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비쑥에서 분리된 Santamarine이 인간 각질형성세포에서 UVB에 의해 유도되는 Matrix Metalloproteinase 발현 저해와 MAPK/AP-1 경로 억제에 미치는 효과

오정환[1,2] · Fatih Karadeniz[3] · 김혜란[4] · 공창숙[5,6] · 서영완[7†]

Santamarine Isolated from *Artemisia scoparia* Inhibits UVB-induced Matrix Metalloproteinase Expression *via* Repression of MAPK/AP-1 Pathway in Human Keratinocytes

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ABSTRACT

Background: *Artemisia scoparia* has a widespread distribution and can be found commonly in Eurasia. In traditional Korean medicine, *A. scoparia* leaves and flowers are used against urethral complications, phlogistic problems, and in the treatment of hepatitis.
Methods and Results: In the present study, the protective effect of santamarine isolated from *A. scoparia* was evaluated in Ultraviolet B (UVB)-damaged keratinocytes. Results showed that santamarine suppressed the production of reactive oxygen species in a concentration-dependent manner. Treating cells with santamarine decreased the generation of Matrix Metalloproteinase (MMP)-1 compared to that in cells treated with UVB alone. Additionally, the mRNA levels of MMP-1 and MMP-3 were remarkably lower in cells treated with santamarine than those in cells with UVB irradiation. Furthermore, upregulated protein levels of MMP-1, MMP-2, MMP-3, and MMP-9 following UVB exposure were ameliorated by the introduction of santamarine. Investigation of the mechanisms underlying the photoprotective effect of santamarine showed enhanced inhibition of MAPK/AP-1 signaling following santamarine treatment. The presence of santamarine also recovered the UVB-induced decrease in collagen amount.
Conclusions: Overall, these results demonstrated that santamarine has a potential protective effect against UVB-induced skin photoaging in keratinocytes *in vitro*. The mechanism behind this effect was suggested to be *via* suppression of MAPK/AP-1 signaling.

Key Words: *Artemisia scoparia*, Keratinocytes, Matrix Metalloproteinases, Santamarine, Ultraviolet B

INTRODUCTION

Ultraviolet (UV) irradiation alters various molecular pathways in the skin, therefore leading to sunburn, immunosuppression, carcinogenesis and photoaging in human skin. UV-light is divided into UVA, UVB and UVC. Among them, UVC is the most dangerous to the skin, but it is absorbed by the ozone layer. UVA and UVB reach earth's surface and induce photochemical damage in human skin. UVB, in particular, is more genotoxic than UVA (Yaar and Gilchrest, 2007). UVB irradiation is known to cause oxidative stress through the production of reactive oxygen species (ROS).

Enhanced ROS levels induce the activation of matrix metalloproteinases (MMPs), a class of extracellular matrix-

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degrading enzymes, which can impair the extracellular matrix (ECM) components and suppress collagen synthesis in human skin (Fisher *et al*., 2002). Hence, reducing ROS accumulation is a contributing factor to skin photoprotection.

UVB irradiation is also known to change multiple molecular cascades in the skin, thereby inducing photoaging. The damage mechanisms occur via oxidative stress-induced DNA damage and activate the signal pathways related to photoaging. Recent studies have demonstrated that UV radiation stimulates the mitogen-activated protein kinases (MAPKs) such as p38, extracellular signal-regulated kinase (ERK) 1/2 and c-Jun N-terminal kinase (JNK) proteins, which affect the regulation of activating protein-1 (AP-1) (Afaq, 2011).

Increased AP-1, a hetero dimer comprised of c-Jun and c-Fos, promoted MMP expression and induced collagen breakdown in the skin. MMPs expression is controlled by their natural inhibitors, tissue inhibitors of metalloproteinases (TIMPs). Both MMPs and TIMPs are closely related to degrading collagen, but their effects are opposite (Zaid *et al*., 2007). MMPs play a main role in ECM degradation and wrinkle formation, characteristics of photoaging, while TIMPs inhibit the activity of MMPs and prevent the breakdown of the ECM (Visse and Nagase, 2003).

Artemisia scoparia is a medicinal plant mainly distributed in China, Korea, Japan, India, Saudi Arabia and Iran. It has been used as a traditional medicine ingredient for diuretic and antiphlogistic activities as well as to treat hepatitis (Ding *et al*., 2021).

The halophyte *A. scoparia* lives in coastal salt marshes under high-salt stress and thus might contain different and diverse secondary metabolites compared to terrestrial plants. Several studies reported that *A. scoparia* has active constituents including flavonoids, chromones, coumarins, phenolic acids and terpenoids (Wang *et al*., 2018; Stojanović *et al*., 2020; Ding *et al*., 2021).

Sesquiterpene lactones are common secondary metabolites for halophytes mainly taking roles in defense mechanisms and found ubiquitously found in *Artemisia* species. Santamarine is such sesquiterpene lactone with the chemical formula of $C_{15}H_{20}O_3$. To date, it has been named santamarine (Choi *et al*., 2012), douglanin (Rosselli *et al*., 2012) or balchanin (Zdero *et al*., 1991) in other reports.

Zhang *et al*. (2021) reported an antitumor activity for santamarine while several other reports also presented anti-inflammatory (De Marino *et al*., 2005; Choi *et al*., 2012), bactericidal (Coronado-Aceves *et al*., 2016), anti-fibrotic (Wang *et al*., 2021) and antioxidant (Oh *et al*., 2021) properties for santamarine both *in vitro* and *in vivo*.

Also, a study by Yoshikawa *et al*. (2000) showed that santamarine was able to inhibit orally administered ethanol absorption in rats. In a previous study, santamarine isolated from *A. scoparia* showed antioxidant and photoprotection activities against UVA in fibroblasts (Oh *et al*., 2021). However, it remains unknown whether santamarine can prevent skin photoaging against UVB in human keratinocytes.

Thus, this study further validates the protective effect of santamarine isolated from *A. scoparia* against UVB-induced MMP induction and collagen depletion in HaCaT cells via regulation of the MAPK/AP-1 pathway.

MATERIALS AND METHODS

1. Isolation and characterization of santamarine

Santamarine was isolated from the combination of methanolic and chloride extracts of *A. scoparia*. The leaves and stems of *A. scoparia* were air dried and ground to powder subsequently.

Powdered material (100 g) was extracted with 1 ℓ methylene chloride (CH_2Cl_2) and 3 ℓ methanol (MeOH) separately for 24 h each at room temperature. Obtained extracts were combined and dried with a rotary evaporator. Santmarine was isolated from the future separation of this extract as described earlier (Oh *et al*., 2021).

Characterization of santamarine was carried out *via* comparison of spectral data with published literature. NMR spectral data were recorded on a Bruker Avance II NMR 900 spectrometer (Billerica, MA, USA) and obtained at the Korean Basic Science Institute (Daejeon, Korea).

2. Cell culture and UVB Irradiation

HaCaT immortal keratinocyte was purchased from Cell Line Service (Eppelheim, Germany). Cells were cultured in Dulbecco's modified Eagle medium (DMEM, Gibco-BRL, Gaithersburg, MD, USA) containing 100 ㎍/㎖ penicillin-streptomycin antibiotics, 10% fetal bovine serum (FBS) (Gibco-BRL, Gaithersburg, MD, USA) and maintained at 37℃ in a humidified atmosphere of 5% carbon dioxide.

For UVB irradiation, the culture medium was removed, and the cells were washed with phosphate-buffered saline (PBS). Then the cells were exposed to UVB using a Bio-Sun UV Irradiation System (Vilber Lourmat, Marine, France) with a spectral peak of 312 ㎚. After the UVB irradiation at the range

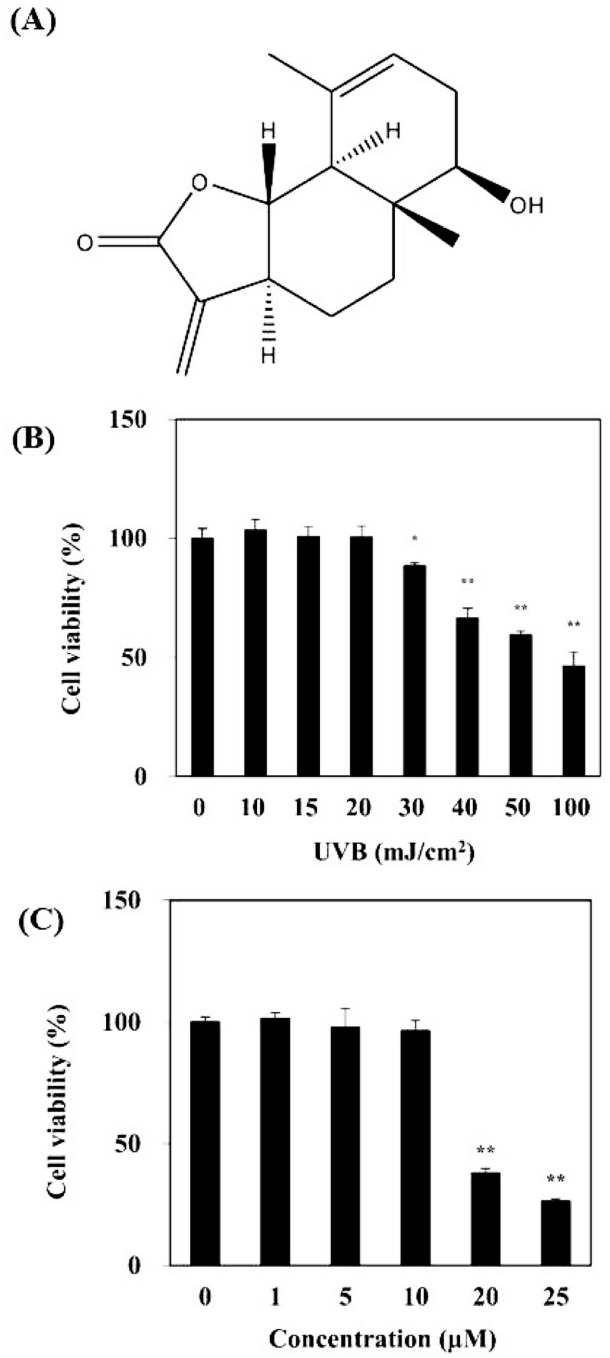

Fig. 1. The effect of UVB (A) and santamarine (B) on the viability of HaCaT cells. The viability of the cells was assessed using MTT assay. Cell viability following santamarine treatment was measured by examining the ability to form MTT-formazan crystals. Viability was quantified as a relative percentage of the untreated group. Data are shown as the means ± standard deviation ($n = 3$). $^{*}p < 0.05$, $^{**}p < 0.01$ vs. non-treated group.

of 10 mJ/㎠ - 100 mJ/㎠, cells were maintained with DMEM without FBS for 24 h. UVB exposure at higher than 30 mJ/㎠ dose significantly decreased the cell viability, therefore intensity of UVB was selected at 20 mJ/㎠ for the current study (Fig. 1).

3. Cell viability assay

The viability of cells was analyzed by MTT assay. Briefly, cells were counted and seeded in 96-well plates and incubated for 24 h. Then, the cells were treated with five different concentrations (1, 5, 10, 20 and 25 μM) of santamarine and introduced with serum-free medium. After incubation for 24 h, the cell culture supernatant was removed and replaced with 100 ㎕/well of 1 ㎎/㎖ MTT solution (AMRESCO, Solon, OH, USA), followed by incubation for 4 h. Cells were then rinsed in PBS and dimethyl sulfoxide was added to dissolve the formazan crystals.

The absorbance was read on a GENios® microplate reader (Tecan Austria GmbH, Grödig, Austria) at 540 ㎚. The viability of cells was quantified as a relative percentage compared with the untreated control group. Each sample group was tested by three independent repeats.

4. Determination of intracellular ROS generation

The generation of intracellular ROS was determined using an oxidation-sensitive dye, 2′,7′-dichlorofluorescein diacetate (DCFH-DA). The cells were seeded in fluorescence microtiter 96-well plates and cultured for 24 h. The cells were stained with 20 μM DCFH-DA for 30 min and treated with samples for 2 h in the dark. Immediately after washing three times with PBS, 500 μM H_2O_2 was added to the wells.

The 2′,7-dichlorofluorescin (DCF) fluorescence intensity was measured at the excitation wavelength at 495 ㎚ and the emission wavelength at 630 ㎚ using a fluorometer (Tecan Group Ltd., Mannedorf, Switzerland).

5. Measurement of MMP-1 production

UVB-induced changes in the MMP-1 production in HaCaT cells were determined by ELISA. HaCaT cells were pre-incubated in six-well plates, and after 24 h of treatment with or without santamarine, the culture medium was collected.

The amount of MMP-1 in the culture medium was calculated with the help of a commercial ELISA kit (R&D systems, Minneapolis, MN, USA) in accordance with the manufacturer's protocol.

6. Reverse transcriptase polymerase chain reaction analysis

Total RNA was isolated using Trizol reagent from HaCaT

cells treated with or without santamarine for 24 h following UVB irradiation (Invitrogen Co., Carlsbad, CA, USA).

Complementary DNA (cDNA) was synthesized from 2 μg RNA using cell Script All-in-One cDNA Master Mix (CellSafe, Yongin, Korea).

PCR amplification of the cDNA template was performed with PCR premix (Bioneer, Daejeon, Korea) and the following gene-specific primer pairs: forward 5'-GAT-GTG-GAG-TGC-CTG-ATG-TG-3' and reverse 5'-TGC-TTG-ACC-CTC-AGA-GAC-CT-3' for MMP-1; forward 5'-ATT-CAG-TCC-CTC-TAT-GGA-CCT-CC-3' and reverse 5'-CTC-CAG-TAT-TTG-TCC-TCT-AC-3' for MMP-3; forward 5'-AGC-CAT-GTA-CGT-AGC-CAT-CC-3' and reverse 5'-TCC-CTC-TCA-GCT-GTG-GTG-GT-3' for β-actin.

Amplification conditions consisted of 30 cycles as follows: 2 min denaturation at 95℃, 45 sec annealing at 60℃, and 1 min extension at 72℃. The reaction was performed with the T100 Thermo Cycler (Bio-Rad Laboratories Ltd., Watford, England). PCR products were separated by 2.0% agarose gel and stained with ethidium bromide.

Photographs were detected using a Davinch-Chemi imager™ (Davinch K, Seoul, Korea). mRNA levels were then divided by the corresponding â-actin band, respectively.

7. Immunoblotting

Total cellular protein was collected with the RIPA buffer (Sigma-Aldrich Co., Saint Louis, MO, USA) containing protease inhibitor cocktail (Thermo Scientific Inc., Waltham, MA, USA). Protein concentrations were determined by bicinchoninic acid (BCA) method. Aliquots of 25 μg total proteins were separated by 10% SDS-PAGE.

Proteins on gels were transferred onto a nitrocellulose membrane (Amersham Pharmacia Biotech UK Ltd., Amersham, England) and hybridized at 4℃ overnight with primary antibodies against the following proteins: MMP-1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), MMP-3 (Santa Cruz biotechnology, Santa Cruz, CA, USA), p38 (Cell Signaling Technology Inc., Danvers, MA, USA), phospho(p)-p38 (Cell Signaling Technology Inc., Danvers, MA, USA), ERK (Cell Signaling Technology Inc., Danvers, MA, USA), p-ERK (Cell Signaling Technology Inc., Danvers, MA, USA), JNK (Thermo Scientific Inc., Waltham, MA, USA), pJNK (Cell Signaling Technology Inc., Danvers, MA, USA), TIMP-1 (Cell Signaling Technology Inc., Danvers, MA, USA), TIMP-2 (Cell Signaling Technology Inc., Danvers, MA, USA) and β-actin (Cell Signaling Technology Inc., Danvers, MA, USA).

After hybridization with HRP-labeled secondary antibodies for 1 h, the protein bands were visualized with an ECL illuminate (Amersham Pharmacia Biotech UK Ltd., Amersham, England) using a Davinch-Chemi imager™ (Davinch K, Seoul, Korea).

8. Immuno fluorescence Staining

To detect collagen I expression in UVB-irradiated HaCaT cells, immunofluorescence staining was conducted using an Immunofluorescence Application Solutions kit (Cell Signaling Technology Inc., Danvers, MA, USA) as follows.

HaCaT cells were cultured on the glass cover slips and exposed to UVB. Then, the cells were treated with the santamarine for 24 h. After the treatment, HaCaT cells were fixed with 4% paraformaldehyde, permeabilized with methanol and stained with anti-collagen I (Abcam, Cambridge, England) antibody at 4℃ overnight. The cells were then incubated with an Alexa Fluor 488-conjugated secondary antibody at room temperature in the dark and mounted with a drop of mounting medium containing the 4',6-diamidino-2-phenylindole (DAPI) (Cell Signaling Technology Inc., Danvers, MA, USA).

9. Statistical analysis

Data werepresented as means ± SD. All experiments were conducted at least three times. The statistical analysis of the result performed by One-way Analysis of Variance (ANOVA), followed by Duncan's Multiple Rage Tests (DMRT) using the SPSS 12.0 (SPSS Inc., Chicago, IL, USA). The significance of differences was defined significant when $^{*}p < 0.05$ or $^{**}p < 0.01$.

RESULTS

1. Cytotoxicity of santamarine on cell viability and UVB irradiation in HaCaT cells

Prior to *in vitro* analysis of protective effect of santamarine against UVB-induced photoaging, the cytotoxic effects of treatment with various concentrations of santamarine and UVB intensity were examined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay.

To determine the UVB-induced phototoxic effects on HaCaT cell line, UVB irradiation was carried out within a range of 10 mJ/㎠ - 100 mJ/㎠. UVB radiation at higher than 20 mJ/㎠ significantly reduced the cell viability (Fig. 1B).

Therefore, an intensity at 20 mJ/㎠ was selected as appropriate

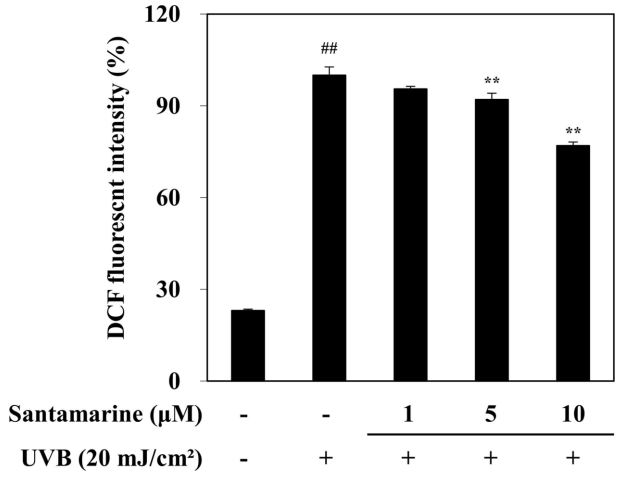

Fig. 2. The effect of santamarine on the intracellular ROS production in HaCaT cells treated with H_2O_2. ROS scavenging effects of santamarine was evaluated after 2 h treatment by the DCFH-DA assay. Data are shown as the means ± standard deviation ($n = 3$). $^{**}p < 0.01$ vs. H_2O_2 treated group. $^{\#\#}p < 0.01$ vs. non-treated group.

level for further experiments. When the cells were treated with santamarine, the cell viability was dose-dependently reduced. However, treatment with santamarine did not have any significant cytotoxicity up to a concentration of 10 μM (Fig. 1C).

2. Effect of santamarine on UVB-induced intracellular ROS generation in HaCaT cells

Excessive exposure to UVB causes oxidative stress, generates reactive oxygen species (ROS) including singlet oxygen, hydrogen peroxide, superoxide and hydroxyl free radicals and induces the activation of the MMP, which is directly related to the skin's collagen degradation.

The first aim of the study was to determine whether santamarine modulates the UVB-induced ROS production. Therefore, the intracellular ROS changes were evaluated using DCFH-DA, a ROS sensitive dye. Based on the results in Fig. 2, exposure to UVB considerably enhanced ROS generation by 77.0% compared with the non-irradiated cells, whereas treatment with santamarine greatly reduced the UVB-induced ROS generation in a dose-dependent manner. This result demonstrates that santamarine has antioxidant effect through its own ROS scavenging ability.

3. Effect of santamarine on MMP-1 secretion and MMPs expression in UVB-irradiated HaCaT cells

Since santamarine showed antioxidant effect on UVB-induced

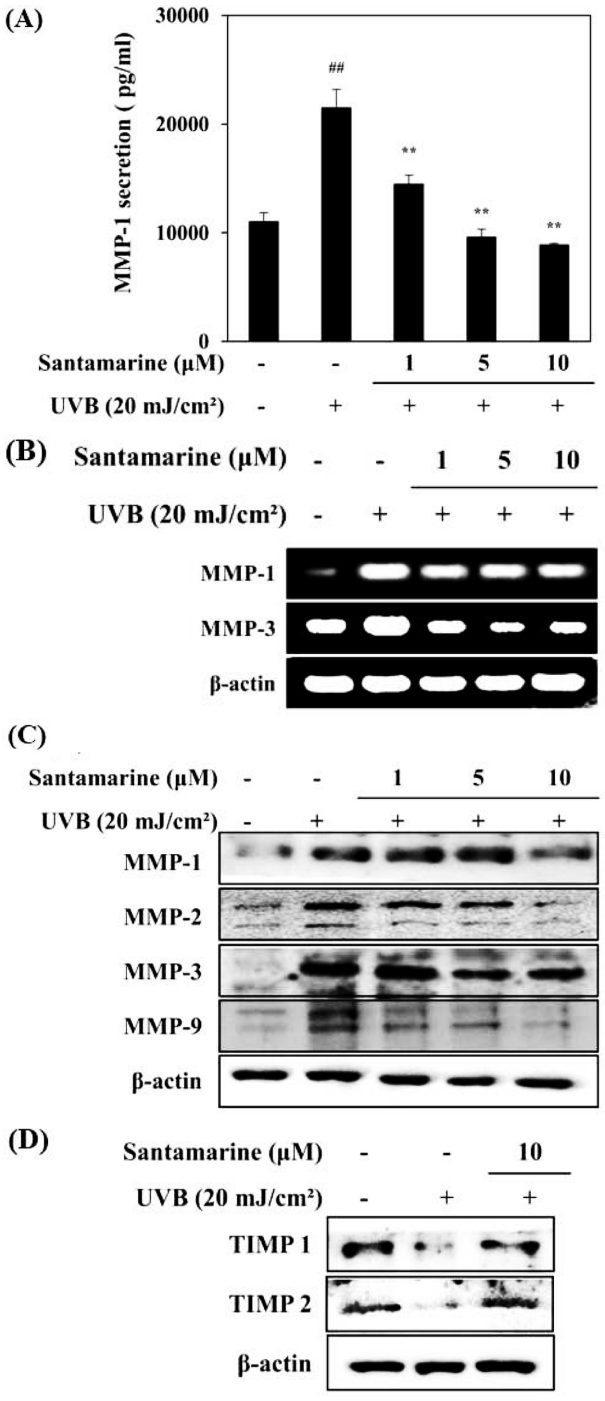

Fig. 3. The effect of santamarine on MMP-1 secretion (A), the mRNA levels (B) and the protein expression levels of MMPs and TIMPs (C and D) in UVB-irradiated cells, respectively. (A) MMP-1 secretion in UVB-irradiated HaCaT cells was analyzed by conducting ELISA following 24 h treatment. The mRNA and protein expression levels were detected by conducting reverse transcription-polymerase chain reaction and western blot, respectively. β-actin was used as an internal standard. Data are shown as the means ± standard deviation ($n = 3$). $^{**}p < 0.01$ vs. UVB treated group. $^{\#\#}p < 0.01$ vs. non-treated

oxidative stress, the effect of santamarine on the MMPs, which are provoked by ROS generation was addressed next.

As exhibited in Fig. 3A, exposure to UVB expectedly elevated MMP-1 secretion to 21,481.67 pg/㎖ from 11,021.67 pg/㎖ of non-treated blank group. Santamarine decreased the secretion of MMP-1 in UVB-irradiated cells in a dose-dependent manner. In particular, santamarine at 10 μM concentration lowered UVB-mediated MMP-1 release to 8,865.8 pg/㎖, which was a 58.7% decrease compared with the UVB-treated control group.

The result obtained from ELISA analysis was consistent with the mRNA and protein expression results. UVB irradiation stimulated the mRNA (Fig. 3B) and protein (Fig. 3C) levels of MMPs. The expression levels of MMP-1 and MMP-3 mRNA were dose-dependently inhibited by treatment with santamarine in UVB-irradiated cells. In parallel with the mRNA, santamarine downregulated the induction of MMP-1, MMP-2, MMP-3 and MMP-9 proteins in a dose-dependent fashion.

These findings speculated santamarine suppressed the UVB-mediated activation of MMPs through depletion of oxidative stress. Moreover, cells treated with 10 ìMsantamarine recovered the downregulated level of TIMP-1 and TIMP-2 proteins, evidencing the photoprotector capacity of santamarine.

4. Effect of santamarine on MAPKs and AP-1 pathway in UVB-irradiated HaCaT cells

In order to study the action mechanism behind the suppression of UVB-induced MMP regulation, the effect of santamarine on MAPKs signaling pathway was investigated with western blotting. Notably, santamarine downregulated the phosphorylation of p38, JNK and ERK compared to only UVB-irradiated cells.

This indicates that santamarine might inhibit the UVB-induced MMPs expression by suppressing the MAPKs pathway (Fig. 4A). UV radiation promotes MAPK pathway, which then activates its downstream transcription factor, AP-1.

The results seen in Fig 4B show that treatment with santamarine significantly reduced the protein levels of phosphorylated c-Fos and phosphorylated c-Jun, which form the AP-1 transcription factor complex. Thus, the inhibition of UVB-induced p38, JNK and ERK phosphorylation by santamarine is involved in the suppression of AP-1 activity in HaCaT cells, indicating that santamarine can suppress the photoaging-related signaling pathways.

5. Effect of santamarine on collagen Ⅰ in UVB-irradiated HaCaT cells

Next, to confirm the capability of santamarine to increase collagen production in UVB-irradiated HaCaT cells, fluorescent staining of cellular collagen was conducted. As seen in Fig. 5, UVB irradiation resulted in a considerable decrease in the collagen amount compared with the UVB-untreated group. However, treatment with 10 μM santamarine effectively enhanced the UVB-induced decrease in collagen amount.

DISCUSSION

MMPs play a predominant role in the physiological mechanisms

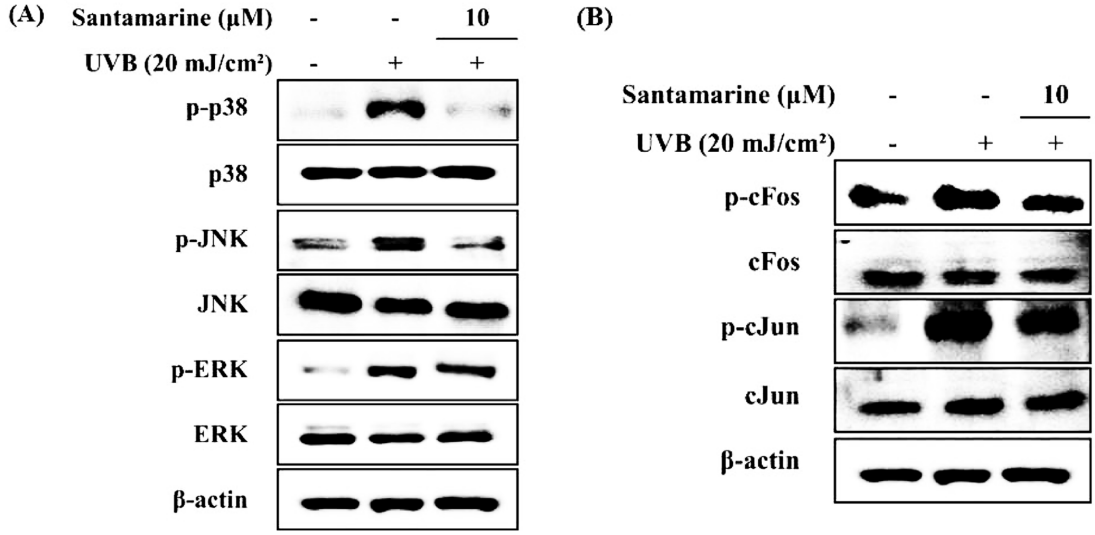

Fig. 4. The effect of santamarine on the phosphorylation of MAPKs (A) and AP-1 (B) signaling pathway in UVB-irradiated HaCaT cells. The protein expression levels were detected by western blot. β-actin was used as an internal standard.

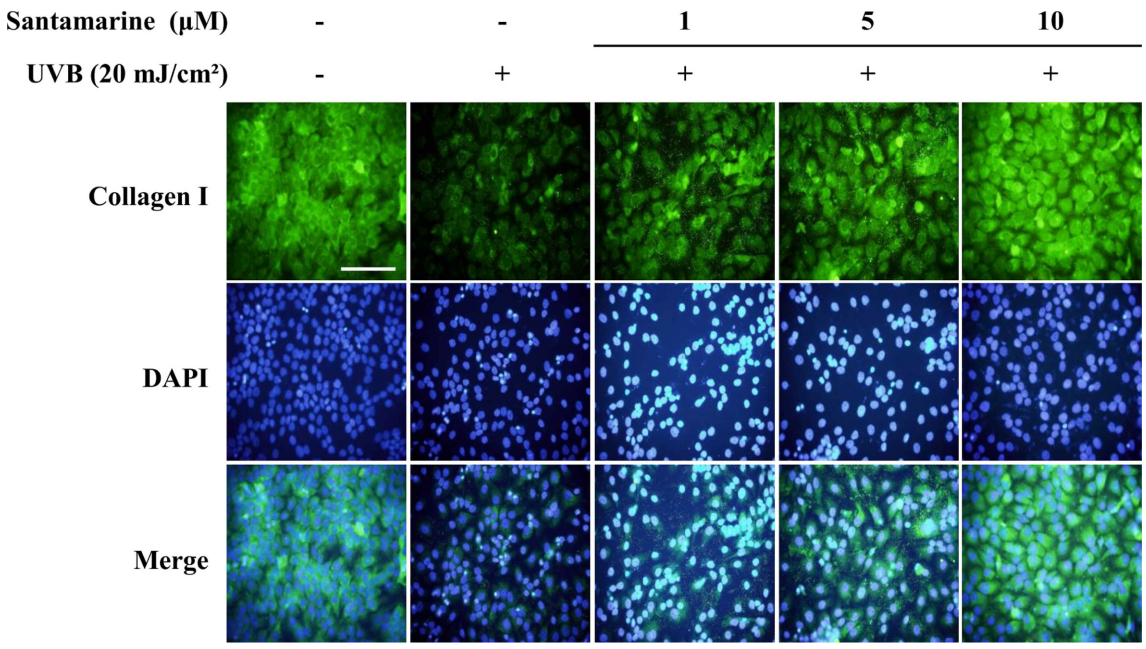

Fig. 5. The effect of santamarine on the collagen I in UVB-irradiated HaCaT cells. Intracellular production of collagen was observed by immunofluorescence through an overlay of green collagen Ⅰ staining, and blue DAPI staining. Scale bar, 100 ㎛.

of photoaging. UV rays react with the mammalian cells and induce the degradation of ECM proteins such as collagens, elastins, fibronectin, gelatin, and matrix glycoproteins by upregulation of MMPs (Visse and Nagase, 2003).

Therefore, regulation of UV-inducible MMPs has been studied to attenuate photoaging in terms of protection from ECM degradation. Of the various types of MMPs, MMP-1 is strongly upregulated in UV-damaged skin, and cleaves interstitial collagen I, II and III. After fragmentation by MMP-1, collagen peptides can be further digested by MMP-3 and MMP-9 (Fisher *et al*., 1996; Brennan *et al*., 2003).

A coordinated decomposition of ECM by MMPs is precisely controlled by endogenous TIMPs. Especially, TIMP-1 conjugates specifically to MMP-2, whereas MMP-9 conjugates to TIMP-1. Therefore, deterioration of the equilibrium between the MMP and TIMP synthesis, resulting in excess of MMPs, is thought to be a major phenotype of the photoaged human skin.

Accumulating evidence suggested that antioxidants suppress the activity of UV-induced MMPs (Kim *et al*., 2015; Sun *et al*., 2016; Xuan *et al*., 2017). Thus, the present study hypothesized that santamarine, which is known to have antioxidant capability (Choi *et al*., 2012), might reverse UVB-induced MMP induction.

In this study, treatment with santamarine markedly lessened ROS generation (Fig. 2), which may cause the inhibition of MMP-1 production (Fig. 3A) in UVB-irradiated cells. Prior studies showed that UV irradiation significantly enhanced the expression of MMPs such as MMP-1, MMP-2, MMP-3 and MMP-9 in human skin (Krengel *et al*., 2002; Quan *et al*., 2009). Our results indicated that treatment with santamarine quenched the UVB-induced increase of MMP-1 and MMP-3 at mRNA (Fig. 3B) level.

Furthermore, santamarine was shown to inhibit UVB-induced upregulations of MMP-1, MMP-2, MMP-3 and MMP-9 proteins (Fig. 3C). Relevant reports found that TIMPs irreversibly conjugate to MMPs to inhibit their activity as they participate in remodeling of ECM components (Visse and Nagase, 2003; Kim *et al*. 2012). Especially, TIMP-1 and TIMP-2 proteins are considered to be specific endogenous inhibitors that control the activity of MMP-2 and MM-9 (Kong *et al*., 2010).

As described in Fig. 3D, treatment with santamarine abolished the UVB-induced suppression on TIMPs expression. These data demonstrated that santamarine attenuated UVB-mediated MMPs expressions via enhanced TIMPsexpression. Overexpression levels of various MMPs are mainly regulated by MAPK/AP-1 signaling pathway which results in promoted collagen deficiency (Lu *et al*., 2018; Chaiprasongsuk *et al*., 2017).

This study confirmed that 10 μM santamarine effectively inhibited UVB-induced activation of MAPKsubfamilies such as p38, JNK and ERK (Fig. 4A). As a downstream effector, the

transcriptional activity of AP-1 which is composed by c-Fos and c-Jun is directly stimulated by the phosphorylation of p38, ERK and JNK. UVB-stimulated phosphorylation of c-Fos and c-Jun was lowered in santamarine treated cells (Fig. 4B).

AP-1-induced production of MMPs breaks down collagen and other ECM in human skin. Treatment with santamarine blocked the degradation of collagen in UVB-irradiated cells suggestively via suppressed activity of AP-1 complex (Fig 5). These findings suggest that santamarine inhibits UVB-induced MMPs expressions and degradation of collagen by suppressing MAPK/AP-1 pathway in human keratinocytes.

In conclusion, our study confirmed that the protective effect of santamarine against UVB-damaged keratinocytes through ROS scavenging, down regulating MMPs expressions and up regulating TIMPs proteins. The dominant action mechanism by santamarine was associated with inhibition of MAPK/AP-1 pathway. Therefore, santamarine isolated from *A. scoparia* should be viewed as a potential candidate against photoaging with its capability to protect skin against UVB damage. However further studies are needed to elucidate its anti-photoaging effects *in vivo*.

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