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자외선이 조사된 사람 피부 섬유아세포에서 권백의 항산화와 MMP 발현에 미치는 영향에 대한 연구

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Antioxidation and Inhibition of Matrix Metalloproteinase in UV-irradiated Human Dermal Fibroblast by *Selaginella tamariscina*

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요 약: 다양한 약용식물의 피부노화에 미치는 영향을 평가하였으며, 이 중 권백(*Selaginella tamariscina*)은 동양에서 암환자 치료를 위한 전통약용식물로 알려져 있다. 우리는 피부노화에 대한 화장품 소재로 권백에 대한 다양한 생물학적 평가를 하였다. 권백의 항산화 효과를 알아보기 위해 DPPH radical과 superoxide anion radical 소거효과를 측정하였다. 그 결과 DPPH radical의 IC_{50} 값은 65.1 μg/mL이고, xanthine/xanthine oxidase에 의한 superoxide anion radical의 IC_{50} 값은 40.9 μg/mL이었다. 세포내 활성산소 소거평가를 위해 사람 진피 섬유아세포(human dermal fibroblast)를 배양하여 UVB (20 mJ/cm^2)에 의해 증가된 세포내 활성산소(ROS)가 권백을 처리함으로써 활성산소 소거효과가 증가하였다. 사람 진피 섬유아세포에서 UVA에 의해 발현된 MMP-1 단백질과 mRNA가 권백에 의해 농도 의존적으로 감소하였다. 뿐만 아니라, 권백은 zymography와 semi-quantitative RT-PCR을 이용하여 UVA조사된 사람 진피 섬유아세포에 MMP-2 (gelatinase)의 활성 감소를 확인하였다. 결론적으로 권백은 자외선에 의한 세포손상을 보호하여 항노화 화장품의 새로운 소재로 이용될 것으로 사료된다.

Abstract: In this study, we evaluated anti-aging activity of medical plants that protect the skin cell damage induced by UV irradiation. We have investigated diverse biological activities of *Selaginella tamariscina* as an anti-aging ingredient of cosmetics. *S. tamariscina* was found to show scavenging activities of radicals and reactive oxygen species (ROS) with the IC_{50} values of 65.1 μg/mL against 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical and 40.9 μg/mL against superoxide radicals in the xanthine/xanthine oxidase system, respectively. For testing intracellular ROS scavenging activity, the cultured human dermal fibroblasts were analyzed by increase in dichlorofluorescein (DCF) fluorescence upon exposure to UVB 20 mJ/cm^2 after treatment of *S. tamariscina*. UVA-induced MMP-1 protein and mRNA expression in human dermal fibroblasts were reduced in a dose-dependent manner by *S. tamariscina*. Moreover, *S. tamariscina* inhibited MMP-2 (gelatinase) activity in UVA-irradiated human dermal fibroblasts assayed by zymography and semi-quantitative RT-PCR. Taken together, these results suggest that *S. tamariscina* may act as an anti-aging agent by increasing collagen and preventing the skin cell damage induced by UV irradiation, and imply that *S. tamariscina* may be useful as a new ingredient for anti-aging cosmetics.

Keywords: *Selaginella tamariscina, anti-aging, ROS, antioxidant activity, MMP*

1. Introduction

Selaginella tamariscina with the popular Korean name Keoun Back, is a traditional medicinal plant for therapy of advanced cancer patients in the Orient which has been shown to modify gene expression and cytokine production and to lower blood sugar and lipid peroxide levels. It has also been reported that biflavones isolated from related *Selaginella species* exert both tumoricidal effects against human tumor cell lines and anti-mutagenic activity[1].

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The matrix metalloproteinases (MMPs) are a family of 20 zinc-dependent endoproteinases that capable of degrading almost of the components of the extracellular matrix[2]. MMPs can be divided into four categories based on substrate preference: collagenases, gelatinases, stromelysins, and membrane-associated matrix metalloproteinases. Human fibroblasts have the capacity to produce several MMPs including interstitial collagenase (collagenase-1, MMP-1) and 72 kDa gelatinase (MMP-2). The expression of MMPs is low in unstimulated cells, but some are induced by various extracellular stimuli including growth factors, cytokines, tumor promoters, and ultraviolet. MMPs are necessary for tissue remodeling and the healing cascade under normal physiological condition. The aging process of skin can be divided into intrinsic aging and photoaging[3]. Clinically, naturally aged skin is smooth, pale and finely wrinkled. In contrast, photoaged skin is coarsely wrinkled. Alterations in collagen, the major structural component of skin, have been suggested as a cause of the changes, such as skin wrinkling and loss of elasticity, observed in naturally aged and photoaged skin[4].

To investigate the potentials as antioxidative and anti-wrinkle ingredients, the skin cell protection properties and various biological activities for anti-aging cosmetics were tested.

2. Material and Methods

2.1. Free Radical Scavenging Activity Assay (DPPH Test)

1,1-diphenyl-2-picrylhydrazyl (DPPH) is a stable free radical using determination of radical scavenging assay. The radical scavenging activity was determined by a previous report of Haraguchi *et al.*[5]. Then, its antioxidative activity (%) was calculated as compared with blank control.

2.2. Superoxide Radical Scavenging Activity assay (NBT Test)

Superoxide dismutase (SOD) activity was measured using xanthine-xanthine oxidase system as a source of superoxide and nitroblue tetrazolium (NBT) as a scavenger for this radical. SOD activity was determined as described by K. Furuno [6] by measuring percent inhibition of NBT reduced by SOD.

2.3. Cell Cultures and UV Irradiation

Human dermal fibroblasts (HDFs) from newborn foreskin were acquired from Korea Cancer Center Hospital. HDFs were maintained in Dulbecco's Modified Eagle's Media (DMEM)/F12 (3 : 1) with 10% fetal bovine serum (FBS) and kept in a humidified 5% CO_2 atmosphere at 37℃. HDFs from passage 6 to 10 were used in the experiments.

UV irradiation doses were 6.3 J/cm^2 (UVA), 20 mJ/cm^2 (UVB) and the radiation intensity was measured using UV radiometer (International light Inc., Newburyport, MA, USA).

2.4. Measurement of ROS Accumulation

HDFs (1.5×10^5 cells/mL) were seeded into 96-well plates and cultured overnight. 5-(and 6-) chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H_2 DCFDA), non-fluorescent compound, is able to react with free radical compound, especially with hydrogen peroxide and to generate fluorescent DCF. For the detection of ROS, HDFs were loaded with 4.0 μM CM-H_2DCFDA (Molecular Probe, Eugene, OR, USA) and incubated 37℃ for 30 min. Fluorescence was determined using a Luminescence spectrophotometer (Perkin Elmer, UK) using an excitation wavelength of 488 nm and emission wavelength of 525 nm.

2.5. Determination of MMP-1 by ELISA

HDFs (3×10^4 cells/well) were seeded into 48-well plates and cultured overnight. The culture media were replaced with DMEM/F12 (3 : 1) containing sample. After 24 h scultivation, MMP-1 secreted into the cultured medium was detected by enzyme-linked immunosorbent assay (ELISA) method.

2.6. Determination of MMP-2 by Zymography

Enzyme activity of MMP-2 was assayed by gelatin zymography according to the method of Herron *et. al.* [7]. The conditioned media of 1.5×10^5 cells were separated by electrophoresis on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) containing 0.1% gelatin. The electrophoresed gel was washed twice with washing buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 2.5% triton

X-100, followed by a brief rinsing in washing buffer without triton X-100. Then, the gel was incubated with incubation buffer of 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM $CaCl_2$, 0.02% NaN_3 at 37℃. After incubation, the gel was stained with coomassie brilliant blue solution for 10 min and destained with 10% acetic acid for 1 hr to visualize the bands of proteolytic activity.

2.7. Reverse Transcription Polymerase Chain Reaction (RT-PCR)

RNA was extracted using RNeasy MiNi Kit (Qiagen, Maryland, USA) according to the supplier's instruction. RT-PCR was performed using One Step RT-PCR kit (Qiagen, Hilden, Germany). PCR primers were produced by custom oligonucleotide synthesis service (Bioneer, Korea). Target gene mRNA levels were quantified based on standard and normalized to β-actin mRNA level.

2.8. Statistical Analysis

Results were presented as means ± S.E. Experimental results were statistically analyzed by using a Student't-test. P values < 0.05 were regarded as indicating significant differences.

3. Results and Discussion

3.1. Antioxidative Activity of *S. tamariscina*

Antioxidative activity was measured by NBT test and DPPH test. Figure 1 represents the results of DPPH test on *S. tamariscina* compared with other well-known antioxidants to measure free radical scavensing effect. The activity of *S. tamariscina* was comparatively as good as or higher than vitamin C. Figure 2 represents the results of NBT test on *S. tamariscina* to measure superoxide radical (O_2) scavenging effect and validate the correlation with DPPH test. As both of the results, *S. tamariscina* showed very effective radical scavenging activities.

3.2. Free Radical Scavenging Activity of *S. tamariscina* after UV Irradiation in HDF

For testing intracellular ROS scavenging activity, oxidative stress also analyzed by following the increase in DCF fluorescence upon exposure to UVB 20 mJ/cm^2.

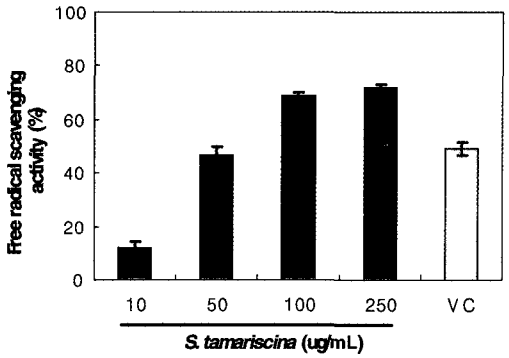

Figure 1. Antioxidant effect of *S. tamariscina* and vitamin C (VC, 60 μM) in the DPPH assay. The results were expressed as the average of triplicate samples with S.D.

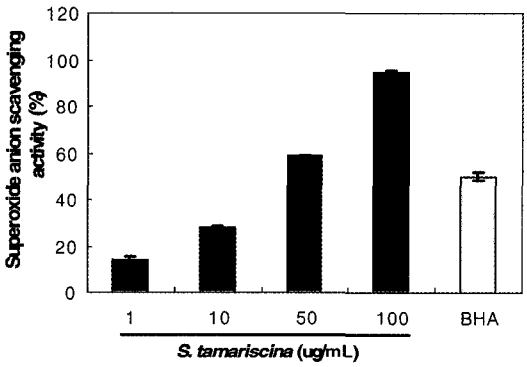

Figure 2. Antioxidant effect of *S. tamariscina* in the NBT assay. The results were expressed as the average of triplicate samples with S.D. (BHA, 0.2 mM 3-*t*-butyl-4-hydroxyanisole).

The basal and UVB-induced levels of fluorescence for CM-H_2DCFDA loaded HDFs after *S. tamariscina* treatment are shown in Figure 3.

3.3. *S. tamariscina* Reduced UVA-Induction of MMP-1 Expression

UV-irradiation damages human skin and causes premature skin aging (photoaging) through the activation of matrix metalloproteinases which are responsible for the degradation of collagen, gelatin and other components of the extracellular matrix[8].

To evaluate the anti-aging effect of *S. tamariscina*, we examined the inhibitory effects on expression of MMP-1. The results are summarized in Figure 4. The level of secreted MMP-1 was measured in cell media by an enzyme-linked immunsorbent assay (ELISA). Twenty four hours of exposure to 100 μg/mL *S.*

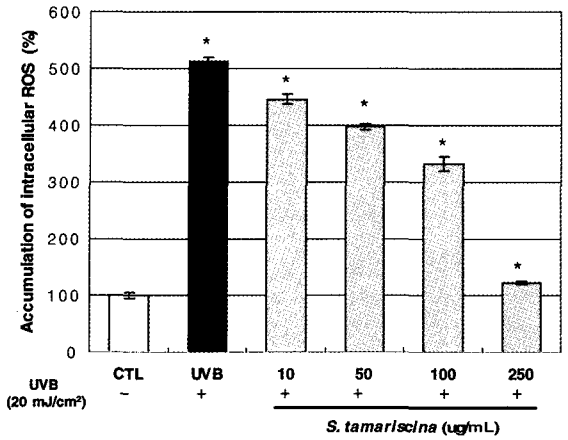

Figure 3. The effect of *S. tamariscina* on the production of intracellular reactive oxygen species (ROS) in human dermal fibroblasts. The values of intracellular ROS are significant ($*p < 0.05$). Values are expressed as mean ± S.E.M.

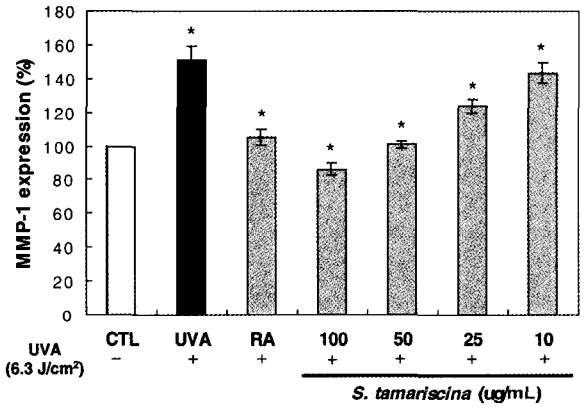

Figure 4. The effect of *S. tamariscina* on the production of MMP-1 by the UV irradiated human dermal fibroblast. The results were expressed as the average of triplicate samples with S.D. * $p < 0.05$ compared with control (RA, 3.5 μM retinoic acid).

tamariscina decreased the levels of MMP-1 by about 43%, compared to UV-irradiated control.

Several applications have been made to elucidate the influence of UVA irradiation on the stimulation of interstitial collagenase and gelatinase mRNA and their corresponding proteins in cultured HDF[9].

Thus, in the present study, the effect of *S. tamariscina* on UVA irradiation was examined by measuring the steady-state MMP-1 mRNA level in relation to β-actin mRNA levels. As shown in Figure 5, the expression of MMP-1 mRNA in UVA-irradiated HDF

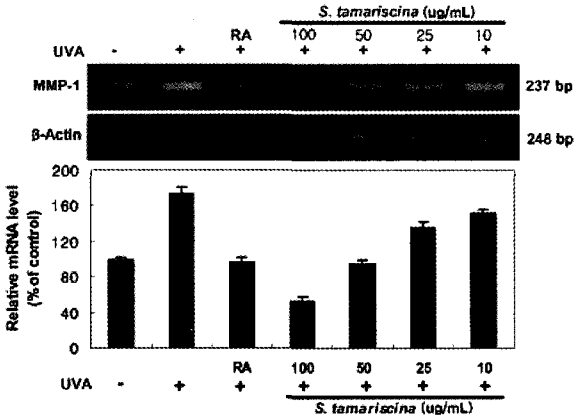

Figure 5. Effect of *S. tamariscina* on MMP-1 mRNA expression in human dermal fibroblast irradiated with UVA (RA, 3.5 μM retinoic acid).

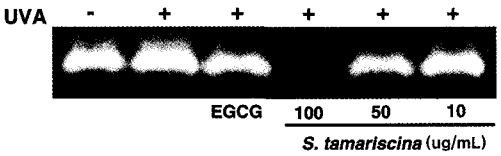

Figure 6. Effect of *S. tamariscina* on MMP-2 gelatinase activity from UVA irradiated human dermal fibroblasts (EGCG, 10 μM).

was significantly reduced by *S. tamariscina* in a dose-dependent manner while β-actin mRNA was in constant.

3.4. Effect of *S. tamariscina* on UVA-Induced MMP-2

The skin is the most susceptible organ to damage by UV irradiation as it is directly exposed to UV light. Molecular mechanisms of skin wrinkles are probably due to the loss of macromolecules making up the dermal matrix, among which collagen is the major component. In UV-irradiated skin, the level of MMPs that are important enzymes for the proteolysis of extracellular matrix proteins is elevated. Among them, MMP-2 (gelatinase A, 72 kDa) secreted as proenzyme, play an important role in degrading type IV collagen. Inhibition of induction of MMP has been reported to alleviate UV-induced photoaging by preventing from collagen destruction.

The effect of *S. tamariscina* on MMP-2 gelatinase activities was confirmed by zymography using supernatant from UVA irradiated HDF. MMPs, the super-

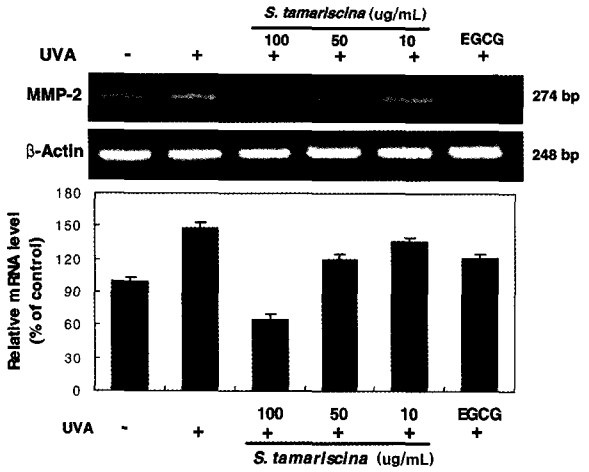

Figure 7. Effect of *S. tamariscina* on MMP-2 mRNA expression in human dermal fibroblast irradiated with UVA (EGCG, 10 μM).

natant from UVA irradiated HDFs were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 10% polyacrylamide gel containing 0.1% gelatin. The gels were regenerated, washed and incubated (18 h) with *S. tamariscina* (100 μg/mL, 50 μg/mL, 10 μg/mL) or EGCG (10 μM) in the incubation buffer. As shown in Figure 6, MMP-2 gelatinase activities were inhibited in a dose-dependent manner when *S. tamariscina* was added to the incubation medium. Also, MMP-2 mRNA expression was inhibited in a dose-dependent manner when *S. tamariscina* was added to the incubation medium (Figure 7). Therefore *S. tamariscina* was able to significantly inhibit MMP-2 expression in protein and mRNA level.

4. Conclusion

In this study, *S. tamariscina* showed effective free radical, superoxide radical scavenging activities and inhibition of ROS generation in UVB-irradiated cultured human dermal fibroblasts. *S. tamariscina* had potent inhibitory effect of MMP-1 and MMP-2 expression from UVA-irradiated human dermal fibroblasts.

This study demonstrated that *S. tamariscina* was able to prevent skin photoaging, and useful as an ingredient for cosmetics.

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