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Anti-oxidative and skin barrier effects of natural plants with a supercritical extract

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초임계 추출을 적용한 식물추출물의 항산화 및 피부장벽 효과

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Abstract

In this study, we searched for bioactive compounds from natural resources with a supercritical extract. We selected the extracts of *Chrysanthemum zawadskii, Lufa cylindrica, Paeonia lactiflora, Gardenia jasminoides* and *Scutellaria baicalensis*, as natural materials, and evaluated the effects of their skin barrier function. We found that these extracts increased the transactivation activity of the PPAR-responsive element (PPRE) and the anti-oxidation with different priorities, respectively. In addition, these extracts promoted the expression of proteins related to cornified envelope (CE) formation, such as involucrin. From these results, we suggest that natural materials from supercritical extracts will be pertinent candidates for the improvement of the epidermal permeability barrier function.

Key words : peroxisome proliferators activated receptors, anti-oxidation, supercritical extract

Introduction

Natural resources are currently the focus of a great deal of attention as functional materials, which are used for food, drug, and cosmetics. The epidermis is a stratified squamous epithelium that acts as a barrier against chemical, physical and biological agents (1). This 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 (2). 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 (3).

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 (4,5). 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 (6,7). Thus, PPAR-α agonists have been extensively studied in keratinocytes differentiation and in the epidermal permeability barrier, and it has been demonstrated that topical treatment with PPAR ligands promotes differentiation in the murine epidermis (8). 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 (9). 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. Therefore, a search for a new bioactive natural product is required to determine whether activators of PPAR-α can alter the rate

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of keratinocyte differentiation.

In order to identify natural products for the improvement of skin barrier function, we performed supercritical carbon dioxide extraction (SCE) on natural products. 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 anti-oxidant quality (10). In the present study, we extracted 5 native plant resources: *Scutellaria baicalensis* (roots) *Chrysanthemum zawadskii* (leaves, stems) *Lufa cylindrica* (fruits) *Gardenia jasminoides* (fruits) *Paeonia lactiflora* (roots) in Jellanam-do Korea using the SCE method, and evaluated cell cytotoxicity, anti-oxidant effects, PPAR-α activities and expression of protein related to CE formation as biomarker.

Materials and Methods

Sample preparation and supercritical carbon dioxide extraction

Scutellaria baicalensis (roots) *Chrysanthemum zawadskii* (leaves, stems) *Lufa cylindrica* (fruits) *Gardenia jasminoides* (fruits) *Paeonia lactiflora* (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 hr 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 ground particles at a predetermined pressure at 50℃. Raw materials were extracted from a separator for 5 hr, and the extracted raw materials were dissolved in the mixture of purified water and butylene glycol (7:3, v/v) at 40℃. An appropriate amount of soluble extract was used in this experiment.

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). CV-1 cells were purchased from Korea Cell Line Bank (KCLB, Seoul, Korea) and cultured in Dulbecco's Modified Eagle's Medium (Gibco-BRL/Life Technologies) 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℃. The medium was renewed twice weekly. Cells were treated using 5 natural materials, the PPAR-α activator WY14643, or $CaCl_2$, respectively (Sigma-Aldrich Co., St. Louis, MO, USA). All the other reagents used were of the highest purity.

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 hr. The media was replaced with degassed serum-free media, and the samples were treated using 5 natural materials and 2% TritonX-100 as a positive control. Then, the samples were incubated at 37℃ for 24 hr. 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.

Anti-oxidant assay

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay was used to determine the anti-oxidant capacity of 5 natural materials. 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 (11). Ascorbic acid was used as an anti-oxidant standard to define the EC_{50} parameters.

DNA constructs, transient transfection, and PPRE transactivation assay

PPAR-α transcription activity was performed using the PPRE transactivation method (12) 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) and normalized luciferase activity was determined.

Immunoblot analysis

Normal human keratinocytes pretreated with 5 native plant resources were cultured for 48 hr. Cells were washed once in PBS and lysed in a lysis buffer (Cell Signaling, Danvers, MA, USA). Protein concentrations were determined using the Bradford protein assay kit (BioRad, Hercules, CA, USA). Proteins were separated on a 12% SDS-polyacrylamide gel and transferred to PVDF membranes (Millipore Co., Billerica, MA, USA). After blocking the membrane with Tris-buffered saline and Tween 20 (TBS-T, 0.1% Tween 20), containing 5% non-fat dried milk, for 1 hr at room temperature, membranes were washed twice with TBS-T and incubated with primary antibodies for 1 hr at room temperature or overnight at 4℃. The following primary antibodies were used: rabbit anti-involucrin (Santa Cruz Biotechnology, Carlsbad, CA, USA), and mouse anti-β-actin (Sigma-Aldrich Co.). The membrane was washed 3 times with TBS-T for 10 min, followed by incubation for 1 hr at room temperature with horseradish peroxidase (HRP)-conjugated secondary antibodies. After extensive washing, the bands were detected by enhanced chemiluminescence (ECL) reagent (Santa Cruz Biotechnology). β-actin was used as the standard for normalizing protein samples. The band intensities were quantified using a Photo-Image System (Molecular Dynamics, Uppsala, Sweden).

Statistical analysis

Data are presented as means±standard deviation (SD) from more than 3 separate experiments performed in triplicate. The representative experiment is depicted at instances where results of the blots are shown. Comparisons between multiple groups were performed using one-way analysis of variance (ANOVA) with Bonferroni's test. Statistical significance was defined as $p<0.01$.

Results and Discussion

Effect of 5 native plant resources on cell cytotoxicity

To investigate normal human keratinocyte cytotoxicity on 5 native plant resources, LDH activity was measured. The result indicated that the cytotoxicity of keratinocytes in the extracts-treated groups was high depending on the concentration (Fig. 1). The IC_{50} values of *Chrysanthemum zawadskii, Lufa cylindrica, Paeonia lactiflora, Gardenia jasminoides,* and *Scutellaria baicalensis* were 29.9, 154.0, 46.8, 28.9, and 41.2 mg/L, respectively.

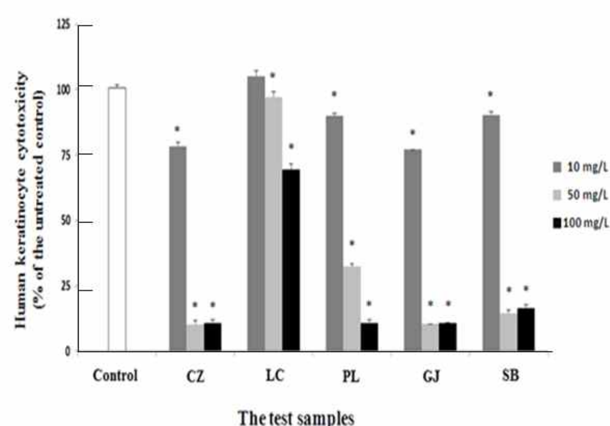

Fig. 1. Human keratinocyte cytotoxicity of 5 natural materials extracts.

Normal human keratinocytes cultured in medium containing the 5 extracts were measured at 490 nm using spectrophotometer with LDH enzymes. A solution of 2% triton-X100 was treated as a positive control. CZ, *Chrysanthemum zawadskii*; LC, *Lufa cylindrica*; PL, *Paeonia lactiflora*; GJ, *Gardenia jasminoides*; SB, *Scutellaria baicalensis*. Values are presented as mean±SD *$p<0.01$ compared to the control group.

Anti-oxidant effect of 5 native plant resources

To determine the anti-oxidant capacity for the raw materials extracted, a 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay was used. As shown in Fig. 2, the radical scavenging activities of the 5 natural materials increased according to concentration. In particular, *Scutellaria baicalensis* had the highest anti-oxidant activity. The EC_{50} values of *Chrysanthemum zawadskii, Lufa cylindrica, Paeonia lactiflora, Gardenia jasminoides,* and *Scutellaria baicalensis* were 140.1, no effect, 168.1, 586.7, and 22.9 mg/L, respectively.

Transactivation of PPRE

We determined the transactivation activity of PPAR-responsive element (PPRE) on 5 native plant resources. Renilla and firefly luciferase activities were measured using a luminescence spectrometer. These results showed that the PPAR-α activities of the 5 natural extracts increased according

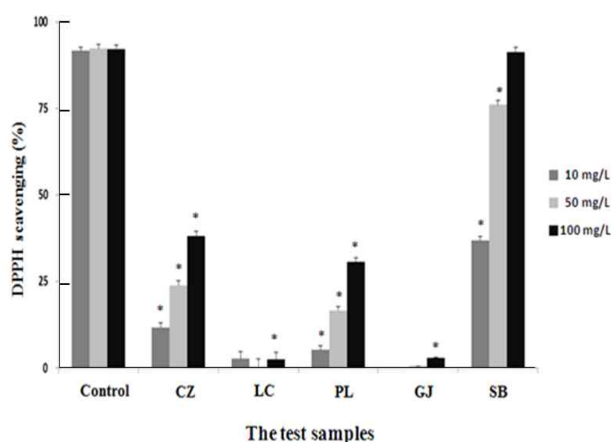

Fig. 2. Anti-oxidant effects of 5 natural materials extracts.

Anti-oxidant effects were confirmed using a DPPH radical scavenging method. Ascorbic acid was used as a positive control. CZ, *Chrysanthemum zawadskii*; LC, *Lufa cylindrica*; PL, *Paeonia lactiflora*; GJ, *Gardenia jasminoides*; SB, *Scutellaria baicalensis*. Values are presented as mean±SD. *p<0.01 compared to the control group.

to the concentration in the following order: *Scutellaria baicalensis> Chrysanthemum zawadskii > Lufa cylindrica > Gardenia jasminoides> Paeonia lactiflora* (Fig. 3). These results indicate that the 5 natural extracts have higher PPAR-α activities related to the differentiation stage of the keratinocytes. In present, it is known that PPAR-α activated in combination with a ligand promotes the differentiation of keratinocytes and reconstructs the damaged skin barrier. Among the PPAR isoforms, PPAR-α has an important function in the regulation of differentiation, as well as in the regulation of inflammatory mediators, cell proliferation, and metabolism of glucose, lipids, and hormones (6). In addition, 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 (8,12). Therefore, we attempted to identify new natural materials as PPAR-α agonists, and these 5 natural extracts can be potential candidates for epidermal permeability barrier recovery, furthermore, they may have an advantage as natural compounds which have no adverse effects on the human skin with a low environmental impact.

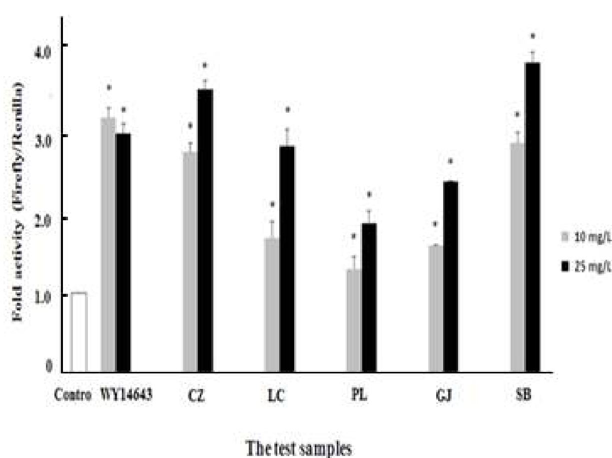

Fig. 3. Transactivation of a PPAR response element (PPRE) from 5 natural materials extracts.

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. CZ, *Chrysanthemum zawadskii*; LC, *Lufa cylindrica*; PL, *Paeonia lactiflora*; GJ, *Gardenia jasminoides*; SB, *Scutellaria baicalensis*. Values are presented as mean±SD. *p<0.01 compared to the control group.

Expression of involucrin related to keratinocyte differentiation

We also confirmed changes in the protein expression related to CE formation by determining the expression of involucrin. As shown in Fig. 4, in the untreated group, the protein expression levels of involucrin were increased by 5 native plant resources in the following order: *Scutellaria baicalensis> Paeonia lactiflora > Lufa cylindrica > Gardenia jasminoides> Chrysanthemum zawadskii,* but the levels were somewhat lower than that in the group treated using 1.2 mM $CaCl_2$. These results indicate that 5 native plant resources significantly activate epidermal barrier homeostasis related to CE formation. These results suggest that these materials can be appropriate candidates for epidermal permeability barrier recovery, and furthermore, that these candidates may have advantages as it is a natural compound with no adverse effects on human skin. The epidermal barrier is composed of stratum corneum (SC) and intact epidermal layers that

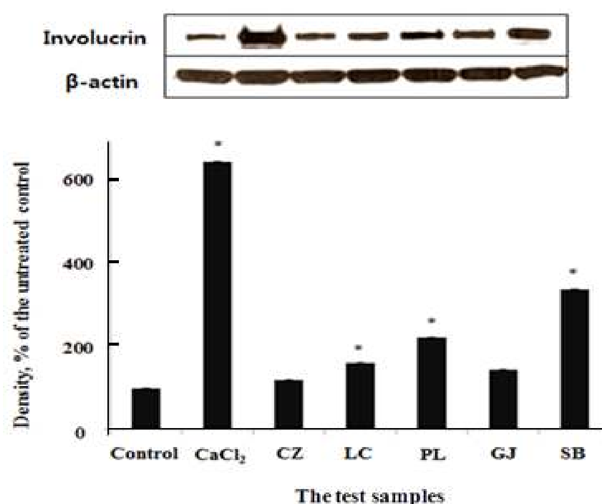

Fig. 4. Involucrin expression rate by western blot analysis.

Each signal was quantified by scanning densitometry. β-Actin was used as an internal standard. 1.2mM CaCl2 was used as a positive control and no treatment was used as a negative control. 10mg/L of the 5 natural extracts was treated. CZ, *Chrysanthemum zawadskii*; LC, *Lufa cylindrica*; PL, *Paeonia lactiflora*; GJ, *Gardenia jasminoides*; SB, *Scutellaria baicalensis*. Values are presented as means±SD. *p<0.01 compared to the untreated group.

differentiate into corneocytes of the SC as a first level of the skin barrier, which are regulated by specific gene such as involucrin, bind to the keratin to form CE. Additionally, increase expression of this protein occurs during barrier repair (13). Therefore, involucrin is an essential cell envelope component and can be used as biomarker for screening of new natural materials for the improvement skin barrier function. This study demonstrated that natural materials by SCE may be potential materials for the improvement of epidermal permeability barrier function. In further studies, attempts will be made to investigate the efficacy of 5 natural extracts in vivo.

요 약

본 연구에서는 다양한 식물추출물의 항산화성 및 미용관련 기능성을 알아보기 위하여 기존의 용매추출에 비해 안전성이 높은 것으로 알려진 초임계 추출방식을 적용하여 구절초(*Chrysanthemum zawadskii*), 수세미(*Lufa cylindrica*), 작약(*Paeonia lactiflora*), 치자나무(*Gardenia jasminoides*) 및 황금(*Scutellaria baicalensis*)의 천연식물 초임계 추출물을 확보하고, 이들의 항산화력 및 피부장벽 향상 효과를 조사하였다. 초임계 식물추출물의 피부장벽기능 향상 효과를 조사하기 위하여 항산화 활성 외 proliferator-activated receptor(PPAR)-α 활성, cornified envelope(CE)에 관련된 단백질인 involucrin의 발현량을 측정하였다. 이들 추출물 중 황금추출물이 가장 높은 항산화 활성을 보였으며, 모든 추출물에서 대조군과 비교하여 유의적으로 높은 수준의 PPAR-α 활성을 나타내었다. 또한, 피부장벽 기능 지표 단백질인 involucrin 발현량도 모든 추출물에서 높게 나타났으며, 황금추출물이 가장 높은 단백질 발현 양상을 보였다. 따라서 본 연구에서 조사한 5개 식물의 초임계 추출물은 항산화 및 피부장벽 기능개선과 같은 기능성 생물활성 소재로 활용 될 수 있을 것으로 판단된다.

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