Protective Effects of Standardized *Siegesbeckia glabrescens* Extract and Its Active Compound Kireno l against UVB-Induced Photoaging through Inhibition of MAPK/NF-κB Pathways

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**Introduction**

Skin aging occurs because of exposure to extrinsic factors, such as ultraviolet (UV) irradiation, physical stimulation, and various chemicals; skin aging due to prolonged exposure to UV irradiation is termed photoaging. The photoaging symptoms include oxidative stress, dryness, wrinkles, and hyperpigmentation [1, 2]. Once UVB-induced reactive oxygen species (ROS) are formed in the skin, the extracellular matrix (ECM) components, including collagens, gelatins, elastic fibers, and glycosaminoglycans, are broken down and disorganized [3, 4].

Increased ROS in UVB-irradiated skin induce matrix metalloproteinase (MMP) expression and collagen degradation in the ECM, resulting in photodamaged skin. UVB-induced oxidative stress stimulates protein kinase cascades, such as mitogen-activated protein kinases (MAPKs), activated protein 1 (AP-1), and nuclear factor kappa B (NF-κB), ultimately enhancing MMP secretion and collagen degradation. Thus, ROS-scavenging antioxidants in natural materials have been proposed as photoprotective agents [5, 6].

The ECM in the skin is broken down by MMPs containing zinc-binding catalytic domains [7]. Depending on the substrates, MMPs can be categorized into various subgroups, including collagenases, gelatinases, and stromelysins. Collagenases (MMP-1 and -13) degrade native collagens, whereas gelatinases (MMP-2 and -9) cleave the basement membrane. On the other hand, stromelysins (MMP-3 and -10) confer broader substrate specificity than that of the first two subgroups mentioned [8].

UV-induced photoaging stimulates MMPs by several pathways, including the MAPK and NF-κB pathways [9]. UV irradiation activates three MAPKs: extracellular signal-regulated kinase (ERK), p38, and c-Jun N-terminal kinase (JNK) [10]. The MAPK activation results in the heterodimerization of c-Jun/c-Fos and the formation of the AP-1 complex, which promotes collagen degradation by regulating MMP transcription [11]. Furthermore, UV irradiation activates the NF-κB pathway, stimulating the
production of the proinflammatory cytokines, such as interleukin (IL)-6 and -8. Then, IL-6 and -8 can upregulate MMP expression, epidermal proliferation, inflammation, and various UV-induced cellular responses in human skin [12, 13].

Siegesbeckia glabrescens, a subspecies in Herba Siegesbeckiae, has been traditionally used to treat inflammation, arthritis, and hypertension [14]. It is known that S. glabrescens has antiasthma [15], anticancer [16], and antibacterial effects [17]; however, its anti-photoaging effect remains to be elucidated. Kirenol (Fig. 1) is a diterpenoid component found in Herba Siegesbeckiae [18], exhibiting anti-adipogenesis [19], antiarthritic [20], and anti-inflammatory effects [21]; however, its anti-photoaging activity remains unknown. Here, the anti-photoaging effects of S. glabrescens extract and kirenol were investigated in UVB-induced photoaging in vitro and in vivo model, respectively.

Materials and Methods

Preparation of Standardized S. glabrescens Extract (SGE)

The edible aerial parts of S. glabrescens were purchased from Kyungdong market (Korea). Dried edible aerial parts were ground and extracted with 95°C hot water for 3 h. Then, a vacuum rotary evaporator (Laborata 4000-efficient; Heidolph Instruments GmbH & Co. KG., Germany) was used to concentrate the extract with a yield of 12% (w/w), including 2% (w/w) of kirenol as a bioactive compound.

Chemical Reagents

Kirenol was obtain from the Natural Product Bank of the Institute for Korea Traditional Medical Industry (Korea). Dulbecco’s modified Eagle’s medium (DMEM) was supplied from Hyclone Laboratories, Inc. (USA). Fetal bovine serum (FBS) was supplied from Gibco (USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and protease inhibitor cocktails were purchased from Sigma-Aldrich (USA). Primary antibodies directed against MMP-1, -2, -9, phospho- and total-ERK, phospho- and total-JNK, phospho- and total-p38, phospho- and total-c-jun, c-Fos, catalase, NF-κB, and α-tubulin were purchased from Cell Signaling Technology (USA). Horseradish peroxidase-linked secondary anti-rabbit and anti-mouse antibodies were purchased from Bethyl Laboratories (USA).

Animal Experiments

Fifteen 5-week-old female albino hairless mice (SKH-1; Daehan Biolink Ltd., Korea) were maintained in a humidity-, temperature-, and light-controlled specific pathogen-free room (55 ± 10% humidity, 23 ± 2°C, and 12 h light/dark cycle) at Yonsei Laboratory Animal Research Center (Korea). All protocols for animal experiments were in compliance with the guideline of the Institutional Animal Care and Use Committee at Yonsei University (Permit No. 201404-147-02). The hairless mice were randomly grouped into three groups (5 mice/group): (i) normal group (Normal), (ii) UVB-irradiated control group (UVB), and (iii) UVB-irradiated and 600 mg/kg/day SGE treatment group (UVB + SGE 600). Mice received SGE daily for 13 weeks by oral gavage and were concurrently exposed to UVB irradiation on their dorsal surface. UVB irradiation was carried out as described in our previous publication [9]. UVB radiation was performed using a CL-1000M UV Crosslinker (UVP, USA) which delivered UVB energy at a wavelength of 302 nm. Mice were exposed to UVB radiation at a dose of 75 mJ/cm² during the first week. The dose of UVB radiation was increased weekly from 1 minimal erythema dose (MED) to 3 MEDs, which was maintained until the end of the experiment. The mice were intraperitoneally euthanized with a 1:1 mixed solution of rompun and zoletil (One Bio, Korea). The dorsal skin sections of the hairless mice were collected and kept at −80°C.

Evaluation of Skin Wrinkle Formation

Replicas of mouse dorsal skin were prepared using the Silflo kit (CuDerm Corporation, USA) and analyzed with Visioline VL650 (Courage + Khazaka Electronic GmbH, Germany). The skin wrinkle parameters were measured in terms of total wrinkle area, length, depth, and number.

Evaluation of Skinfold Thickness and Skin Elasticity

Skinfold thickness of the dorsal skin was measured at the mid-back level with a caliper (Ozaki MFG Co., Ltd., Japan) after 13 weeks. The dorsal skin of hairless mice was picked up by finger by pinching at the neck and base of the tail, and skinfold thickness was measured at the mid-back level. More than three measurements were taken at close proximity, and the mean value was calculated. Skin elasticity was measured with the Cutometer MPA580 (CK Electronics GmbH).

Histological Analysis

Skin samples of the mice were fixed with 10% formalin for 24 h. Hematoxylin and eosin (H&E) staining for measuring skin thickness,
Masson’s trichrome (M&T) staining for measuring collagen fiber, and Verhoeff-van Gieson’s staining for measuring elasticity were performed on skin tissue sections. The stained skin area was examined using the Eclipse TE2000U Inverted Microscope with twin CCD cameras (Nikon, Japan).

**Hydroxyproline Assay**

The hydroxyproline content was determined using a hydroxyproline content assay kit of Quickzyme Biosciences (Netherlands), according to the manufacturer’s instructions.

**Cell Culture and UVB Irradiation**

Hs68 human skin fibroblasts (American Type Culture Collection, USA) were maintained in DMEM supplemented with 10% FBS and 1% antibiotics (penicillin and streptomycin) under a humidified atmosphere of 5% CO₂ at 37°C. The fibroblasts were pretreated in a serum-free medium with or without kirenol for 24 h. After pretreatment, the fibroblasts were exposed to 302 nm UVB light (25 mJ/cm²). Western Blot Analysis

Equal 30 μg amounts of protein were size-fractionated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to membranes. The membranes were incubated for 1 h in blocking solution with Tris-buffered saline with Tween-20 containing 5% skim milk. They were then incubated overnight with primary antibodies at 4°C. The membranes-bound primary antibodies were again incubated with horseradish peroxidase-linked secondary anti-rabbit or anti-mouse antibodies for 2 h. Antibody signals were visualized with ECL detection solution (GE Healthcare, Sweden) and were detected using the G:BOX Image Analysis System (Syngene, UK).

**Cell Viability Assay**

Cell viability was assessed by MTT assay. Hs68 human skin fibroblasts were treated to various doses of kirenol (0 to 60 μM). No significant cytotoxicity was shown at various concentrations of kirenol ranging from 10 to 40 μM (data not shown). The following experiments were conducted with or without kirenol in serum-free medium for 24 h.

**Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis**

Total RNA of homogenized skin sections and Hs68 cells was isolated with Trizol reagent (Invitrogen, USA). cDNA was synthesized with 1 μg of total RNA, oligo(dT), and Reverse Transcription Premix (ELPIS-Biotech, Korea) in a 20 μl reaction mixture. The sequences of primers were as follows: mouse COL1A1 (forward, 5'-AGC ACA GGA GCA GGT GTA GA-3'; reverse, 5'-TAG CAG GTT ATC GTA AA-3'), mouse MMP-13 (forward, 5'-CAT CCA TCC CTT GTC CCT-3'; reverse, 5'-GAC TGA CTC TCA CAA TGC GA-3'), mouse β-actin (forward, 5'-GCT CCG GCA TGT CTT GCG-3'; reverse, 5'-AGG ATC TTC ATG AGG TAG T-3'), human COL1A1 (forward, 5'-GGG GGA GCC CCA CAC AT-3'; reverse, 5'-TAC GGG CAA AAA CCG CCA GC-3'), human COL3A1 (forward, 5'-GCC GAC TCA GCC TCC TGT GGG TG-3'; reverse, 5'-ACC TTC TGG GCA GTA AAC CCG AA-3'), human IL-6 (forward, 5'-ATG AGG AGA CTT GCC TGG TG-3'; reverse, 5'-ACA ATA TCA TGA GGT GCC CA-3'), human MMP-13 (forward, 5'-CAC GGA AGA AAC CAG AAC AA-3'; reverse, 5'-CCT CTT CAC CCA CTT TGT CT-3'), human GAPDH (forward, 5'-GCA TGA CTC TCA CAA T-3'), and human β-actin (forward, 5'-GCT CCG GCA TGT CTT GCG-3'; reverse, 5'-ACC TTC TGG GCA GTA AAC CCG AA-3').

**Statistical Analysis**

Descriptive results are reported as the mean ± standard deviation (SD). Statistical analysis was performed with SPSS21.0 (SPSS, Inc., USA). The differences of groups were evaluated by unpaired Student’s t-test for in vivo data. One-way analysis of variance followed by Schelke’s test was performed for in vitro data. *p < 0.01, **p < 0.05, and ***p < 0.01 were considered to be statistically significant.

**Results and Discussion**

**Effect of SGE on Wrinkle Formation and Skin Thickness In Vivo**

UV-irradiated skin exhibits clinical and histological
changes, including wrinkling, elastosis, and skin thickening. Clinical symptoms of UV-irradiated skin photoaging include dryness, roughness, hyperpigmentation, coarse elastosis, and laxity [9]. This study investigated whether SGE protects UVB-induced photoaging by assaying histological and clinical changes in hairless mice. UVB irradiation increased the formation of wrinkles in the dorsal skin of control mice; however, SGE treatment significantly reduced it, as seen in the UVB + SGE 600-treated mice (Fig. 2A). To quantify wrinkle formation, we measured the total wrinkle area, length, depth, and number (Fig. 2B). UV irradiation stimulates MMP production and inhibits collagen synthesis in the ECM, which results in increasing skin thickness by excessive epidermal proliferation and wrinkle formation by degradation of dermal ECM components [22]. In contrast, SGE treatment led to decreased abnormal skinfold thickness (Fig. 2C) and inhibition of abnormal elastic fiber accumulation compared with those observed in the UVB-irradiated control group, thereby preventing a decrease in skin elasticity (Fig. 2D). Thus, SGE could be beneficial in the prevention of photoaging by reducing wrinkle formation and skin thickness, and preventing abnormal elastic fiber accumulation.

Effect of SGE on Collagen Synthesis and MMP Expression In Vivo

Collagen, a predominant component of the skin’s connective tissue, regulates functional properties of skin in the dermis [9]. In the present study, UVB markedly decreased collagen in the UVB-irradiated mice, as indicated by the decreased hydroxyproline contents (total collagen levels) (Fig. 3A) and the reduced blue-stain section (Fig. 3B), compared with that in the normal mice, whereas SGE treatment significantly increased collagen synthesis. The
mRNA expression of COL1A1, COL3A1, COL4A1, and COL7A1 was remarkably elevated in the SGE-treated mice compared with that in the control group (Fig. 3C). UVB irradiation increased the protein levels of MMP-2 and -9 and the mRNA levels of MMP-3 and -13, in the control group, whereas SGE treatment inhibited MMP protein and mRNA expression (Figs. 3D and 3E). Most collagen fibers in the dermis primarily consist of type I and III collagens, which are degraded by MMP-3 and -13. Type VII collagen, which is crucial in forming the dermal epidermal junction, is cleaved by MMP-3 [4, 9]. In contrast, types IV and VII collagens are degraded by MMP-2 and -9 [9]. SGE greatly suppressed MMP-3 expression in UVB-damaged skin of hairless mice. MMP-3 is a known activator of other MMPs, such as MMP-1, -2, and -9 [23], suggesting that SGE has the potential to inhibit the activation of downstream MMPs. Thus, the protective effect of SGE against the collagen degradation may be regulated by the suppression of MMP expression in UVB-irradiated skin tissue.

**Effect of SGE on Catalase and the MAPK/NF-κB Pathways In Vivo**

Accumulation of intracellular ROS by UVB irradiation causes inactivation of major antioxidant enzymes, such as catalase, glutathione peroxidase, and superoxide dismutase [24, 25]. UVB irradiation reduced catalase expression in the UVB-irradiated control mice, whereas SGE treatment significantly increased the expression of catalase (Fig. 4A). The antioxidant enzyme catalase is a key differentiation marker in the epidermal layer of UVB-induced oxidative skin, playing a preventive role against photooxidative stress [9, 26]. Thus, SGE may act as an effective ROS scavenger by increasing the catalase expression.

This study showed that the SGE treatment group
Fig. 4. Effect of *Siegesbeckia glabrescens* extract (SGE) treatment on catalase and the MAPK/NF-κB pathways in vivo. Mice received SGE (600 mg/kg/day) for 13 weeks by oral gavage and were concurrently exposed to UVB irradiation on their dorsal surface. The protein expression of (A) catalase, (B) p-ERK, t-ERK, p-JNK, t-JNK, p-p38, and t-p38, (C) p-c-Jun, t-c-Jun, c-Fos, and (D) NF-κB was assessed by western blotting. α-Tubulin was utilized as a loading control for protein levels. Data represent the mean ± SD from five mice per group. *p < 0.01 (Normal vs. UVB control) and **p < 0.01 (UVB control vs. SGE-treated mice).

Fig. 5. Effect of kirenol treatment on collagen synthesis and MMP expression in vitro. Human dermal fibroblasts were pretreated with or without kirenol for 24 h. The cells were exposed to UVB light (25 mJ/cm²) and then additionally treated with or without kirenol for 24 h. (A) mRNA expression of COL1A1, COL3A1, and COL7A1 assessed by RT-PCR. (B) Protein expression of MMP-1, -2, and -9 assessed by western blotting. GAPDH was utilized as a loading control for mRNA levels and α-tubulin for protein levels. Data represent the mean ± SD from triplicate experiments. *p < 0.01 (Normal vs. UVB control); *p < 0.05 and **p < 0.01 (UVB control vs. kirenol-treated cells).
demonstrated lower UVB-induced phosphorylation of MAPK components, including ERK, JNK, and p38, than the control group (Fig. 4B). Furthermore, the SGE treatment decreased the protein levels of AP-1 complex components, such as c-Jun and c-Fos, compared with the UVB-irradiated control (Fig. 4C). Wrinkle formation is in close relation with MMP production regulated by c-Jun and c-Fos, which are components of the AP-1 complex. Activated JNK simulates the transcriptional activity of c-Jun through phosphorylation [22].

SGE dramatically suppressed the UVB-induced inflammatory pathway by downregulating NF-κB expression (Fig. 4D). UVB-induced inflammation is another mechanism by which the skin photoages. It is well evidenced that oxidative stress induces NF-κB associated with MMP expression in the skin [9]. It was reported that *S. glabrescens*, which has been long used as an herbal medicine to treat inflammatory diseases, inhibited inducible nitric oxide synthase and cyclooxygenase-2 expression in lipopolysaccharide-activated RAW 264.7 macrophages [27]. Accordingly, it is conceivable that SGE prevents UVB through activation of the MAPK and NF-κB pathways.

**Effect of Kirenol on Collagen Synthesis and MMP Expression In Vitro**

Collagen degradation in UV-damaged skin is a result of the upregulation of MMP expression, leading to an aged appearance of skin [28]. In the present study, UVB reduced the mRNA levels of COL1A1, COL3A1, and COL7A1, whereas kirenol significantly increased the expression of these collagen genes in Hs68 human dermal fibroblasts (Fig. 5A). UVB increased the protein levels of MMP-1, -2, and -9; however, kirenol reduced these levels dose-dependently (Fig. 5B). Because MMPs are crucial in the pathophysiological photaging mechanisms of skin, natural biomaterials inhibiting MMP expression in UV-damaged skin are desirable.

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**Fig. 6.** Effect of kirenol treatment on catalase and the MAPK/NF-κB pathways in vitro.

Human dermal fibroblasts were pretreated with or without kirenol for 24 h. The cells were exposed to UVB light (25 mJ/cm²) and then additionally treated with or without kirenol for 24 h. The protein expression of (A) catalase, (B) p-ERK, t-ERK, p-JNK, t-JNK, p-p38, and t-p38, (C) p-c-Jun, t-c-Jun, c-Fos, and (D) NF-κB was assessed by western blotting. (E) mRNA expression of IL-6 and IL-8 assessed by RT-PCR. GAPDH was utilized as a loading control for mRNA levels and α-tubulin for protein levels. Data represent the mean ± SD from triplicate experiments. *p < 0.05 (Normal vs. UVB control); **p < 0.01 and ***p < 0.001 (UVB control vs. kirenol-treated cells).
skin can successfully prevent photoaging [9, 22]. Thus, kirenol can mediate its anti-photoaging activity by promoting collagen synthesis and inhibiting MMP expression.

**Effects of Kirenol on Catalase and MAPK/NF-κB Pathways In Vitro**

Protein expression of catalase was reduced after UVB irradiation; however, catalase was highly elevated after kirenol treatment, indicating that kirenol has strong antioxidant activity (Fig. 6A). These data suggest that kirenol can mediate protection against photoaging, in part, by upregulation of catalase. As shown in Fig. 6B, kirenol reduced the phosphorylation of ERK, JNK, and p38 induced by UVB. The protein levels of c-Jun and c-Fos were markedly upregulated in the control cells, whereas kirenol significantly decreased the expression of c-Jun and c-Fos (Fig. 6C). The UVB-irradiated control cells expressed high levels of inflammatory mediators (NF-κB, IL-6, and IL-8); however, kirenol effectively inhibited their expression dose-dependently (Figs. 6D and 6E). These data demonstrate that kirenol protects fibroblasts from UVB-induced inflammation. It was reported that kirenol was also found to confer anti-inflammatory activity, as it reduced proinflammatory cytokine secretion and increased anti-inflammatory cytokine production of type II collagen-proinflammatory cytokine secretion and increased anti-inflammatory activity, as it reduced levels of inflammatory mediators (NF-κB).

This study was the first to demonstrate that SGE, and its active compound kirenol, prevents UVB-induced photoaging by regulating MMP expression and collagen degradation, in addition to suppressing oxidative stress and the associated intracellular MAPK/NF-κB pathways. It is anticipated that *S. glabrescens* and the active compound kirenol can be employed as effective natural anti-photoaging agents. Further human studies are required to clarify whether SGE is clinically effective as a natural anti-photoaging supplement.

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**References**


