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Effect of *Scutellaria baicalensis* extract on skin barrier function via peroxisome proliferator-activated receptor-α

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Abstract–*Scutellaria baicalensis* extract (SBE) was investigated to determine its effects on the transactivation activity of peroxisome proliferator-activated receptors (PPAR)-responsive element (PPRE) and anti-oxidant activity for improvement of skin barrier function. The treatment with SBE resulted in a significant increase in the transactivation activity of PPRE such as PPAR-α and effect in anti-oxidant and anti-microbial activity. In addition, SBE promotes the expression of protein related to cornified envelope (CE) formation such as involucrin. Therefore, these results indicate that SBE can promote keratinocyte differentiation and restore skin barrier homeostasis, and is suggested to be an appropriate skin therapeutic agent for improving epidermal permeability barrier function.

Keywords: Peroxisome Proliferators Activated Receptors, Anti-oxidative Activity, *Scutellaria baicalensis* Extract

INTRODUCTION

Skin barrier lies in the outermost layer of the epidermis, in the stratum corneum (SC), which consists of two major structural components, the corneocytes and intercorneocyte lipids [1]. Thus, the formation of the SC, layers of terminally differentiated cornified cells in the outermost epidermis, is responsible for the barrier properties of the skin [2]. Xerosis (dry skin), which is believed to be one of the major diseases of modern society, has been proved to be the most important cause of skin barrier dysfunction [3]. Although moisturizers such as ceramides or their derivatives have been developed for their moisture holding capacity and have been widely used in the cosmetic industry, they provide only a temporary relief of symptoms rather than being a fundamental treatment option [4]. Therefore, the development of materials that regenerate after barrier dysfunction is urgently needed.

Peroxisome proliferator-activated receptors (PPAR) are ligand-activated transcription factors that belong to the steroid nuclear receptor family. Three different PPARs have been identified in mammals: PPAR-α, PPAR-β/δ, and PPAR-γ. PPAR-α is expressed in many tissues, including the heart, kidney, liver and epidermis, where it is an important regulator of lipid metabolism [5,6]. Among its isoforms, PPAR-α has an important role in the regulation of differentiation, the regulation of inflammatory mediators, cell proliferation, metabolism of glucose, lipids, and hormones [7,8]. Thus, PPAR-α agonists have been extensively studied in keratinocytes differentiation and in the epidermal permeability barrier [9], and it has been demonstrated that topical treatment with PPAR ligands promotes differentiation in the murine epidermis [10]. Moreover, the topical treatment with PPAR-α agonists restores epidermal homeostasis in the event of essential fatty acid deficiency and in permeability barrier disruption models [11]. The selective PPAR-α agonists WY14643, fenofibrate, and clofibrate increase the expression of cornified envelope (CE)-associated proteins such as involucrin, filaggrin, and transglutaminase etc [12]. Therefore, a search for a new bioactive natural product is required to determine whether activators of PPAR-α can alter the rate of keratinocyte differentiation.

S. baicalensis has been known as medicinal plants for treatment of inflammation, cancer, infectious diseases, hypercholesterolemia, and hypertension [13,14]. However, compared to many pharmacological studies, the effect of *S. baicalensis* extract on skin barrier function as skin therapeutic agent has not been reported. To investigate new bioactive characteristics of *S. baicalensis*, we performed supercritical carbon dioxide extraction (SCE) on *S. baicalensis*. SCE can provide several benefits compared to conventional solvent extraction: faster extraction time, improvement of the yield, a low environmental impact, and in the optimum process for obtaining extracts with high quality [15]. We examined PPAR-α, anti-oxidative, anti-microbial activity, and expression of protein related to CE formation with *S. baicalensis* extract (SBE). We report here that SBE is a potent stimulator for epidermal skin homeostasis.

MATERIALS AND METHODS

1. Sample Preparation and Supercritical Carbon Dioxide Extraction

S. baicalensis (Roots) in Jellanam-do Korea were prepared. For SCE, the supercritical carbon dioxide extraction system and components were acquired from ILSHIN Co. (Daejeon, Korea) series supercritical fluid extractor, included the following: 500 mL extraction vessel, temperature control unit, high-pressure pump, back pressure regulator. The natural plant resources were dried for 24 h and milled to 200 meshes. The extractor was filled with a measured quantity of milled natural plant resources, and carbon dioxide was pumped into the extractor up to a pressure of 400 bar at a flow rate of 30 mL/min to 60 mL/min. After ensuring the pressure, a steady stream of butylene glycol was allowed to pass upward through the bed of

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ground particles at a predetermined pressure at 50 °C. Raw materials were extracted from a separator for 5 h, and the extracted raw materials were dissolved in the mixture of purified water and butylene glycol (7 : 3, v/v) at 40 °C. An appropriate amount of soluble extract was used in this experiment.

2. Cell Culture and Materials

Normal human keratinocytes were purchased from Cascade Biologics (Portland, OR, USA) and maintained in EPI-500 medium containing human keratinocyte growth factor (Gibco-BRL/Life Technologies, Grand Island, NY, USA). Normal human fibroblasts were cultured in 106 medium with low serum growth factor (Gibco-BRL/Life Technologies, Grand Island, NY, USA). CV-1 and Hep3B cells were purchased from Korea Cell Line Bank (KCLB), Seoul, Korea) and cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco-BRL/Life Technologies, Grand Island, NY, USA) and RPMI medium, respectively with 10% fetal bovine serum (FBS), antibiotics (62.5 μg/mL penicillin and 100 μg/mL streptomycin sulfate) in a humidified atmosphere of 5% CO_2 at 37 °C. RAW264.7 cells were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA) and maintained in DMEM with 5% heat-inactivated FBS, antibiotics (62.5 μg/mL penicillin and 100 μg/mL streptomycin sulfate) in 5% CO_2 atmosphere. The medium was renewed twice weekly. Cells were treated using *S. baicalensis* extracts, the PPAR-α activator WY14643, respectively (Sigma-Aldrich Co., St. Louis, MO, USA). All the other reagents used were of the highest purity.

3. Cell Cytotoxicity

Cytotoxicity was determined using the lactate dehydrogenase (LDH) detection assay, according to manufacturer's instruction (Clontech, Mountain View, CA, USA). Normal human keratinocytes were plated in triplicate wells of 96-well plates at a density of 4×10^3 per well, and cultivated for 24 h. The media was replaced with degassed serum-free media, and the samples were treated using 10, 50, 100 mg/L SBE and 2% Triton X-100 as a positive control. Then, the samples were incubated at 37 °C for 24 h. After the incubation period, the medium was harvested and the supernatant from each well was transferred to corresponding wells on the new plate. The reaction solution was added to each well and the cells were incubated at room temperature for 30 min. Absorbance was then measured at 490 nm by using a spectrophotometer.

4. Anti-oxidant Assay

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay was used to determine the anti-oxidant capacity of SBE. The DPPH radical scavenging activity is generally quantified in terms of inhibition percentage of the pre-formed free radical by anti-oxidants and the EC_{50} (concentration required to obtain a 50% anti-oxidant effect) is a typically employed parameter to express the anti-oxidant capacity [16]. Ascorbic acid was used as an anti-oxidant standard to define the EC_{50} parameters.

5. DNA Constructs, Transient Transfection, and PPRE Transactivation Assay

PPAR-α transcription activity was performed using the PPRE transactivation method [17] with slight modifications. A commercial PPAR-α expression vector was purchased from Promega (Mannheim, Germany) and transformed into *Escherichia coli* competent cells. PPAR-α DNA constructs were extracted using a DNA preparation kit (Qiagen, Hilden, Germany). CV-1 cells were prepared at a density of 4×10^4 per well and cultured as described in the Materials and methods. The reporter construct (SA Bioscience, Hilden, Germany) and PPAR-α expression vector were co-transfected into CV-1 cells by using Lipofectamine (Invitrogen, Carlsbad, CA, USA). Transactivation assays were performed using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA), and normalized luciferase activity was determined.

6. Immunohistochemical Analysis

The effect of *S. baicalensis* extracts on involucrin synthesis was analyzed using EpiDerm™, a three-dimensional model of skin equivalents purchased from MatTek Corporation (Ashland, MA, USA). The skin equivalents were stabilized in EPI-100-New maintenance medium (NMM, MatTek Co. Ashland, MA, USA) for 24 h and cultured in DMEM (Gibco-BRL/Life Technologies, Grand Island, NY, USA). The medium was changed and treated with 50 mg/L of *S. baicalensis* extracts. After 12 days of culture, mature skin equivalents were harvested for immunohistochemical studies. The skin fragments were embedded in paraffin, and placed at 60 °C for 1 h. The skin fragments were exposed twice with xylene for 10 min, twice with 100% ethanol for 5 min, and twice with 70% ethanol for 5 min, followed by incubation for 10 min with Tris-buffered saline (TBS). After incubation, the fragments were placed with 3% H_2O_2 for 30 min and then washed three times with TBS. The skin fragments were stained with rabbit anti-involucrin antibody (Santa Cruz Biotechnology, Carlsbad, CA, USA) for 1 h at 37 °C. After reaction, the fragments were washed three times with TBS, followed by incubation for 1 h at room temperature with horseradish peroxidase (HRP)-conjugated secondary antibodies. After extensive washing, the fragments were placed in 3,3′-diaminobenzidine (DAB) buffer containing DAB chromogen (Dako, Glostrup, Denmark) for 2 min and then stopped in distilled water. The prepared samples were observed by microscope (Carl Zeiss Inc., Oberkochen, Germany). The area was analyzed by involucrin with image quantitative analysis software (NIH images, version 1.61.).

7. Anti-microbial Assay

Staphylococcus aureus strains were used for anti-microbial assays and were first grown in LB (Luria-Bertani) broth to an $OD_{600\,nm}$ of 0.8. A 10 μL aliquot of the strain was then taken and added to 8 mL of fresh LB broth with 0.7% agar and poured over a 90 mm Petri dish containing 25 mL of 1.5% agar in LB broth. After the top agar hardened, WY14643 and SBE were dissolved in 10% DMSO, and then a 15 μL aliquot of the control (10% DMSO), WY14643, and SBE filtered on a 0.22 μm Millipore filter was dropped onto the surface of paper disc and incubated overnight at 37 °C. If the sample examined had anti-microbial activity, a clear zone would form around paper disc, representing inhibition of strains growth.

8. Statistical Analysis

Data are presented as means±standard error of the mean (SEM) from more than three separate experiments performed in triplicate. The representative experiment is depicted at instances where results of the blots are shown. Multiple groups were compared by using one-way analysis of variance (ANOVA) with Bonferroni's test. Statistical significance was defined as $P<0.01$.

RESULTS AND DISCUSSION

1. Effect of SBE on Cell Cytotoxicity

To investigate normal human keratinocyte cytotoxicity on SBE,

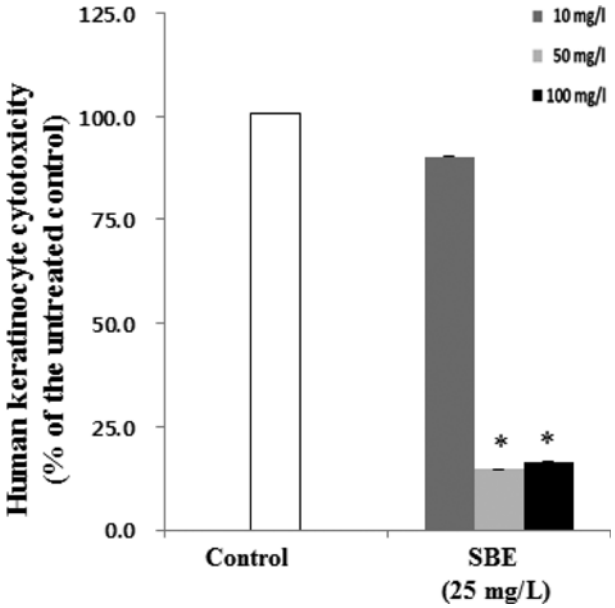

Fig. 1. Human keratinocyte cytotoxicity of SBE. Normal human keratinocytes cultured in medium containing SBE were measured at 490 nm using spectrophotometer with LDH enzymes. A solution of 2% triton-X 100 was treated as a positive control. SBE was treated at 10, 50, and 100 mg/L concentration. Values are presented as mean±S.E.M. *P<0.01 compared to the control group.

LDH activity was measured. As shown in Fig. 1, the cytotoxicity of keratinocytes in the SBE-treated groups (50, and 100 mg/L) was lower than that in the untreated control group. No significant difference was observed between the control group and 10 mg/*l* doses.

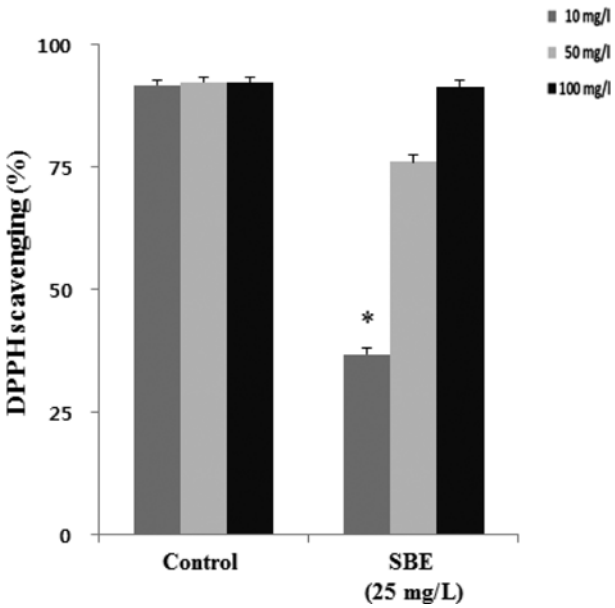

Fig. 2. Anti-oxidant effect of SBE. Anti-oxidant effects were confirmed using a DPPH radical scavenging method. Ascorbic acid was used as a positive control. Ascorbic acid and SBE were treated at 10, 50, and 100 mg/L concentration. Values are presented as mean±S.E.M. *P<0.01 compared to the control group.

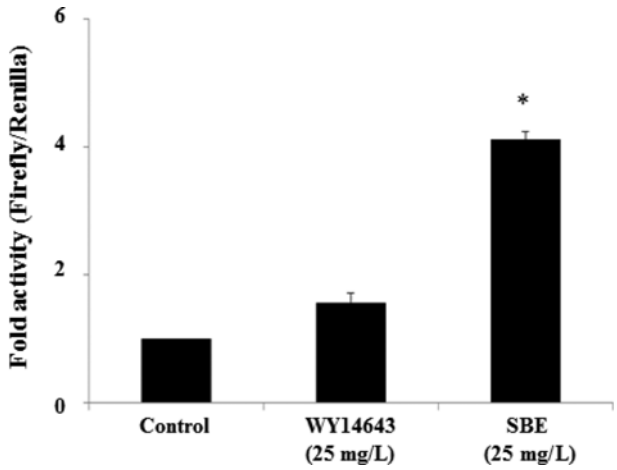

Fig. 3. Transactivation of a PPAR response element (PPRE) by SBE. A CV-1 cell line was transfected with PPRE luciferase. WY14643 was used as a positive control and no treatment was used as a negative control. WY14643 and SBE were treated at 25 mg/L concentration. Values are presented as mean±S.E.M. *P<0.01 compared to the control group.

The IC_{50} value of SBE was 41.2 mg/L. We used concentrations in these ranges (below 25 mg/L) with sufficiently low cytotoxicity for the following experiments. Our results showed that SBE has low cell cytotoxicity for utilization as cosmeceuticals.

2. Anti-oxidant Effect of SBE

To determine the anti-oxidant capacity for SBE, a 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay was used. The radical scavenging activities of SBE increased according to concentration. SBE had highly anti-oxidant activity depending on the concentration (Fig. 2). The EC_{50} value of SBE was 22.9 mg/L. The anti-oxidant effect of SBE was not significantly low compared to ascorbic acid as a positive control. These results indicate that SBE has the anti-oxidant capacity.

3. Transactivation of PPRE

We determined the transactivation activity of PPAR-responsive element (PPRE) on SBE. Renilla and firefly luciferase activities were measured using a luminescence spectrometer. As shown in Fig. 3, the SBE largely influenced PPAR-α ligand binding activity, and 25 mg/L SBE-treated group produced a significant increase compared to the untreated control. Additionally, the group treated SBE showed high level of transactivation activity compared to the group treated using WY14643. It has been reported that PPAR-α activity plays an important role in healing skin wounds, and PPAR-α agonists such as WY14643 and clofibrate have been confirmed to promote differentiation of keratinocytes and recovery of the skin barrier [10]. Our results indicate that PPAR-α was increased by treatment of SBE.

4. Effect of SBE on Expression of Involucrin in a 3D Skin Equivalent Model

Immunohistochemical analysis of protein marker related to keratinocyte differentiation was determined. Involucrin is an essential CE component that can be used as biomarker for screening of new candidates for the improvement skin barrier function. As shown in Fig. 4, the treatment by SBE (50 mg/L) showed a significant increase of involucrin expression compared with treatment by 0.2%

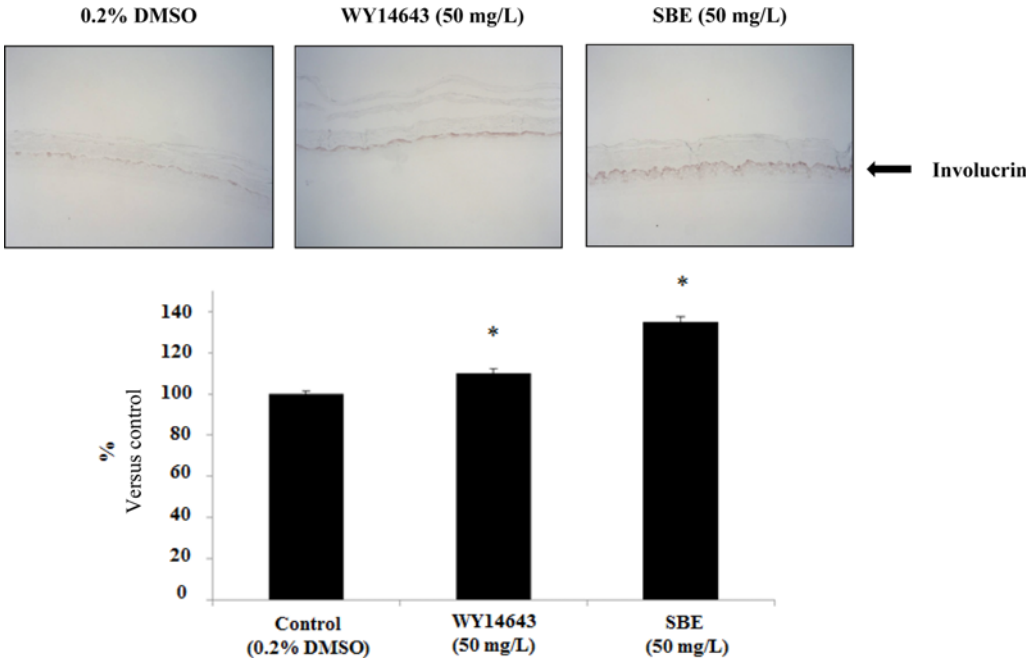

Fig. 4. Immunohistochemical analysis of involucrin in a 3D skin equivalent model. 0.2% DMSO was treated as a control. WY14643 and SBE were treated at 50 mg/L concentration. The skin fragments were subjected to immunohistochemical analysis by using anti-involucrin antibody and observed by microscope. The area was analyzed by involucrin with image quantitative analysis software (NIH images, version 1.61.).

DMSO as a control in skin equivalents, In addition, the group treated with SBE showed similar level in the expression of involucrin compared to the group treated with the same concentration of WY14643. These results indicate that SBE showed similar activities as those by the synthetic compounds.

5. Anti-microbial Effect of SBE

The skin of atopic dermatitis patients has a high susceptibility to *Staphylococcus aureus* [18]. As shown in Fig. 5, a clear zone was only formed in the SBE-treated group. These results indicate that SBE has the anti-microbial capacity. It has been reported that extract of *S. baicalensis* has anti-viral activity by inhibiting virus replication [19]. Therefore, SBE may be used as skin therapeutic agent.

Many studies have shown that natural plants have positive biological effects such as therapeutic agents [20]. However, compared to many pharmacological studies, the effect of *S. baicalensis* extract on skin barrier function as skin therapeutic agent has not been reported. We suggest that SBE can promote keratinocyte differentiation and restore skin barrier homeostasis such as skin therapeutic agent.

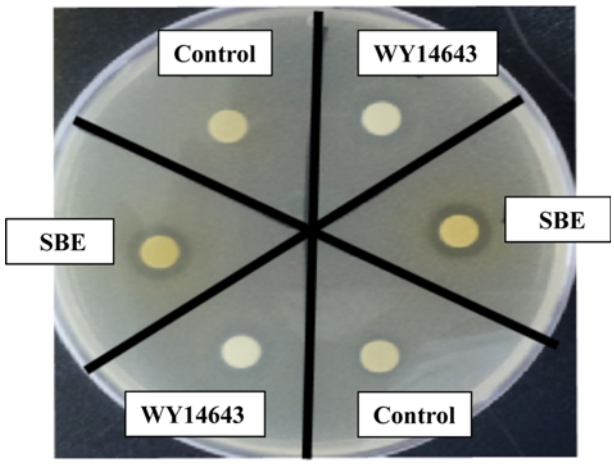

Fig. 5. Anti-microbial effect of SBE. *Staphylococcus aureus* strains were used for anti-microbial assays. 10% DMSO was used as a negative control. WY14643 and SBE were dissolved in 10% DMSO, and then a 15 μL aliquot of the control (10% DMSO), WY14643, and SBE filtered on a 0.22 μm Millipore filter was dropped onto the surface of a paper disc and incubated overnight at 37 °C. A clear zone was examined around the paper disc.

CONCLUSIONS

We investigated the effects of *S. baicalensis* roots extract by supercritical carbon dioxide extraction (SCE) method on the function of the epidermal permeability barrier and on the activity of PPAR-α, anti-oxidative, anti-microbial activity, and expression of protein related to CE formation. SBE possessed a high PPAR-α, anti-oxidative, and anti-microbial activity. Moreover, the expression of involucrin by SBE was a significant high in a 3D skin equivalent model. These results suggest that SBE can be an appropriate candidate for epidermal permeability barrier recovery, and this candidate may have advantages as it is a natural compound with no adverse effects on human skin. Consequently, SBE may be an appropriate material for improving skin barrier function as a cosmeceutical.

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