*p < 0.05$ and $**p < 0.005$.

Results and Discussion

Effects of RCE on cell viability of UVB-induced CCD-986sk cells

Before investigating the preventive effect of the RCE and RCE-fractions on UVB-induced MMP-1 production and type I procollagen decline, we first verified the cytotoxicity of the RCE in the CCD-986sk cells using an MTT assay. Result in Fig. 1, the cells were treated with indicated concentrations of the RCE and RCE-fractions; the extract did not show any toxic effects up to 20 $\mu\text{g}/\text{mL}$. For the investigation of the effect of RCE on UVB-induced MMP-1 production and type I pro-collagen decline, we used concentrations between 5 and 20 $\mu\text{g}/\text{mL}$ in the subsequent experiments.

Effect of RCE on MMP-1 inhibition and type I pro-collagen synthesis

MMPs are directly responsible enzymes for the degradation of ECM components, and they specifically denatured collagens (Kim et al. 2011) therefore, UV irradiation degrades ECM by increasing the MMPs production in the skin. We demonstrated the effects of the RCE and RCE-fractions on UVB-induced MMP-1 expression in CCD-986sk cells (Fig. 2). The cells were treated with 5, 10 and 20 $\mu\text{g}/\text{mL}$ concentrations of RCE and RCE-fractions for 48 h and the MMP-1 concentration was determined using an ELISA kit.

The RCE and RCE-fractions suppressed MMP-1 expression in those cells exposed to UVB; in particular, the RC-E/E fraction reduced MMP-1 expression by 12 % at 5 $\mu\text{g}/\text{mL}$ and 22 % at 10 $\mu\text{g}/\text{mL}$. The highest concentration of the RC-E/E fraction prevented the expression of MMP-1 up to 50 % in a dose-dependent manner. Previous reports have shown that UVB exposure stimulates collagen degradation and the inhibition of type I procollagen production in fibroblasts (Fisher et al. 1996) therefore, we investigated the protective effect of the RCE extracts against UVB. As shown in Fig. 3, the extract of RC-E/E significantly increased type I procollagen synthesis with restorations of 8 and 17 % at 10 and 20 $\mu\text{g}/\text{mL}$, respectively. Overall, the RC-E/E extract suppressed the expressions of UVB-induced MMP-1 and type I collagen protein in the human fibroblast cell (CCD-986sk).

RCE decreased expressions of type I procollagen and inhibition of MMP-1 protein in CCD-986sk cells

We next examined the effects of the RC-E/E fraction on the decrease of type I procollagen and the increase of MMP-1 protein that are induced by UV. As depicted in Fig. 4, the reductions of the expressions of type I procollagen and inhibition of MMP-1 protein were significantly induced at 48 h in those fibroblasts exposed to UVB, as expected. Pretreatment with the RC-E/E extract significantly restored the suppression of the type I procollagen protein expression and also inhibited MMP-1 protein expression in those fibroblasts exposed to UVB, there by showing a pattern alike to the regulation of mRNA expression. All of these results suggest that RC-E/E pretreatment prevents UV-induced changes of type I procollagen and MMP-1 expression at the transcriptional level.

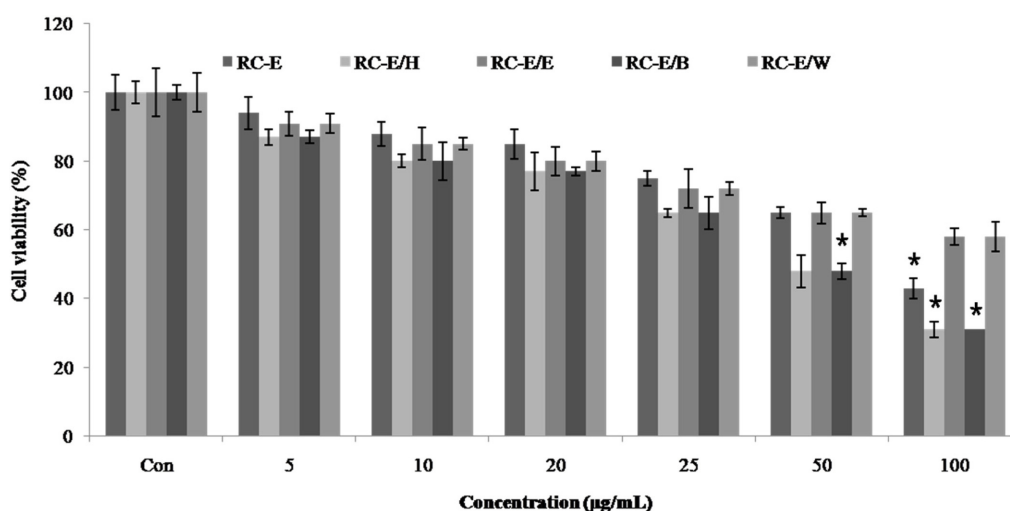


Fig. 1 Cell viability of fibroblast CCD-986sk cells after treatment with *Rubus occidentalis*-seed extract. The fibroblast cells were exposed to UVB for 20 mJ/cm^2 and treated with varying concentrations of 70 % EtOH and fraction extracts obtained from the *R. occidentalis* seed. Cell viability was measured using an MTT assay. Error bar indicates average mean of triple replicates. RC-E: 70 % EtOH extracts; RC-E/H: Hexane fraction of *R. occidentalis*-seed extracts, RC-E/E: Ethyl acetate fraction of *R. occidentalis*-seed extracts; RC-E/B: Butanol fraction of *R. occidentalis*-seed extracts; RC-E/W: Water fraction of *R. occidentalis*-seed extracts

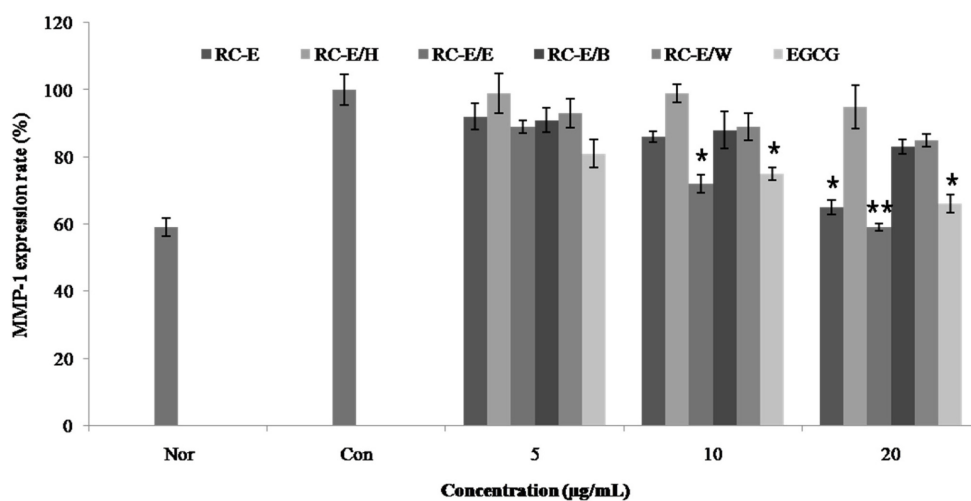


Fig. 2 Inhibition of MMP-1 expression of CCD-986sk fibroblast cells after treatment with *R. occidentalis*-seed extract. Fibroblast cells were exposed to UVB for 20 mJ/cm², and were then treated with varying concentrations of 70 % EtOH and fraction extracts obtained from the *R. occidentalis*-seed. MMP-1 expression was measured using an ELISA kit. Error bar indicates average mean of triple replicates. RC-E: 70 % EtOH extracts; RC-E/H: Hexane fraction of *R. occidentalis*-seed extracts, RC-E/E: Ethyl acetate fraction of *R. occidentalis*-seed extracts; RC-E/B: Butanol fraction of *R. occidentalis*-seed extracts; RC-E/W: Water fraction of *R. occidentalis*-seed extracts

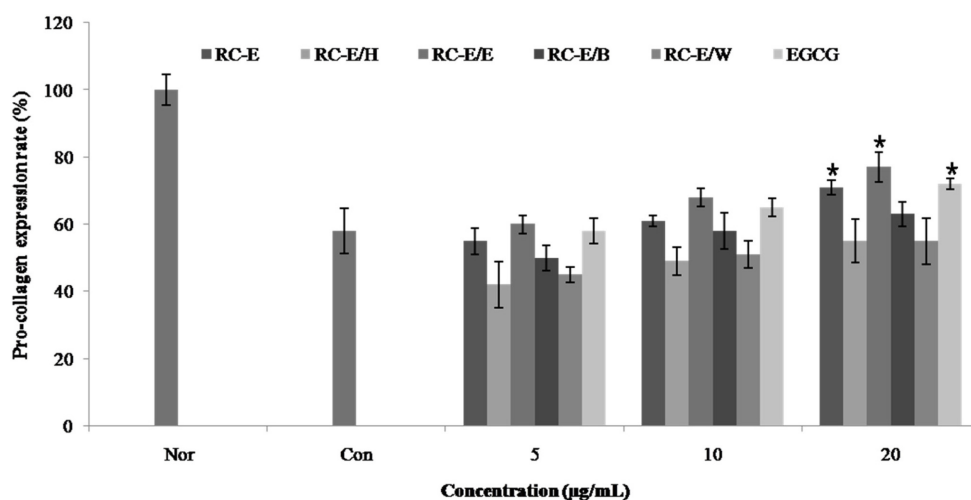


Fig. 3 Inhibition of pro-collagen expression of CCD-986sk fibroblast cells after treatment with *R. occidentalis*-seed extract. Fibroblast cells were exposed to UVB for 20 mJ/cm², and were then treated with varying concentrations of 70 % EtOH and fraction extracts obtained from the *R. occidentalis*-seed. Procollagen expression was measured using an EIA kit. Error bar indicates average mean of triple replicates. RC-E: 70 % EtOH extracts; RC-E/H: Hexane fraction of *R. occidentalis*-seed extracts, RC-E/E: Ethyl acetate fraction of *R. occidentalis*-seed extracts; RC-E/B: Butanol fraction of *R. occidentalis*-seed extracts; RC-E/W: Water fraction of *R. occidentalis*-seed extracts

Preventive effects of the RC-E/E on UVB-suppressed type I procollagen and MMP-1 mRNA expression

When epidermis of human and experimental animal is penetrated by UVB wavelengths it induces photoaging (Debacq-Chainiaux et al. 2005). In addition, UVB irradiation decreases type I procollagen synthesis rate in human fibroblasts. It has also been reported that UVB irradiation leads to the activation of MMP expression and collagen degradation in the human dermis (Chen et al. 2011). We therefore confirmed in our study that UVB exposure promotes the induction of MMP-1 mRNA expression and the inhibition of type

I procollagen mRNA expression in CCD-986sk cells. To determine the protective effect of RC-E/E on MMP-1 and type I procollagen mRNA expression, the mRNA level was measured by the RT-PCR, as described in manufacture's manual. As shown in Fig. 5, the UVB-induced increased mRNA level of MMP-1 was inhibited by the RC-E/E in a dose-dependent manner. Further, we also confirmed the effect of the RC-E/E on UVB-reduced type I procollagen expression, identifying that the RC-E/E restored the contraction of type I procollagen that is caused by UVB exposure. These data suggest that the RC-E/E prevents MMP-1 mRNA

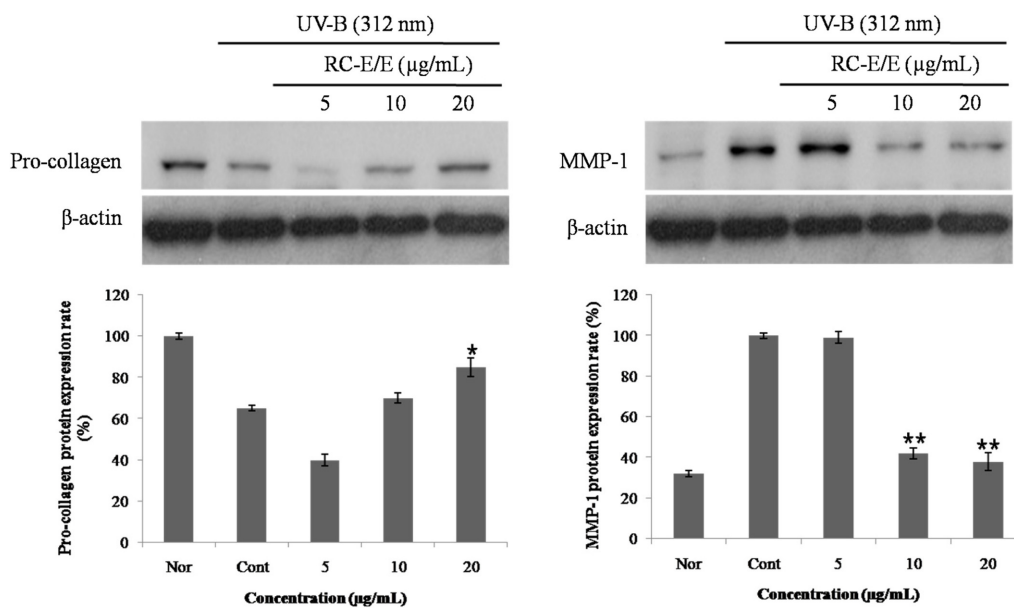


Fig. 4 Expressions of MMP-1 and type I procollagen evaluated by protein level in CCD-986sk fibroblasts after EtOAc-fraction treatment. CCD-986sk cells (5×10^4) were grown and exposed to UVB for 20 mJ/cm²; the cells were then subject to 5, 10 and 20 µg/mL of the EtOAc-fraction treatment. The mRNA levels were normalized using Image Quant software.

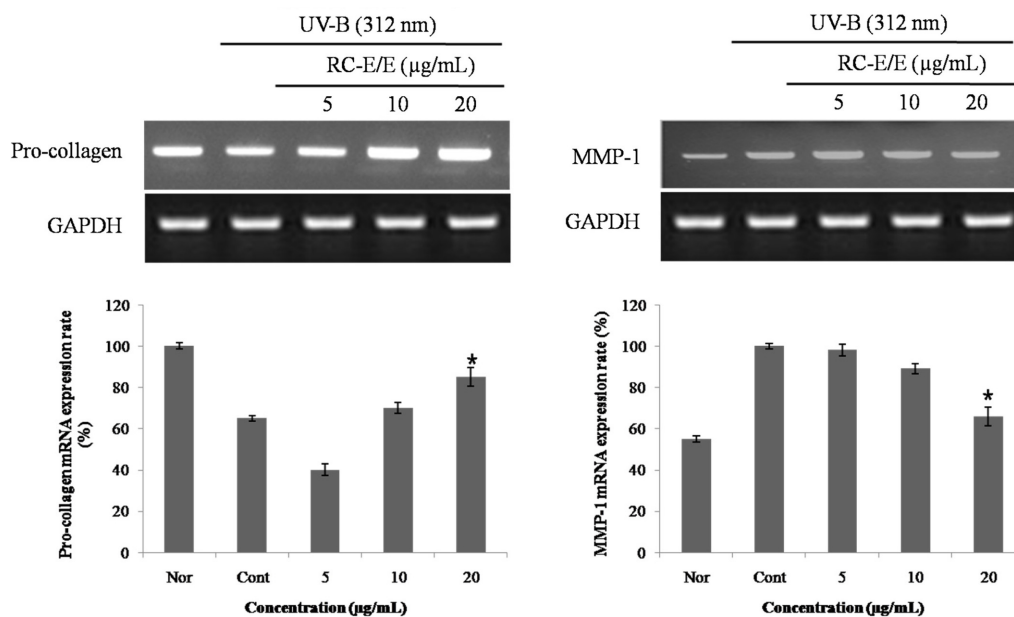


Fig. 5 Expression of MMP-1 and type I procollagen evaluated by mRNA level in CCD-986sk fibroblasts after EtOAc-fraction treatment. The CCD-986sk cells (5×10^4) were grown and exposed to UVB for 20 mJ/cm²; the cells were then subject to 5, 10 and 20 µg/mL of EtOAc-fraction treatment. The protein levels were normalized using Image Quant software.

expression and the reduction of UVB-induced type I procollagen mRNA expression in CCD-986sk cells. The effects of the RC-E/E fraction on UVB stimulated MMP-1 production and type I procollagen degradation in the CCD-986sk cells.

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