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Chrysanthemum zawadskii extract activates peroxisome proliferator-activated receptor-α and has an anti-inflammatory activity : Potential interest for the skin barrier function

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Abstract−*Chrysanthemum zawadskii* extract (CZE) was investigated to determine its effects on the transactivation activity of peroxisome proliferator-activated receptors (PPAR)-responsive element (PPRE) and activity of anti-inflammatory for improvement of skin barrier function. The treatment with CZE resulted in a significant increase in the transactivation activity of PPRE such as PPAR-α and suppression in the TNF-α-, IL-6-induced NF-κB luciferase activity and NO production. In addition, CZE promotes the expression of protein related to cornified envelope (CE) formation such as involucrin. Therefore, these results indicate that CZE can restore skin barrier homeostasis and is suggested to be an appropriate skin therapeutic agent.

Keywords: Peroxisome Proliferators Activated Receptors, Anti-inflammation, *Chrysanthemum zawadskii* Extract

INTRODUCTION

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]. 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. 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. Therefore, the development of materials that regenerate barrier dysfunction is urgently needed.

Peroxisome proliferator-activated receptors (PPAR) are ligand-activated transcription factors that belong to the superfamily of nuclear receptors. There are three different PPARs such as 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]. PPAR-α binds to a diverse set of ligands, namely, arachidonic acid metabolites (prostaglandins and leukotrienes) and plasticizers and synthetic fibrate drugs, including WY14643, fenofibrate, and clofibrate [10]. The selective PPAR-α agonists WY14643, fenofibrate, and clofibrate increase the expression of cornified envelope (CE)-associated proteins such as involucrin, TGase-1, and others. The CE completes the epidermal permeability barrier during the terminal differentiation of keratinocytes [11-13]. Therefore, a search for new agonists is required to determine whether activators of PPAR-α can alter the rate of keratinocyte differentiation.

Inflammation is a normal physiological and immune response to tissue injury. Increased blood supply, enhanced vascular permeability, and migration of immune cells occur at damaged sites [14]. The inflammatory process is a protective response that occurs in response to trauma, infection, tissue injury, or noxious stimuli [15, 16]. Inflammation is a complex process regulated by the activation of various immune cells. Specifically, macrophages play a central role in mediating many different immunopathological phenomena during inflammation, including the overexpression of pro-inflammatory cytokine and inflammatory mediators [17,18] such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), nitric oxide (NO), prostaglandin E_2 (PGE_2), and regulation on activation. The expression of these inflammatory mediators can be regulated by activation of the transcription factor nuclear factor kappa-B (NF-κB), which plays a critical role in regulating the expression of various genes, including the cytokines iNOS and COX-2 [19]. Since NF-κB plays a ubiquitous role in the pathogenesis of inflammatory gene expression, the cytokine is a current target for the treatment of various diseases [20]. Therefore, a search for a new bioactive material is required to determine whether activators of PPAR-α and anti-inflammatory activity can improve skin barrier function.

Chrysanthemum zawadskii has been known as traditional medicine for treatment of pneumonia, bronchitis, cough, gastroenteric disorders, and hypertension [21]. However, compared to many phar-

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macological studies, there are no researches for the improvement of skin barrier function. To investigate new bioactive characteristics of *C. zawadskii*, we performed supercritical carbon dioxide extraction (SCE) on *C. zawadskii*. 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 [22]. Natural resources are currently the focus of a great deal of attention as functional materials, which are used for food, drug, and cosmetics. Previous studies showed that the methanolic extract *C. zawadskii* induced an anti-carcinogenic biomarker enzyme for protection liver cells [23] and protected osteoblastic cells highly reducing sugar-induced oxidative damage in rat [24]. However, the effect of *C. zawadskii* extract on skin barrier function as skin therapeutic agent has not been reported. We investigated the effects of *C. zawadskii* leaves extract by supercritical carbon dioxide extraction (SCE) method on the PPAR-α activity, anti-inflammatory activity, and alteration of human keratinocyte differentiation.

MATERIALS AND METHODS

1. Sample Preparation and Supercritical Carbon Dioxide Extraction

C. zawadskii (leaves) 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, including 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 (200 g), 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. The dynamic extraction time was fixed to 150 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 °C with slight modifications [25]. 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

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 *C. zawadskii* extracts, the PPAR-α agonist WY14643, respectively (Sigma-Aldrich Co., St. Louis, MO, USA). The PPAR-γ agonist pioglitazone hydrochloride was negative control (Tokyo chemical Industry Co., LTD. Tokyo, Japan). All the other reagents used were of the highest purity.

3. Anti-oxidant Assay

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay was used to determine the anti-oxidant capacity of CZE. The DPPH radical scavenging activity is generally quantified in terms of inhibition percentage of the preformed free radical by anti-oxidants and the SC_{50} (concentration required to obtain a 50% scavenging effect) is a typically employed parameter to express the anti-oxidant capacity [26]. Ascorbic acid was used as an anti-oxidant standard.

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

PPAR-α transcription activity was performed using the PPRE transactivation method [27] with slight modifications. A commercial PPAR-α expression vector was purchased from Promega (Manheim, 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 without serum. 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.

5. MTT Assay

The MTT assay was used to evaluate the effects of CZE on cell viability. RAW 264.7 cells were plated in 96-well plates at a density of 1×10^4 cells/well for 24 h without serum. The cells were treated with varying concentrations of CZE (5, 10, 25, 50, and 75 μg/mL) for 24 h at 37 °C, followed by 50 μL of 2 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich, St. Louis, MO, USA) solution to each well and further incubation for 4 h at 37 °C. The media were discarded and 100 μL of dimethyl sulfoxide (DMSO) was added to each well to solubilize the formazan. Optical density was measured at 540 nm on a SpectraMax 190 microplate reader (Molecular Devices, Sunnyvale, CA, USA).

6. Nitric Oxide (NO) Assay

Inhibitory effect on NO production was evaluated using a modified method [28]. Briefly, RAW 264.7 cells (2×10^5 cells/well) were seeded in a 96-well plate for 24 h at 37 °C. Cells were treated with 1 μg/mL of lipopolysaccharides (LPS) alone or in combination with different concentrations of CZE for 24 h. After 24 h, 100 μL of the medium was placed in a 96-well plate and an equal volume of Griess reagent (Sigma-Aldrich, St. Louis, MO, USA) was added. The cells in the plate were then incubated for an additional 15 min at room temperature, and the absorbance was measured at 540 nm with the microplate reader. The amount of NO generated was calculated with a sodium nitrite standard curve.

7. Western Blot Analysis

RAW 264.7 cells (1.0×10^7 cells/100 π dish) were treated with 1 μg/mL of LPS alone or in combination with different concentrations of CZE for 24 h. After 24 h, the cells were collected and washed twice with PBS. The cells were then lysed in RIPA buffer and kept on ice for 1 h. Cell lysates were centrifuged at 12,000 ×g at 4 °C for 15 min, and the supernatants were stored at −70 °C until required for analysis. Protein concentrations were measured by using a protein assay kit (Bio-Rad, Hercules, CA, USA). Aliquots of the lysates

(20 μg of protein) were applied to a 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel and transferred onto a polyvinylidene fluoride (PVDF) membrane. After blocking nonspecific sites with 5% non-fat milk powder in Tris-buffered saline with 0.1% Tween-20 (TBST), the membranes were subsequently incubated with specific primary antibodies (rabbit anti-iNOS: ab15323, 1 : 1000; abcam, Cambridge, United Kingdom; mouse anti-β-actin: AC-15, 1 : 5000; Sigma-Aldrich, St. Louis, MO, USA; goat anti-PPAR-α, sc-1982, 1 : 1000; Santa Cruz Biotechnology, Carlsbad, CA, USA) for 3 h at room temperature. The membranes were subsequently incubated for 1 h with peroxide-conjugated secondary antibodies (goat anti-rabbit IgG-HRP: sc-2004, 1 : 5000; rabbit anti-mouse IgG-HRP: sc-358914, 1 : 5000; Santa Cruz Biotechnology, Carlsbad, CA, USA). The immunoreactive proteins were detected using an enhanced chemiluminescence (ECL) western blotting detection kit (Amersham Pharmacia Biotech, NY, USA). β-actin was used as the standard for normalizing protein samples. The band intensities were quantified with a photo image system (Molecular Dynamics, Uppsala, Sweden).

8. NF-κB Activity

The NF-κB activity was measured by using a luciferase reporter assay system according to the manufacturer's instructions (Promega, Madison, WI, USA). Briefly, before transfection, Hep3B cells were prepared at a density of 2×10^4 per well and cultured as described in the Materials and Methods. After cultivation, the cells were washed with serum-free medium and treated with tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and *C. zawadskii* extracts and cultured for 24 h. Transactivation assays were performed with the Dual-Luciferase Reporter Assay System, and normalized luciferase activity was determined.

9. Protein Chip Assay

The RayBio Mouse Cytokine Antibody Array I was purchased from RayBiotech (Norcross, GA, USA) and utilized according to manufacturer's instructions [29]. The array membranes were incubated with blocking buffer followed by undiluted culture supernatants for 1.5 h. Next, the membranes were washed and incubated with biotin-conjugated antibodies for 1.5 h, followed by HRP-conjugated streptavidin for 2 h. The membranes were then incubated in detection buffer and exposed to X-ray film.

10. Immunohistochemical Analysis

The effect of *C. zawadskii* 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 μg/mL of *C. zawadskii* 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.).

11. HPLC Analysis

The HPLC was performed in Agilent 1260 HPLC-DAD system as per the following conditions: injection volume, 10 μL; Capcell pak C18, UG, 5 μm, column 4.6×250 mm, Agilent Technologies Inc., CA, USA), column temperature at 35 °C, mobile phase A being acetonitrile/acetic acid/water (3/0.5/96.5, v/v/v) and mobile phase B acetonitrile/acetic acid/water (50/0.5/49.5, v/v/v), linear gradient elution from 72.5% A/27.5% B (v/v) to 65% A/35% B (v/v) during 0-10 min, from 65% A/35% B (v/v) to 20% A/80% B (v/v) during 10-35 min, from 20% A/80% B (v/v) to 0% A/100% B (v/v) during 35-40 min; mobile phase flow rate 1 mL/min. The samples were monitored at 360 nm. Flavonoids and their derivatives in the tested sample were determined by comparing the retention times and peak areas with those of authentic reference compounds (Fig. 7). The identified compounds were confirmed by internal standard [30].

12. 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. 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

1. Anti-oxidant Effect of CZE

To determine the anti-oxidant capacity for CZE, a 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay was used. Compared to treatment with ascorbic acid as a positive control (SC_{50}=18.7 μg/mL), treat-

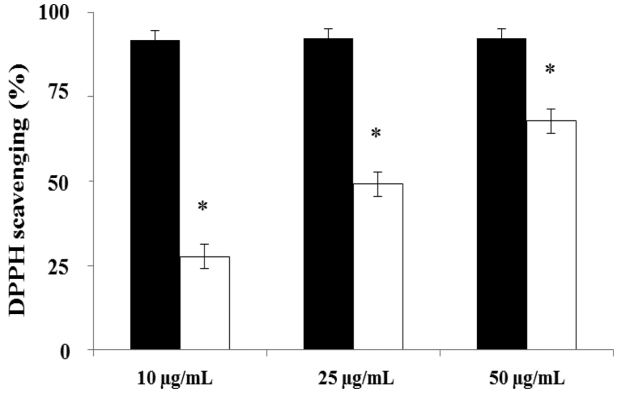


Fig. 1. Anti-oxidant effect of CZE. Anti-oxidant effects were confirmed using a DPPH radical scavenging method. Ascorbic acid was used as a positive control. Ascorbic acid and CZE were used at 10, 25, and 50 μg/mL concentration. Values are presented as mean±S.E.M. *P<0.01 compared to the control group. Black bars, ascorbic acid; white bars, CZE.

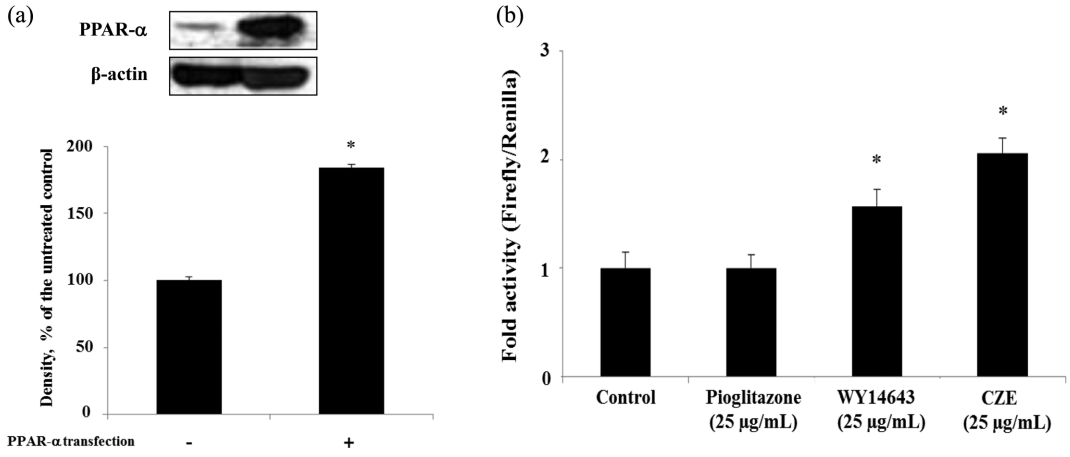


Fig. 2. Transactivation of a PPAR response element (PPRE) by CZE. (a) The expression of PPAR-α was determined by western blot analysis (b) CV-1 cell line was transfected with PPRE luciferase. WY14643 was used as a positive control and no treatment and the PPAR-γ agonist pioglitazone hydrochloride were used as a negative control. WY14643 and CZE were used at 25 μg/mL concentration. Values are presented as mean±S.E.M. *P<0.01 compared to the control (untreated) group.

ment with CZE did not markedly increase DPPH radical scavenging activity (SC_{50}=140.1 μg/mL). But, the radical scavenging activities of CZE were increased according to concentration (Fig. 1).

2. Transactivation of PPRE

We determined the transactivation activity of PPAR-responsive element (PPRE) on CZE. Renilla and firefly luciferase activities were measured with a luminescence spectrometer. As shown in Fig. 2, the CZE largely influenced PPAR-α ligand binding activity, and 25 μg/mL of CZE produced a significant increase compared to the untreated control. Additionally, the CZE-treated group showed high level of transactivation activity compared to the group treated with WY14643 as positive PPAR-α agonists. These results suggest that CZE may have the potential as a novel PPAR-α agonist. Presently, it is known that PPAR-α activated in combination with a ligand promotes the differentiation of keratinocytes and reconstructs the damaged skin barrier. In particular, it was found to inhibit cell proliferation, to promote keratinocyte differentiation in the epidermis, and to improve skin barrier by the induction of lipid metabolism as well as maintaining skin homeostasis by inhibiting the inflammatory response [31,32]. In addition, it has been reported that PPAR-α activity plays an important role in skin wound healing, and PPAR-α agonists such as WY14643 and clofibrate have been confirmed to promote differentiation of keratinocytes and recovery of the skin barrier [8]. CZE increases the transactivation activity of the PPAR-α.

3. Effects of CZE on NO Production in Raw 264.7 Cells

Before testing the effect of CZE on LPS-induced NO production, we examined the effect of CZE on cell viability. After 24 h treatment of RAW 264.7 cells with CZE (5, 10, 25, 50, and 75 μg/mL), cell viability was assessed by the MTT assay. Cell viability was not affected by 50 μg/mL of CZE (Fig. 3(a)). Therefore, all subsequent experiments on inflammatory mediator production were conducted below 50 μg/mL of CZE. To investigate the anti-inflam-

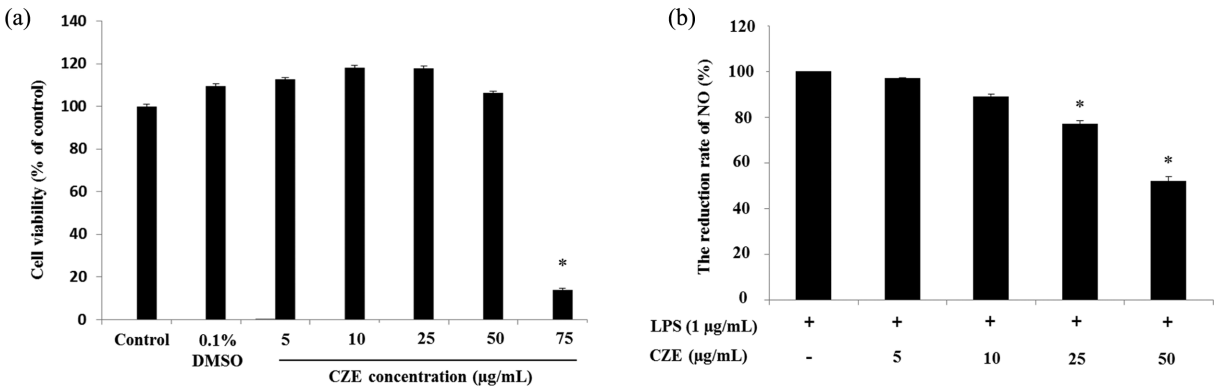


Fig. 3. Inhibitory effects of CZE on LPS-induced NO production in Raw 264.7 macrophages. Cell viability was assessed by the MTT assay, and the results are presented as a percentage of the viability of the control group. RAW 264.7 cells were treated with varying concentrations of CZE for 24 h. The results have been shown as mean±standard error values of triplicate tests. *P<0.01 indicates statistically significant differences of the test group compared to the control (untreated) group (A). RAW 264.7 cells were treated with 1 μg/mL of LPS alone or in combination with varying concentrations of CZE for 24 h. At the end of the incubation period, 100 μL of the culture medium was collected for a nitrite assay. The extracellular medium containing nitrate was analyzed by a Griess regent system. The values are expressed as mean±standard error values of triplicate tests. *P<0.01 indicates statistically significant differences of the test group compared to the LPS alone-treated group (b).

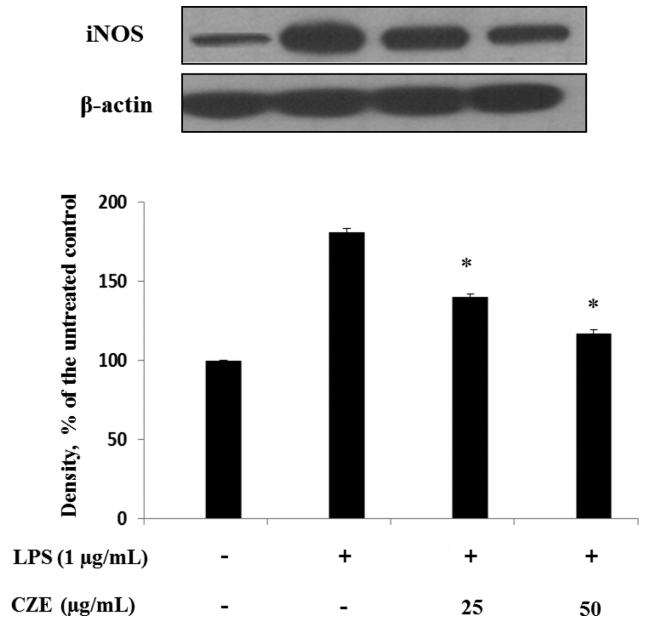


Fig. 4. Effects of CZE on LPS-induced protein expression of iNOS in RAW 264.7 cells. RAW 264.7 cells were treated with 1 μg/mL of LPS alone or in combination with different concentrations of CZE for 24 h. LPS was used as a positive control and no treatment was used as a negative control. Each signal was quantified by scanning densitometry. β-Actin was used as an internal standard. Values are presented as means± standard error of the mean (SEM). *P<0.01 compared to LPS alone-treated group.

matory effects of CZE, we examined its effect on NO production in LPS (1 μg/mL)-induced macrophages. NO is molecular mediator of inflammation process, and the inhibitory effect of CZE on the production of NO was determined. As shown in Fig. 3(b), The CZE significantly inhibited LPS-induced NO production according to concentration.

4. Effects of CZE on LPS-induced iNOS Expression in Raw 264.7 Cells

To elucidate the mechanism by which CZE inhibits LPS-induced NO production, we investigated its effects on iNOS expression. The levels of iNOS protein, which were minimal in RAW 264.7 cells in their resting state, markedly increased upon LPS exposure. Subsequent addition of CZE dose-dependently inhibited LPS-induced iNOS protein expression (Fig. 4). This observation suggests that the inhibitory effect of CZE on LPS-induced NO production is attributable to LPS-induced iNOS expression.

5. Inhibitory Effects of CZE on NF-κB Induced by TNF-α and IL-6 in Hep3B Cells

NF-κB is an important transcription factor to regulate pro-inflammatory mediators such as NO, TNF-α, and IL-6. To investigate the effect of inhibition of NF-κB by CZE, Hep3B cells were stimulated by TNF-α and IL-6, respectively, with or without pretreatment of CZE. The CZE significantly suppressed the TNF-α- and IL-6-induced NF-κB luciferase activity and had a higher inhibitory effect on IL-6-induced NF-κB luciferase activity in comparison to TNF-α-induced NF-κB luciferase activity (Fig. 5). These results indicate that CZE treatment reduced the extent of IL-6- and TNF-α-induced increases in NF-κB-dependent luciferase activity in Hep3B human hepatoma cells. Macrophages participate in inflammatory responses by releasing pro-inflammatory cytokines such as TNF-α and inflammatory factors such as NO [17,18]. Therefore, the inhibition of these inflammatory factors has been considered as a novel candidate for a skin therapeutic agent. In present study, we elucidated the anti-inflammatory effects of CZE by inhibition of NF-κB activity, which is known to induce the expression of inflammatory cytokines. The CZE may be a potential candidate for treating inflammatory symptoms. Many reports suggest that plant-derived compounds have antioxidant properties and act by preventing NF-κB activation, followed by inhibiting iNOS and cytokine gene expression. Our data demonstrate that CZE may exert anti-inflammatory effects via reduction of the pro-inflammatory mediators through suppression of the NF-κB-mediated signaling pathways.

6. Effects of CZE on LPS-induced Cytokine Expression in Raw 264.7 Cells

A mouse cytokine antibody array (Fig. 6(a)) was applied to broadly observe the effects of CZE on LPS-induced cytokine expression. After the cells were incubated with CZE for 24 h, the cytokine expression pattern in the treated cells was differentially compared to that in the control cells (Fig. 6(b)). After LPS treatment, the protein

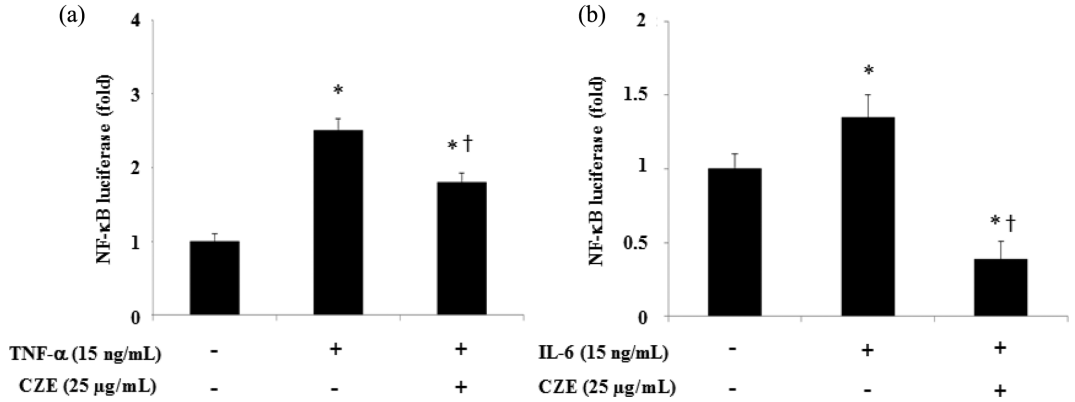
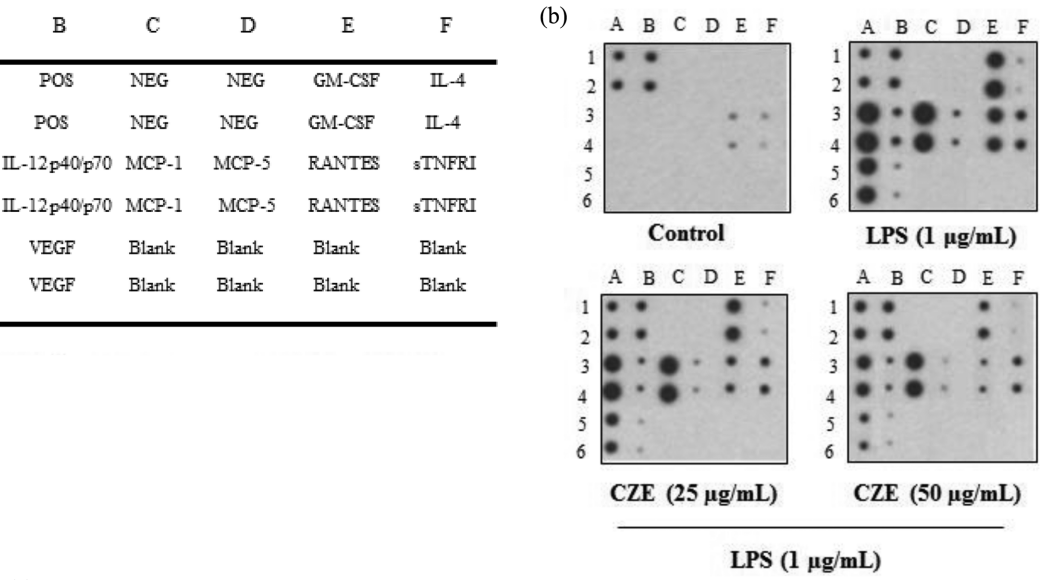


Fig. 5. Inhibitory effect of CZE on TNF-α and IL-6 induced NF-κB activity in Hep3B cells. NF-κB luciferase activity was determined in Hep3B cells. NF-κB luciferase was induced with tumor necrosis factor-α (TNF-α) (a) and interleukin-6 (IL-6) (b). TNF-α and IL-6 was treated at 15 ng/mL. CZE was used at 25 μg/mL concentration. Values are presented as mean±S.E.M. *P<0.01 compared to the untreated group. †P<0.01 compared to TNF-α and IL-6 alone-treated group respectively.

(a)

A	B	C	D	E	F
POS	POS	NEG	NEG	GM-CSF	IL-4
POS	POS	NEG	NEG	GM-CSF	IL-4
IL-6	IL-12p40/p70	MCP-1	MCP-5	RANTES	sTNFRI
IL-6	IL-12p40/p70	MCP-1	MCP-5	RANTES	sTNFRI
TNF-α	VEGF	Blank	Blank	Blank	Blank
TNF-α	VEGF	Blank	Blank	Blank	Blank



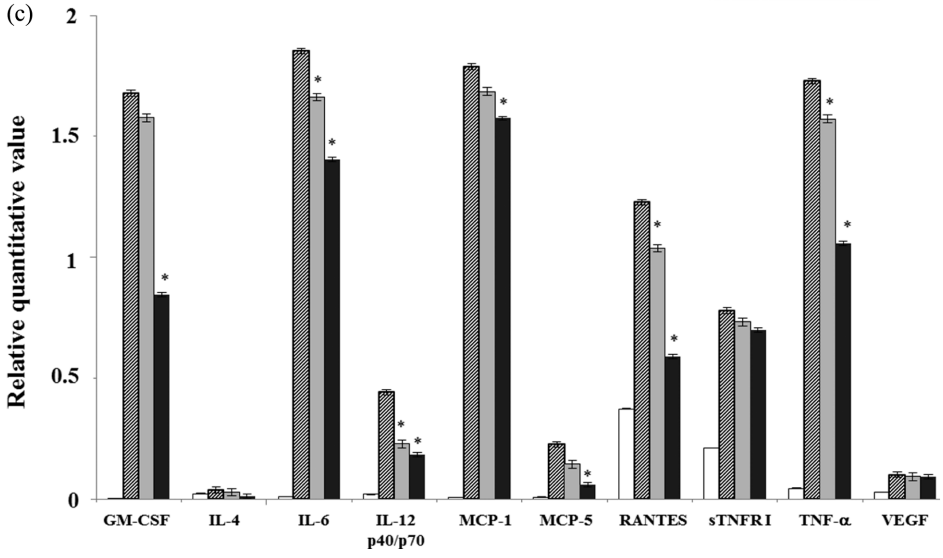


Fig. 6. Cytokine profile of the effect of water chestnut extract on LPS-activated RAW 264.7 cells. (a) Each cytokine is represented by duplicate spots in the location shown. (b) RAW 264.7 cells (1.0×10^7 cells/100 π dish) were treated with 1 μg/ml of LPS only or with different concentrations of water chestnut extract for 24 h. At the end of the incubation period, RAW 264.7 supernatants were collected and assayed for cytokine production using the Mouse Cytokine Array I. The biotin-conjugated IgG produces positive signals (POS), which can be used to identify the orientation and to compare the relative expression levels among the different membranes. A negative control (NEG) in which the sample is replaced with an appropriate mock buffer according to the array protocol was used. (c) The ratio of average intensity of each cytokine spot to each positive spot on the same chip is shown. Values are presented as mean±S.E.M. *P<0.01 compared to the LPS group. White bars, no treatment control group; hatched bars, LPS group; gray bars, CZE group treated at 25 μg/mL after LPS treatment; black bars, CZE group treated at 50 μg/mL after LPS treatment.

expression levels of all cytokines were increased in this study. Each LPS-induced dot in the array was quantified, and the results indicated that CZE inhibited LPS-induced secretion of GM-CSF, IL-6, IL-12, MCP-1, MCP-5, RANTES, and TNF-α but levels of IL-4, sTNFR I, and VEGF did not change significantly (Fig. 6(c)). Our study suggests that CZE inhibits the expression of cytokines induced by LPS.

7. Effect of CZE on Expression of Involucrin in a 3D Skin Equivalent Model

Immunohistochemical analysis of protein marker related to keratinocyte differentiation was determined. As shown in Fig. 7, the treatment by CZE (50 μg/mL) showed a significant increase of involucrin expression compared with treatment by 0.1% DMSO as a control in skin equivalents, In addition, the CZE-treated group showed similar level in the expression of involucrin compared to the group treated with the same concentration of WY14643. These results indicate that CZE showed similar activities as those with the synthetic compounds.

It has previously been shown that synthetic compounds such as clofibrate and WY14643 affect keratinocyte differentiation, such as the CE formation [27,31], and CZE showed similar activities as those with the synthetic compounds. These results suggest that CZE can be an appropriate candidate for epidermal permeability barrier recovery, and furthermore, that this candidate may have advantages

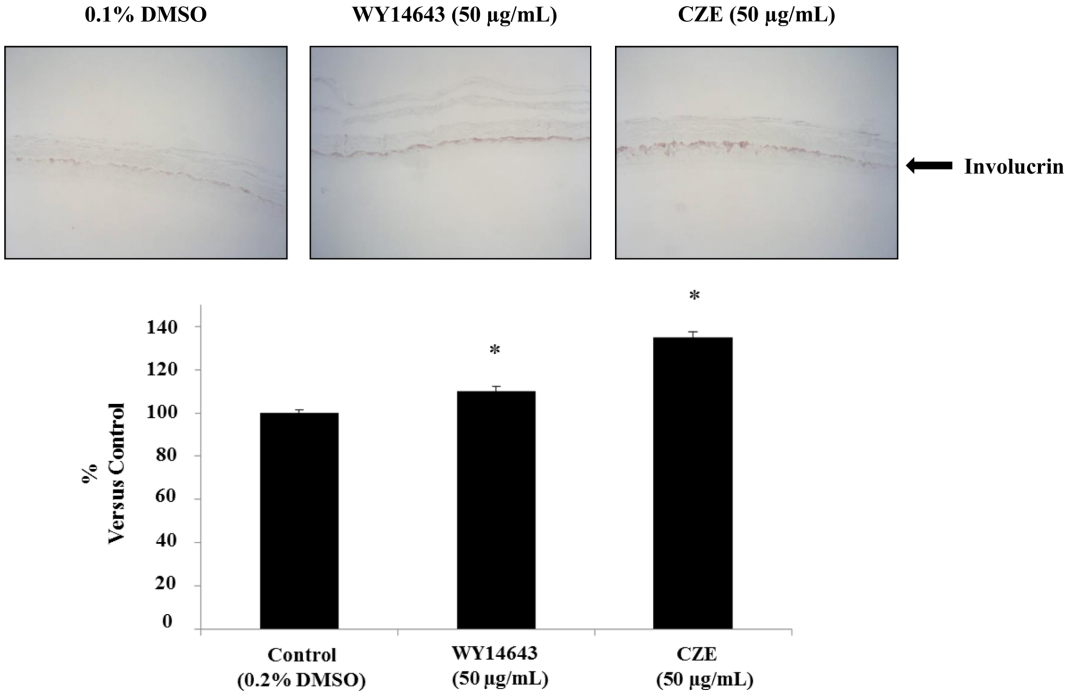


Fig. 7. Immunohistochemical analysis of involucrin in a 3D skin equivalent model. 0.2% DMSO was used as a negative control and WY14643 was used as a positive control. WY14643 and CZE were treated at 50 μg/mL concentration. The skin fragments were subjected to immunohistochemical analysis by using anti-involucrin antibody and observed by microscope. The area was analyzed by involucrin with image quantitative analysis software (NIH images, version 1.61.). Values are presented as mean±S.E.M. *P<0.01 compared to the control group.

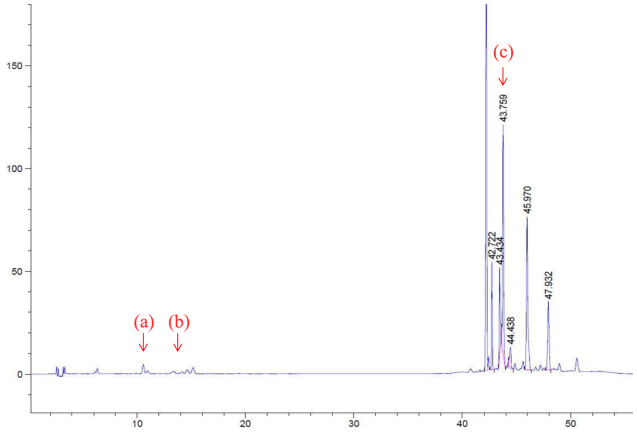


Fig. 8. HPLC analysis of CZE. Each arrow indicates quercetin-3-glucoside (a), quercitrin (b) and kaempferol (c). The other peaks were not identified owing to lack of authentic compounds.

as it is a natural compound with no adverse effects on human skin. In conclusion, the effects of CZE on skin barrier functions were evaluated. This study showed that CZE shows activation of PPAR-α ligand binding, promotes keratinocyte differentiation, and is a novel modulator of skin barrier homeostasis with anti-inflammatory action. Therefore, CZE may be an appropriate material for improving epidermal permeability barrier function as a cosmeceuticals.

8. HPLC Analysis

Several peaks were monitored in the HPLC profile of CZE (Fig. 8). Three peaks were qualitatively identified based on retention times of 30 HPLC reference compounds used and the others were not identified owing to lack of authentic references. Among the identified flavonoids, there were three flavonoids: quercetin-3-glucoside, quercetin, and kaempferol. Kaempferol was the most abundant.

CONCLUSION

We investigated the effects of *C. zawadskii* leaves extract by supercritical carbon dioxide extraction (SCE) method on the function of the epidermal permeability barrier and on the activity of PPAR-α, anti-inflammatory activity, and expression of protein related to CE formation. CZE possessed a high PPAR-α and anti-inflammatory activity. Moreover, the expression of involucrin by CZE was significantly high in a 3D skin equivalent model. These results suggest that CZE 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.

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