

Original Article

Anti-inflammatory effect of *Centella asiatica* phytosome in a mouse model of phthalic anhydride-induced atopic dermatitisPark Ju Ho^{a,b,1}, Jang Jun Sung^{a,1}, Kim Ki Cheon^{a,b}, Hong Jin Tae^{a,*}^a College of Pharmacy and Medical Research Center, Chungbuk National University, 194-31 Osongsangmyeong 1-ro, Osong-eup, Heungduk-gu, Cheongju, Chungbuk 361-951, Republic of Korea^b INIST ST CO., LTD., 500, Sinnae-ro, Geumwang-eup, Eumseong-gun, Chungcheongbuk-do 27644, Republic of Korea

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ABSTRACT

Background: *Centella asiatica* phytosome (CA phytosome) has potent antioxidant and anti-inflammatory properties. However, its anti-dermatitic effect has not yet been reported.

Purpose: We investigated the effects of CA phytosome on inflammatory responses by macrophages in an atopic dermatitis (AD) mouse model.

Study design: The effects of CA phytosome on atopic dermatitis were examined by using phthalic anhydride (PA)-induced AD mouse model and RAW 264.7 murine macrophages.

Methods: An AD-like lesion was induced by a topical application of 5% phthalic anhydride (PA) to the dorsal skin or ear of HR-1 mice. After AD induction, 100 μl (20 μl/cm²) of 0.2% and 0.4% CA phytosome was spread on the dorsal skin and ear of the mice three times a week for four weeks. We evaluated histopathological changes and changes in protein expression by Western blotting for iNOS and COX-2; NF-κB activity was determined by EMSA. We also measured TNF-α, IL-1β, and IgE concentration in the blood of AD mice by ELISA.

Results: Histological analysis showed that CA phytosome inhibited infiltration of inflammatory cells. CA phytosome treatment inhibited the expression of iNOS and COX-2, activity of NF-κB, and release of TNF-α, IL-1β, and IgE. In addition, CA phytosome (5, 10, and 20 μg/ml) potently inhibited LPS (1 μg/ml)-induced NO production as well as iNOS and COX-2 expression in RAW 264.7 macrophage. Furthermore, CA phytosome inhibited LPS-induced DNA binding activities of NF-κB, and this was associated with the discontinuation of IκBα degradation and subsequent decreases in the translocation of p65 and p50 into the nucleus.

Conclusion: From our data, CA phytosome application, which operates via NF-κB signaling inhibition, seems to be a promising AD treatment. Herein, we investigated the effects of *Centella asiatica* phytosome (CA phytosome) on inflammatory responses by macrophages in an atopic dermatitis (AD) mouse model. An AD-like lesion was induced by the topical application of 5% phthalic anhydride (PA) to the dorsal skin or ear of HR-1 mice. After AD induction, 100 μl (20 μl/cm²) of 0.2% and 0.4% CA phytosome was spread on the dorsal skin and ear of the mice three times a week for four weeks. We evaluated dermatitis severity, histopathological changes, and changes in protein expression by Western blotting for iNOS and COX-2; NF-κB activity was determined by gel electromobility shift assay (EMSA). We also measured TNF-α, IL-1β, and IgE concentration in the blood of AD mice by enzyme-linked immunosorbent assay (ELISA). CA phytosome attenuated the development of PA-induced AD. Histological analysis showed that CA phytosome inhibited hyperkeratosis, proliferation of mast cells, and infiltration of inflammatory cells. Furthermore, CA phytosome treatment inhibited the expression of iNOS and COX-2, activity of NF-κB, and release of TNF-α, IL-1β, and IgE. In addition, CA phytosome (5, 10, and 20 μg/ml) potently inhibited lipopolysaccharide (LPS) (1 μg/ml)-induced NO production as well as iNOS and COX-2 expression in RAW 264.7 macrophage cells. Furthermore, CA phytosome inhibited LPS-induced DNA binding activities of NF-κB, and this was associated with the discontinuation of IκBα degradation and subsequent decreases in the translocation of p65 and p50 into the nucleus. From our data, CA phytosome application, which operates via NF-κB signaling inhibition, seems to be a promising AD treatment.

Abbreviations: AD, atopic dermatitis; CA, *Centella asiatica*; PA, phthalic anhydride; iNOS, inducible nitric oxide synthetase; COX-2, cyclooxygenase-2; NF-κB, nuclear factor-κB; EMSA, gel electromobility shift assay; TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1β; IL-6, interleukin-6, IgE, immunoglobulin E; LPS, lipopolysaccharide; DNCB, 2,4-dinitrochlorobenzene

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Introduction

Atopic dermatitis (AD) is a common chronic inflammatory skin disease characterized by itchy lesions and relapsing eczema (Leung and Bieber, 2003). Several genetic and environmental factors and immunological abnormalities cause AD (Hashimoto et al., 2011). Allergic induction of AD involves the secretion of IL-4, IL-5, and IL-13 by Th2 cells and elevated production of serum IgE (Homey et al., 2006; Tokura, 2010). In a mouse model, 2,4-dinitrochlorobenzene-induced AD-like skin lesion reportedly showed increased serum IgE and Th2 cytokines (Harada et al., 2005; Klewicka et al., 2011). These cytokines directly impact epidermal keratinocytes, which produce pro-inflammatory cytokines that induce an inflammatory response through infiltration of immune cells into the skin lesions (Homey et al., 2006). CD4⁺T cells and mast cells in the skin lesions are also involved in AD pathogenesis (Kwak et al., 2013; Sung et al., 2016; Zhang et al., 2016). The IgE-mediated mast cell activation leads to the release of various chemical mediators; these mediators result in the infiltration of inflammatory cells, such as eosinophils and lymphocytes into the skin lesion (Kuramoto et al., 2015).

Keratinocytes participate in inflammatory responses based on the production of pro-inflammatory cytokines that play a crucial role in AD (Brandt and Sivaprasad, 2011; Seo et al., 2015). The stimulation of epidermal keratinocytes with TNF- α and IFN- γ induces the expression of various inflammatory mediators (Nedoszytko et al., 2014; Pastore et al., 2011). Thus, the activation of keratinocytes along with the suppression of these cytokines can be effective for treating AD.

Nuclear factor- κ B (NF- κ B) plays an important role in regulating allergic inflammatory responses in AD. Many studies have shown that it is an important factor associated with various immune responses, such as AD (Wullaert et al., 2011). Facilitated translocation of NF- κ B may exacerbate the allergic inflammation by enhancing the production of inflammatory cytokines and chemokines. Therefore, a compound that suppresses NF- κ B activation could act as a therapeutic agent for allergic disorders. Cyclosporine and tacrolimus (FK506), potent immunosuppressants, have been used to cure AD (Leung, 2000). In addition, numerous strategies to control AD pharmacologically, including topical administration of steroids and the use of NF- κ B inhibitors, have been reported (Leung, 2000; Schakel et al., 2014). Tanaka et al. (2005) reported that the NF- κ B inhibitor IMD-0354 inhibited abnormal proliferation of mast cells and reduced allergic responses. Therefore, the activation of NF- κ B certainly contributes to the progression of allergic inflammation.

Phytosomes are drug carriers used to improve vesicular drug delivery systems wherein they are bound and linked with lipid membranes and phospholipid molecules. Phytosomes are liposome vesicles formed due to the interaction of hydrogen bonds between phospholipids of the lipid membrane and phytomolecules; they facilitate the transdermal permeation of therapeutic agents during transdermal and dermal delivery (Semalty et al., 2010). Although various pharmacological effects of *Centella asiatica* (CA) have been reported, the effect of its phytosome formulation against AD has not been reported yet. Therefore, we investigated its effect against AD and the involved action mechanism in 5% phthalic anhydride (PA)-induced AD in a mouse model as well as in vitro.

Materials and methods

Ethical approval

The experimental protocols were carried out according to the guidelines for animal experiments of the Institutional Animal Care and Use Committee (IACUC) of Laboratory Animal Research Center at Chungbuk National University, Korea (CBNUA-929-16-01). All efforts were made to minimize animal suffering, and to reduce the number of animals used. All mice were housed in cages, three mice per cage, with

automatic temperature control (21–25 °C), relative humidity (45–65%), and 12 h light-dark cycle illuminating from 08:00 a.m. to 08:00 p.m. Food and water were available ad libitum. They were fed a pellet diet consisting of crude protein 20.5%, crude fat 3.5%, crude fiber 8.0%, crude ash 8.0%, calcium 0.5%, phosphorus 0.5% per 100 g of the diet (collected from Daehan Biolink, Chungcheongbuk-do, Korea). During this study, all mice were specifically observed for the normal body posture, piloerection, ataxia, urination, etc 2 times per day.

Preparation and extraction of *Centella asiatica*

Collected aerial parts of *Centella asiatica* were oven-dried at 50 °C and then powdered using a milling machine. The powdered plant (1 kg) was extracted with 75% (v/v) ethanol (3 $\times$ 4 l, 3 days each) at room temperature. The extracts were filtrated with depth-filter coated with active carbon and concentrated at 80 °C under reduced pressure. The concentrate was divided to precipitate and filtrate fraction by filtration. The precipitate fraction was dried at 50 °C to make asiaticoside powder which yielded 0.12% (w/w) of dried plant. Subsequently, the filtrates fraction was hydrolyzed with alkaline solution containing sodium hydroxide (1% w/v) at 80 °C. Subsequently, it was concentrated, precipitated and dried using the procedure described above. The yield of the genins (asiatic acid and madecassic acid) powder obtained from the filtrate fraction was 0.18% (w/w) of dried plant. Both powder extracts were mixed to give the titrated extract of *Centella asiatica*. The components of the titrated extract of *Centella asiatica* were asiaticoside (40%), asiatic acid (30%), and madecassic acid (29–30%) (Table 1). The purity of these commercial products were over 99%.

Preparation of phytosome

There are some methods for production of phytosomes including anti-solvent precipitation (Gupta and Dixit, 2011), solvent evaporation (Li et al., 2008; Peng et al., 2008; Sikarwar et al., 2008), precipitation and anhydrous co-solvent lyophilization (Singh et al., 2012). Phytosomes are obtained by reacting 2–3 mol or 1 mol of phospholipid such as phosphatidylcholine, phosphatidylethanolamine or phosphatidylserine with 1 mol of bioactive components (flavonoids or terpenoids) in an aprotic solvent (dioxane, acetone, methylene chloride, or ethyl acetate). The solvent is evaporated under vacuum or precipitation with non-solvent (aliphatic hydrocarbons), lyophilization (freeze drying) or spray drying; therefore, the complex is isolated (Comoglio et al., 1995). Phytosome production consists of blending biomaterial, inorganic solvent and phospholipid until clear solution creation, solvent evaporation and creating thin layer, hydration and sonication, respectively. Commercial product of centella phytosome prepared from *Centella asiatica* extracts, which are available in the market, is shown in Table 2 (Angelico et al., 2014; Kumar et al., 2014). All solvents used were of commercial grade and obtained from Indena Spa, Milano, Italy.

Animal treatment

The protocols for the animal experiment used in this study were carefully reviewed for ethical and scientific care procedures and approved by the Chungbuk National University-Institutional Animal Care

Table 1
Composition of titrated extracts of *Centella asiatica*.

Extract	Composition of extract
Titrated extract of <i>Centella asiatica</i> (TECA)	Asiaticoside (40%), Asiatic acid (30%), Madecassic acid (29–30%)

Titrated extract of *Centella asiatica* includes 40% of asiaticoside, 30% of Asiatic acid, and 29–30% of madecassic acid.

and Use Committee (Approval Number CBNU-2015-0976). HR-1 mice (eight-week-old, $n = 40$) were randomly divided into one of four groups. In the first group (Vehicle, $n = 10$), 100 μl of AOO (4:1 acetone: olive oil, v/v: AOO) was spread on the dorsum of the ears and back skin three times a week for four weeks. In the second group (PA, $n = 10$), 100 μl (20 $\mu\text{l}/\text{cm}^2$) of 5% phthalic anhydride solution was applied. The third group (CA phytosome 0.2%, $n = 10$) and fourth group (CA phytosome 0.4%, $n = 10$) were applied with PA, and 3 h after 100 μl of 0.2% and 0.4% *Centella asiatica* phytosome (40 μg or 80 $\mu\text{g}/\text{cm}^2$) were applied. Age-matched HR-1 mice were used as the control group (Control, $n = 10$).

Blood cell number measurements

Mice blood was taken by heart puncture. Blood cell number were measured in the laboratory animal research center at Chungbuk National University.

Measurement of body and lymph node weight, and ear thickness

Alterations of body weight during the experimental procedure were measured with an electronic balance (Mettler Toledo, Greifensee, Switzerland) once a week for 4 weeks. Weights of lymph nodes collected from the sacrificed mice were also measured by the same method. Ear thickness was measured using a thickness gauge (Digimatic Indicator, Matusutoyo Co., Tokyo, Japan) to determine the degree of allergic skin inflammation induced by PA treatment.

Histological techniques

Ear and back skins were removed from mice, fixed with 10% formalin, embedded in paraffin wax, routinely processed, and then sectioned into 5 μm thick slices. The skin sections were then stained with hematoxylin and eosin (H&E). The thickness of the epidermis and dermis were also measured using the Leica Application Suite (Leica Microsystems, Wetzlar, Germany).

Enzyme-linked immunosorbent assay (ELISA) for detection of serum IgE concentration

The serum IgE concentration was measured using an ELISA kit (Shibayagi, Inc., Gunma, Japan) according to the manufacturer's instructions. Briefly, capture antibodies were plated in the Nunc C bottom immunoplate supplied in the kit. Next, wells were washed with washing solution (50 mM Tris, 0.14 M NaCl, 0.05% Tween 20, pH 8.0) three times. Then, serum samples and standards diluted with buffer solution were added to the wells, and the plate was incubated for 2 h. The wells were washed again with washing solution, 50 μl of Biotin-conjugated anti-IgE antibodies (1000-fold dilution) were added to each well and incubated further for 2 h to bind with captured IgE. The wells were washed again with washing solution, after which horseradish peroxidase-conjugated detection antibodies (2000-fold dilution) were added to each well and incubated for 1 h. An enzyme reaction was then initiated by adding tetramethylbenzidine (TMB) substrate solution (100 mM sodium acetate buffer pH 6.0, 0.006% H_2O_2) and incubating the plate at room temperature in the dark for 20 min. Finally, the reaction was terminated by adding acidic solution (reaction stopper, 1 M H_2SO_4), and absorbance of yellow product is measured spectrophotometrically at 450 nm. The final concentration of IgE was calculated using the standard curve.

Cytokine assay

By the end of the study period, blood specimens were collected. Serum levels of mouse TNF- α IL-4, IL-13, and IL-1 β were measured by enzyme-linked immunosorbent assay (ELISA) kits provided by Thermo

Fisher Scientific Inc. (Meridian Rd, Rockford, IL USA) according to the manufacturer's protocol.

Western blot analysis

100 mg skin or ear tissues or about 1×10^6 cells were harvested and homogenized with lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 0.02% sodium azide, 0.2% SDS, 1 mM phenyl methylsulfonyl fluoride (PMSF), 10 $\mu\text{l}/\text{ml}$ aprotinin, 1% igapal 630 (Sigma Chem. Co. St. Louis, MO, USA), 10 mM NaF, 0.5 mM EDTA, 0.1 mM EGTA and 0.5% sodium deoxycholate). The extracts were centrifuged at 23,000 g for 1 h. Equal amount of protein (20 μg) was separated on a sodium dodecyl sulfate (SDS)/ 10%-polyacrylamide gel, and then transferred to a nitrocellulose membrane (Hybond ECL, Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA). Blots were blocked for 2 h at room temperature with 5% (w/v) non-fat dried milk in Tris-buffered saline (10 mM Tris (pH 8.0) and 150 mM NaCl) solution containing 0.05% tween-20. The membrane was incubated for 4 h at room temperature with specific antibodies: Mouse monoclonal antibodies directed against JNK, phosphor-JNK, p38, phosphor-p38, ERK, phosphor-ERK (1:1000) (Cell signaling Technology, Beverly, MA, USA), and against p50 (1:500) (Santa Cruz Biotechnology Inc. Santa Cruz, CA, USA), and rabbit polyclonal antibodies against iNOS, COX-2, p65 and I κ B- α (1:500) (Santa Cruz Biotechnology Inc. Santa Cruz, CA, USA) were used in the study. The blot was then incubated with the corresponding conjugated anti-rabbit immunoglobulin G-horseradish peroxidase (Santa Cruz Biotechnology Inc. Santa Cruz, CA, USA). Immunoreactive proteins were detected with the enhanced chemiluminescent (ECL) western blotting detection system.

Gel electromobility shift assay (EMSA)

Gel shift assays were performed according to the manufacturer's recommendations (Promega, Madison, WI, USA). Briefly, 1×10^6 cells were washed twice with $1 \times$ PBS, followed by the addition of 1 ml of PBS, and the cells were scraped into a cold Eppendorf tube. Cells were spun down at 15,000 g for 1 min, and the resulting supernatant was removed. Solution A (10 mM HEPES, pH 7.4, 10 mM KCl, 1.5 mM MgCl_2 , 0.5 mM dithiothreitol, 0.2 $\mu\text{g}/\text{ml}$ phenylmethylsulfonyl fluoride, 1 $\mu\text{g}/\text{ml}$ pepstatin A, 1 $\mu\text{g}/\text{ml}$ leupeptin, 10 $\mu\text{g}/\text{ml}$ soybean trypsin inhibitor, 10 $\mu\text{g}/\text{ml}$ aprotinin, and 0.5% Nonidet P-40) was added to the pellet in a 1:1 ratio (v/v), and allowed to incubate on ice for 10 min. The cells were centrifuged at 15,000 g for 7 min. And then, the supernatant was used as a cytosolic extraction. Solution C (solution A + 20% glycerol, 0.2 mM EDTA, and 420 mM NaCl) was added to the pellet in a 2:1 ratio (v/v), vortexed on ice for 20 min and centrifuged at 15,000 g for 15 min. The oligonucleotide sequences for NF- κ B were 5'-AGT TGA GGG GAC TTT CCC AGG C-3'. Consensus oligonucleotides were end-labeled using T4 polynucleotide kinase and [^{32}P] ATP for 10 min at 37 $^\circ\text{C}$. The ^{32}P -labeled oligonucleotide was reacted with nuclear extracts (10 μg) for 20 min at room temperature. Subsequently, 1 μl of gel loading buffer was added to each reaction and loaded onto a 6% non-denaturing gel and electrophoresis until the dye was three-fourths of the way down the gel. The gel was dried at 80 $^\circ\text{C}$ for 1 h and exposed to

Table 2
Commercial phytosome preparation.

Phytosomes	Phytoconstituents complex with phosphatidylcholine	Indication
Centella phytosome	Terpenes	Vein and skin disorders
Centella phytosome®	Triterpenes from <i>Centella asiatica</i> leaf	Cicatrizing, trophodermic

Commercial product of centella phytosome prepared from *Centella asiatica* extracts which are available in the market.

film overnight at 70 °C. The relative density of the protein bands was scanned by densitometry using MyImage, and quantified by Labworks 4.0 software (UVP Inc., Upland, USA).

Cell culture

The RAW 264.7 murine macrophage cell line was obtained from the Korea Cell Line Bank (Seoul, Korea). These cells were grown at 37 °C in DMEM medium supplemented with 10% FBS, penicillin (100 units/ml) and streptomycin sulfate (100 µg/ml) in humidified atmosphere of 5% CO₂. Cells were incubated with CA phytosome at various concentrations (5, 10, or 20 µg/ml) or positive chemicals and then stimulated with LPS 1 µg/ml for the indicated time in figure legends. Various concentrations of CA phytosome dissolved in dimethylsulfoxide (DMSO)-ethanol solvent (1:1 DMSO: ethanol, v/v) were added together with LPS. The final concentration of DMSO-ethanol solvent used was less than 0.05%. Cells were treated with 0.05% DMSO-ethanol solvent as vehicle control.

Nitrite quantification assay

The NO was determined through the indication of nitrite level in the cell culture media. The RAW 264.7 murine macrophages were seeded in 6-well plates (1×10^6 cells/well) with 2 ml of cell culture media and incubated for 24 h. This was followed by discarding the old culture media and replacing them with the new media to maintain the cells. Different concentrations of CA phytosome (5, 10, and 20 µg/ml) were pretreated with the RAW 264.7 macrophages. Induction of RAW 264.7

macrophages with LPS (1 µg/ml) for all samples was conducted except for the control for another 24 h. Then, 100 µl of the collected supernatants was added with 100 µl of Griess reagent (0.1% NED, 1% sulphanilamide, and 2.5% phosphoric acid) and incubated at room temperature for 10 min in the dark. The absorbance was determined by using microplate reader at 540 nm wavelength. The NO concentration was determined by comparison to the standard curve.

Statistical analysis

The experiments were conducted in triplicate, and all experiments were repeated at least three times with similar results. All statistical analysis was performed with GraphPad Prism 5 software (Version 5.03; GraphPad software, Inc., San Diego, CA). Group differences were analyzed by one-way ANOVA followed by Tukey's multiple comparison test. All values are presented as mean $\pm$ SD. Significance was set at $p < 0.05$ for all tests.

Results

Effects of CA phytosome treatment on ear thickness and morphology

Changes in the body weight were measured during the experimental period. No significant difference in body weight was detected after any treatment (Fig. 1(A)). To investigate whether or not CA phytosome treatment suppresses the changes in ear phenotype induced by PA treatment, the thickness and morphology of the ears were observed.

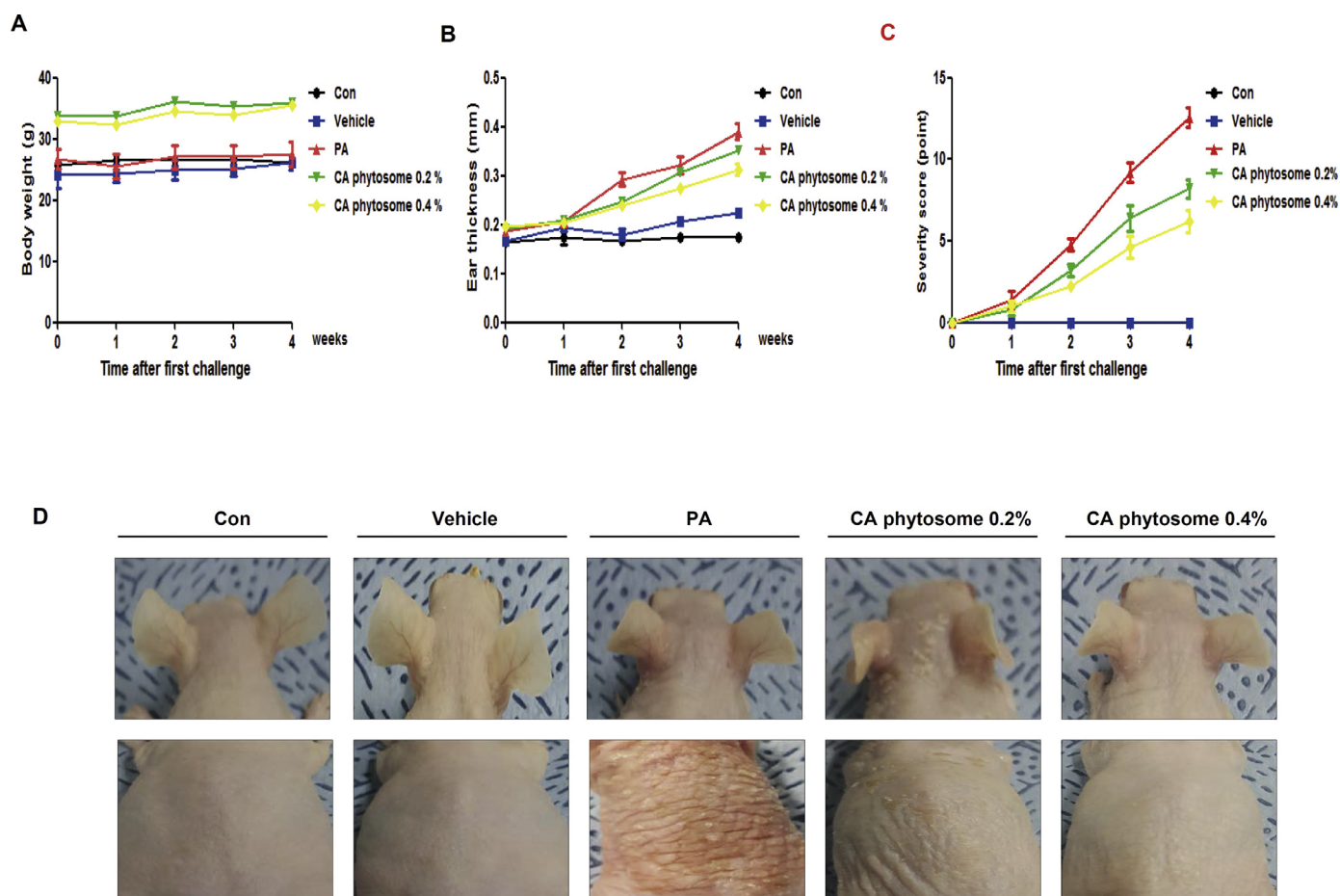


Fig. 1. Differences in body weight, ear thickness, ear phenotypes, and back phenotypes.

Body weights of mice in the five groups were measured with a chemical balance (A). PA solution was repeatedly applied to the dorsum of the ear and back during the topical application of CA phytosome. After four weeks, ear thickness (B) and phenotypes (C) were observed following the procedure described in Materials and Methods. Data shown are the mean $\pm$ SD ($n = 10$).

Increased ear thickness was more rapid in PA treated mice than in control or vehicle-treated mice. Conversely, it only slowly increased in a dose-dependent manner in CA phytosome-treated mice (Fig. 1(B)). Furthermore, unlike the control or vehicle-treated mice, PA-treated mice showed symptoms indicative of erythema, edema, and erosion. These changes of the ear and back morphologies and ear thickness were dramatically reversed upon CA phytosome treatment (Fig. 1(C) and (D)). These effects of phytosome are more effective on skin thickness as well as IL-1 β levels compared to TECA itself (Supplementary Fig. 1).

Effect of CA phytosome treatment on lymph node weight and IgE concentration as well as on expression of iNOS and COX-2

To investigate whether or not CA phytosome could suppress the increases in lymph node weight and IgE concentration, we evaluated the auricular lymph node weight and serum IgE concentration. The former increased more in PA-treated mice than in control or vehicle-treated mice. However, the lymph node weight was significantly reduced in CA phytosome-treated mice (Fig. 2(A)). Hyperproduction of IgE is a characteristic feature of allergic hypersensitivity and is an indicator of the magnitude of the allergic immune response during AD development (Suzuki et al., 2016). Serum IgE concentration and

immune cell number were measured in mice to determine whether CA phytosome suppressed the allergic responses induced by PA treatment. Repeated topical application of PA solution induced a significant increase in serum IgE concentration. However, a significant decrease of IgE concentration was observed in CA phytosome-treated mice (Fig. 2(B)). WBC, neutrophil, lymphocytes, B cells and dendritic cell number were also reduced by treatment of CA phytosome (Supplementary Fig. 2(A)). In addition, protein expression of iNOS and COX-2 was significantly upregulated in PA-treated mice but were significantly suppressed in a dose-dependent manner in CA phytosome-treated mice (Fig. 2(C)).

Effect of CA phytosome treatment on the release of inflammatory cytokines

To determine if CA phytosome treatment can alter the inflammatory cytokine release in PA-induced skin inflammation, the levels of TNF- α and IL-1 β were measured in mouse sera of the control and vehicle treatment, PA treatment, and PA + CA phytosome treatment groups. The levels of TNF- α and IL-1 β were generally higher in the PA treatment group than in the control or vehicle treatment group. However, these levels in the CA phytosome treatment group were dramatically decreased and were comparable to the levels in the control or vehicle

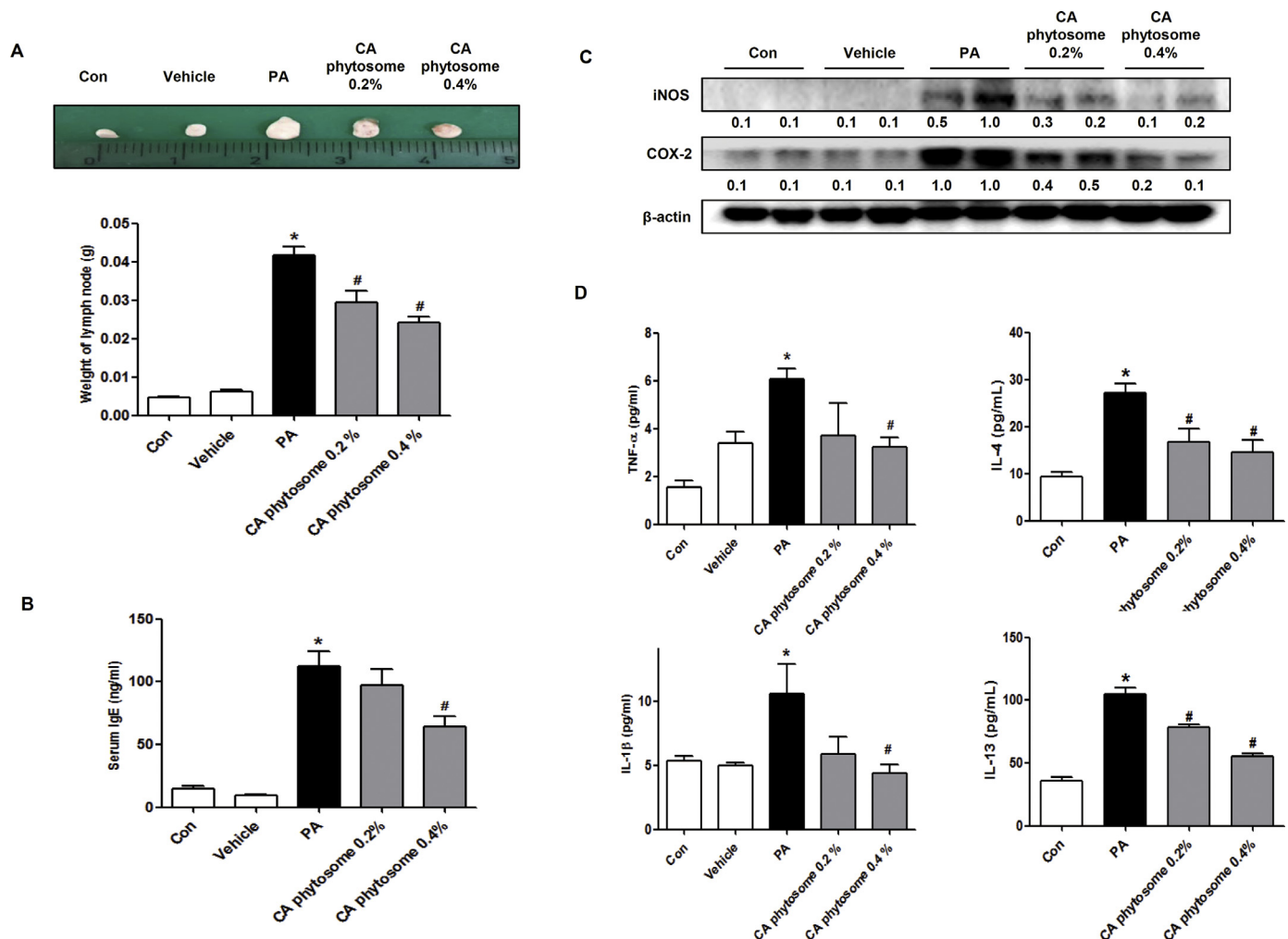


Fig. 2. Changes in the auricular lymph node weight, expression level of iNOS and COX-2 protein in the lymph node, and serum cytokine concentration. After final treatment, mice from each group were euthanized under anesthesia. The auricular lymph nodes were then harvested from the neck regions of the mice using a microscissor, and were weighed thereafter (A). Alterations in the expression of the two proteins was measured by Western blotting (C). The serum used to measure the cytokine concentration was prepared from blood samples collected from the abdominal veins of mice. Serum IgE (B), TNF- α , and IL-1 β (D) concentrations were quantified by ELISA. Data shown are the mean $\pm$ SD ($n = 10$). * $p < 0.05$ is the significance level compared to the control group. # $p < 0.05$ is the significance level compared to the PA treatment group.

treatment group (Fig. 2(D)).

Effect of CA phytosome treatment on inflammatory responses on ear and back skin

To investigate the suppressive effect of CA phytosome treatment on ear and back histology, histological analysis of the dorsal ear and back skin was performed (Figs. 3(A) and 4(A)). The epidermis and dermis of the ear (Fig. 3(B)) and the epidermis of the back (Fig. 4(B)) were thicker in the PA treatment group than in the control or vehicle treatment group. However, the thickness was greatly decreased in the CA phytosome treatment group in a dose-dependent manner. In addition, protein expression of iNOS and COX-2 was significantly upregulated in PA-treated mice but were significantly suppressed in CA phytosome-treated in a dose-dependent manner (Figs. 3(C) and 4(C)).

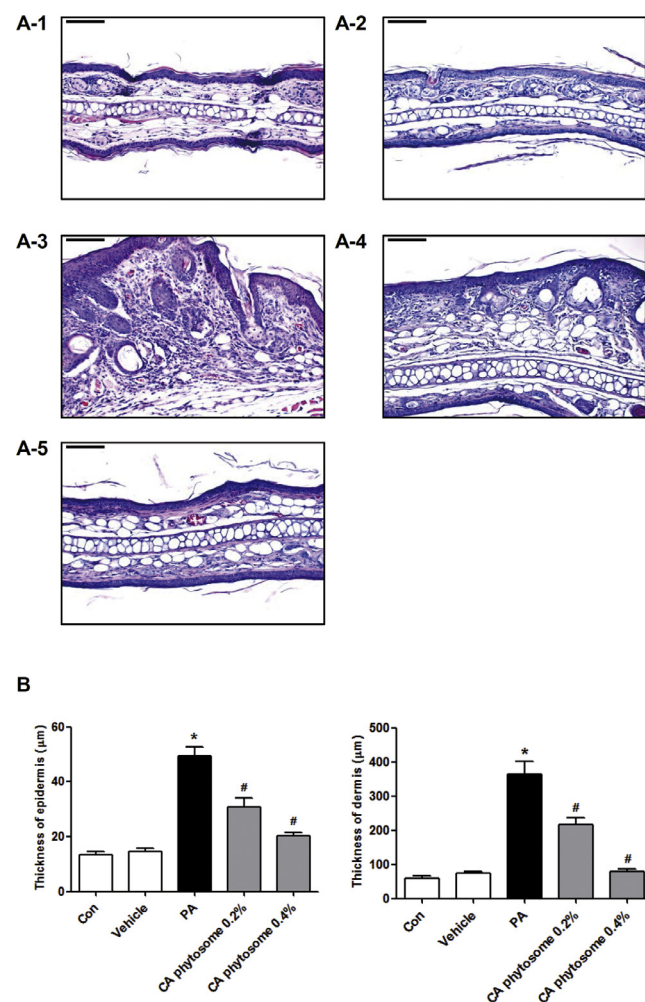
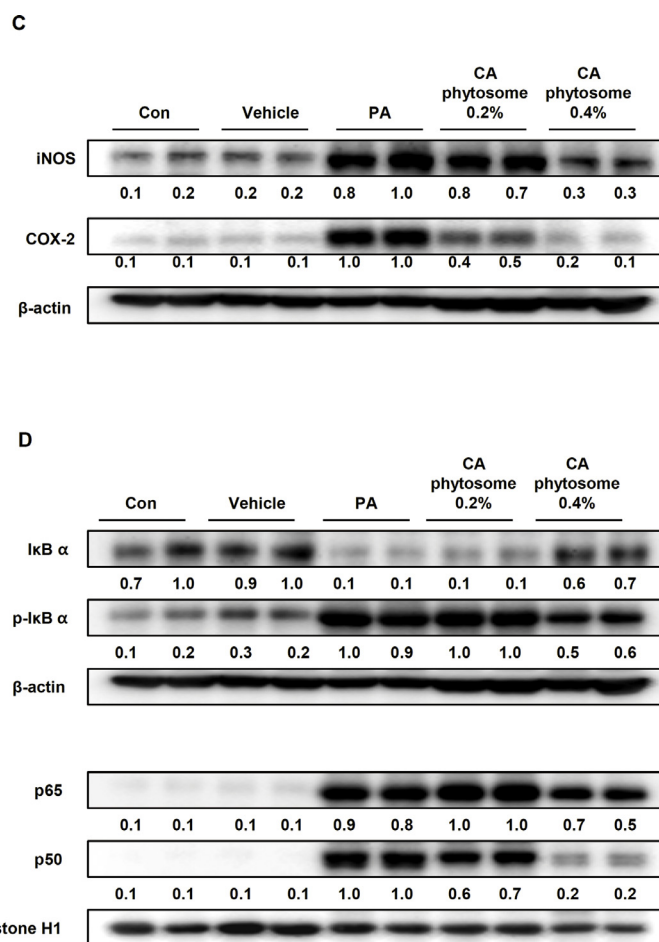


Fig. 3. Histopathological analysis of ear tissue and the inhibitions of NF-κB DNA binding activity by topical application of CA phytosome on ear skin. Histopathology of ear skin in control (A-1), vehicle (A-2), PA (A-3), PA + CA phytosome 0.2% (A-4), and PA + CA phytosome 0.4% (A-5). PA solution was repeatedly applied to the dorsum of ears during the topical application of CA phytosome. Histopathological changes were identified in the slide sections of ear tissues by staining with hematoxylin and eosin followed by observation at 200 × magnification (Scale bars, 100 μm). Histological images (A) and thickness of the epidermis and dermis (B).

Alterations in the expression of iNOS and COX-2 proteins were measured by Western blotting (C). The effect of CA phytosome on translocation of the subunits of NF-κB (p50 and p65) into the nucleus and the phosphorylation of IκBα in cytosol in ear skin (D). Equal amounts of nuclear proteins (20 μg/lane) or total proteins (20 μg/lane) were subjected to 10% SDS-PAGE, and the expression of p50, p65, IκBα, and p-IκBα proteins was detected by Western blotting using specific antibodies. Histone h1 protein and β-actin protein were used here as internal controls. Data shown are the mean ± SD (n = 10). *p < 0.05 is the significance level compared to the control group. #p < 0.05 is the significance level compared to the PA treatment group.

Effect of CA phytosome on NF-κB DNA binding activity and cytokine levels in PA-induced AD mice

NF-κB is implicated in inflammatory responses in AD. To investigate whether CA phytosome can inhibit NF-κB activation in PA-induced AD model, nuclear extract from back skin tissue was prepared and assayed for NF-κB DNA binding by EMSA (Fig. 4(D)). The NF-κB binding activity was significantly higher in PA-treated mice than in control and CA phytosome-treated mice (Fig. 4(D)). In addition, as shown in Figs 3(D) and 4(E), PA-treated mice showed significant IκBα degradation in cytosolic fraction when compared to the control group mice in both ear and back skin. Conversely, IκBα degradation was reduced significantly more in CA phytosome-treated mice than in PA-treated mice. PA treated mice also showed increased nuclear expression of p65 and p50. In contrast, CA phytosome inhibited translocation of p65 and p50 into the nucleus in a dose-dependent manner (Figs. 3(D) and 4(E)). We also measured the cytokine level in the back skin, and found that similar to sera levels, the cytokines TNF-α and IL-1β, IL-8, IL-4 and IL-13 were generally higher in the PA treatment group than in the control or



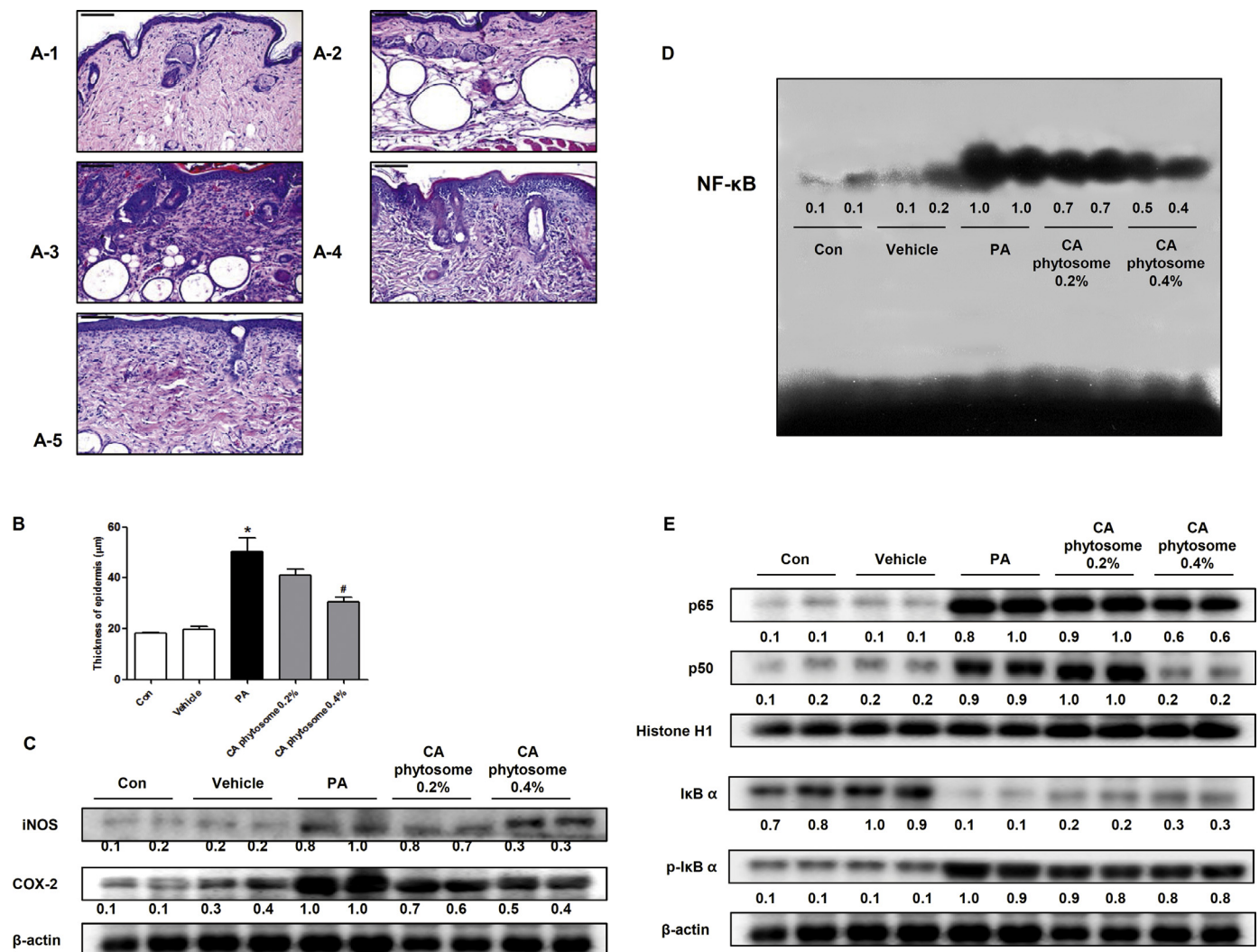


Fig. 4. Histopathological analysis of back tissue and the inhibitions of NF- κ B DNA binding activity by topical application of CA phytosome in back skin. Histopathology of back skin in control (A-1), vehicle (A-2), PA (A-3), PA + CA phytosome 0.2% (A-4), and PA + CA phytosome 0.4% (A-5). PA solution was repeatedly applied to the back skin during topical application of CA phytosome. Histopathological changes in the slide sections of back tissue were identified by staining with hematoxylin and eosin followed by observation at $200\times$ magnification (Scale bars, $100\mu\text{m}$). Histological images (A) and thickness of the epidermis (B). Alterations in the expression of iNOS and COX-2 proteins were measured by Western blotting (C). The effect of CA phytosome on NF- κ B DNA binding activity in back skin (D). The activation of NF- κ B was investigated using EMSA as described in Materials and Methods. Nuclear extracts from homogenized back skin tissue were incubated in the binding reactions of ^{32}P -end-labeled oligonucleotide containing the NF- κ B sequence. The effect of CA phytosome on translocation of the subunits of NF- κ B (p50 and p65) into the nucleus and phosphorylation of I κ B α in cytosol in back skin (E). Equal amounts of nuclear proteins ($20\mu\text{g}/\text{lane}$) or total proteins ($20\mu\text{g}/\text{lane}$) were subjected to 10% SDS-PAGE, and the expression of p50, p65, I κ B α , and p-I κ B α proteins was detected by Western blotting using specific antibodies. Histone h1 protein and β -actin protein were used here as internal controls. Data shown are the mean $\pm$ SD ($n = 10$). * $p < 0.05$ is the significance level compared to the control group. # $p < 0.05$ is the significance level compared to the PA treatment group.

vehicle treatment group, which were dramatically decreased by phytosome treatment (Supplementary Fig. 2(B)).

The effect of CA phytosome on LPS-induced NO production, and iNOS and COX-2 expression in RAW 264.7 cells

The effect of CA phytosome on LPS-induced NO production in RAW 264.7 cells was investigated by measuring the released nitrite in the culture medium using the Griess test. After co-treatment with LPS and CA phytosome (5, 10, and $20\mu\text{g}/\text{ml}$) for 24 h, LPS-induced nitrite concentration in the medium decreased remarkably in a concentration dependent manner (Fig. 5(A)). In addition, to investigate whether CA phytosome inhibits the NO production by inhibiting the corresponding gene expression, we determined iNOS expression by Western blotting. We also determined COX-2 expression since iNOS can be modulated by COX-2. As shown in Fig. 5(B), under unstimulated conditions, the cells

expressed extremely low levels of iNOS and COX-2 protein. However, these levels markedly increased in response to LPS ($1\mu\text{g}/\text{ml}$) after 24 h. Treatment with CA phytosome (5, 10, and $20\mu\text{g}/\text{ml}$) caused concentration-dependent decreases in LPS-induced iNOS and COX-2 expression in RAW 264.7 cells (Fig. 5(B)).

Effect of CA phytosome on NF- κ B DNA binding activity in RAW 264.7 cells

Because activation of NF- κ B is critical for induction of both iNOS and COX-2 by LPS or other inflammatory cytokines, we determined whether CA phytosome can suppress NF- κ B activation in LPS-activated RAW 264.7 cells. These cells were co-treated with LPS and CA phytosome for 1 h each, which is the estimated time to activate NF- κ B maximally by LPS treatment (data are not shown). Nuclear extracts from co-treated cells were prepared and NF- κ B DNA binding was assayed by EMSA. In RAW 264.7 cells, LPS induced a strong NF- κ B

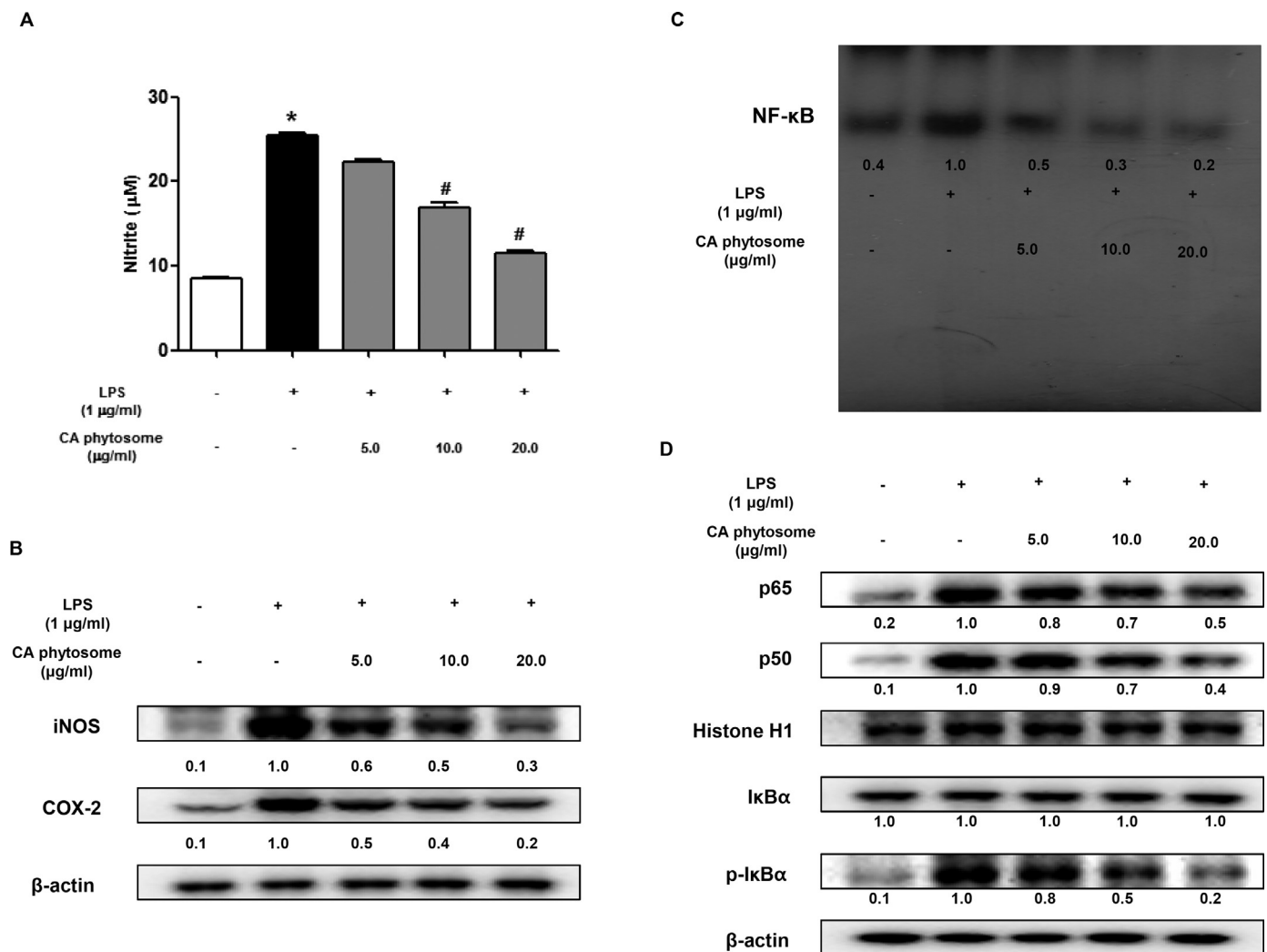


Fig. 5. Effects of CA phytosome on NO production and protein expression of iNOS and COX-2 in LPS-treated RAW 264.7 macrophages. The cells were treated with 1 µg/ml of LPS alone (LPS) or LPS with different concentrations (5, 10, and 20 µg/ml) of CA phytosome for 24 h. At the end of incubation, 50 µl of the medium was removed to measure NO production (A). Control values were obtained in the absence of LPS. In the culture medium, NO production was measured by the Griess reaction as described in Materials and Methods. The cells were treated with 1 µg/ml of LPS alone or LPS with the indicated concentration of CA phytosome for 24 h. Equal amounts of total proteins (20 µg/lane) were subjected to 10% SDS-PAGE, and alterations in the expression of iNOS and COX-2 proteins were detected by Western blotting using specific antibodies (B). β-actin protein was used here as an internal control. The effect of CA phytosome on LPS-induced NF-κB DNA binding activity in RAW 264.7 cells (C). The cells were treated with 1 µg/ml of LPS alone or LPS with the indicated concentration of CA phytosome for 1 h. The activation of NF-κB was investigated using EMSA as described in Materials and Methods. Nuclear extracts from RAW 264.7 cells treated with LPS alone (1 µg/ml) or with indicated concentration of CA phytosome (5, 10, and 20 µg/ml) were incubated in the binding reactions of ³²P-end-labeled oligonucleotide containing the NF-κB sequence. The NF-κB DNA binding activity was determined by EMSA. The effect of CA phytosome on the LPS-induced translocation of the subunits of NF-κB (p50 and p65) into the nucleus and the phosphorylation of IκBα in cytosol (D). Equal amounts of nuclear proteins (20 µg/lane) or total proteins (20 µg/lane) were subjected to 10% SDS-PAGE, and the expression of p50, p65, IκBα, and p-IκBα proteins was detected by Western blotting using specific antibodies. Histone h1 protein and β-actin protein were used here as internal controls. Data shown are the mean ± SD (n = 10). *p < 0.05 is the significance level compared to the control group. #p < 0.05 is the significance level compared to the PA treatment group.

binding activity, which was markedly inhibited in a concentration-dependent manner by co-treatment with CA phytosome (Fig. 5(C)). We further investigated the inhibitory effect of CA phytosome on the NF-κB subunit translocation and IκB phosphorylation. Consistent with the inhibitory effect on NF-κB activity, CA phytosome also inhibited the nuclear translocation of p65 and p50 and LPS-induced phosphorylation of IκBα, both in a concentration-dependent manner (Fig. 5(D)).

Discussion

AD is a chronic inflammatory skin disease characterized by skin inflammation with eczema-like lesions, itching, and dry skin (Park et al., 2013). Common treatments for AD are topical application of corticosteroids and administration of immunosuppressive drugs

because of their great anti-inflammatory and anti-allergic activities; however, they cause irreversible side-effects (Cohen and Heidary, 2004). Immunosuppressive drugs are available for oral administration but not for topical application owing to their molecular weight and the consequent transdermal impermeability (Pacor et al., 2004). Conversely, natural products, including various plants, herbs, flowers, yeasts, and fungi, with smaller molecular weights can pass through the skin barrier; thus, they have been effective against AD when applied at the affected skin sites without any scratch wounds (Gianni and Sulli, 2001). Therefore, to avoid any unexpected side effects, topical application is preferred over systemic administration for the treatment of AD, and the use of natural products as anti-allergic agents, such as herbal medicines, is being emphasized (Tan et al., 2013; Yamashita et al., 2013). It has been proven that *C. asiatica* has an

excellent effect on deposition of collagen (Choi et al., 2016; Hashim et al., 2011) and inhibition of the inflammatory phase of wound healing (Nhiem et al., 2011).

Herein, topical application of CA phytosome ameliorated typical and histological changes of AD, such as dermatitis severity, ear thickness, epidermal hyperplasia, and infiltration of inflammatory cells. We found that CA phytosome effectively reduces both skin inflammation and PA treatment-induced allergic responses via reduced activation of NF- κ B signaling. In an in vitro assay, we also found that through reduced NF- κ B DNA binding activity, CA phytosome inhibited LPS-induced inflammatory responses in RAW 264.7 macrophages.

Phytosomes are innovative formulations that improve the bioavailability of hydrophilic flavonoids. Phytomolecules have been reported to show a better absorption profile and more suitable lipid solubility, making them competent to cross biological membranes (Damle and Mallya, 2016). Silymarin, an antioxidant, shows increased specific activity in the phyto-liposome complex and is more stable and durable than as a free constituent (El-Gazayerly et al., 2014). Moreover, bioavailability and activity of curcumin (Ali et al., 2016; Appendino et al., 2011; Di Pierro and Settembre, 2013), green tea (Di Pierro et al., 2009; Hulbert and Else, 2005), and grape seed extracts (Carini et al., 2001; Maffei Facino et al., 1998) in phytosome complexes have been reported. In our present study, we found that half a dose of CA in phytosome formulation had similar effects with CA. Thus, topical application of CA phytosome could be more effective than its original formulation for treatment of AD.

Topical application of CA phytosome significantly reduced the typical histopathological phenomena of AD and the number of immune cells infiltrating the skin lesions in the PA-treated AD mice. Furthermore, increase in IgE levels is a marker of AD development and is usually noticed in response to specific allergens (Dokmeci and Herrick, 2008). Several studies have shown that AD is a typical Th2-type immune disorder and is characterized by increased serum IgE levels and Th2-type cytokine expression (Chen et al., 2004; Neis et al., 2006). In the present study, we demonstrated that Th2 type cytokine such as sera IL-4 and IL-13 was also reduced by topical application of CA phytosome.

NF- κ B is a prime component of the intracellular signaling pathways responsible for the up-regulation of pro-inflammatory cytokines which are instrumental in AD advancement (Barnes and Karin, 1997). Activation of NF- κ B has also been observed after IgE-induced production of TNF- α and IL-6 in mast cells (Jeong et al., 2002). Although a direct relation between NF- κ B and AD pathogenesis has not been reported, some reports have speculated the role of NF- κ B in the immunological disturbance observed in AD. NF- κ B is activated immediately after aggregation of Fc ϵ RI on mast cells and dendritic cells, leading to the production of inflammatory cytokines and chemokines (Marquardt and Walker, 2000). NF- κ B is also activated in B and T cells when they are stimulated via CD40 and T cell receptors, respectively, indicating the critical roles of NF- κ B in both Ig production and cell activation (Matsumoto et al., 2002). Moreover, many studies have shown that compounds that suppress NF- κ B activation could act as therapeutic agents for AD (Park et al., 2015; Wullaert et al., 2011). In the present study, CA phytosome decreased the degradation of I κ B α and nuclear translocation of NF- κ B in lymph node as well as tissues accompanied with reduced cytokine levels.

Our data demonstrated that CA phytosome attenuates the activation of NF- κ B, contributing to the reduced levels of TNF- α and IL-1 β and reduced expression of iNOS and COX-2. Therefore, our data suggest that CA phytosome should be considered a candidate agent for AD therapy.

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Conflict of interest

The authors declare that there is no duality of interest associated with this manuscript.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.phymed.2018.04.013.

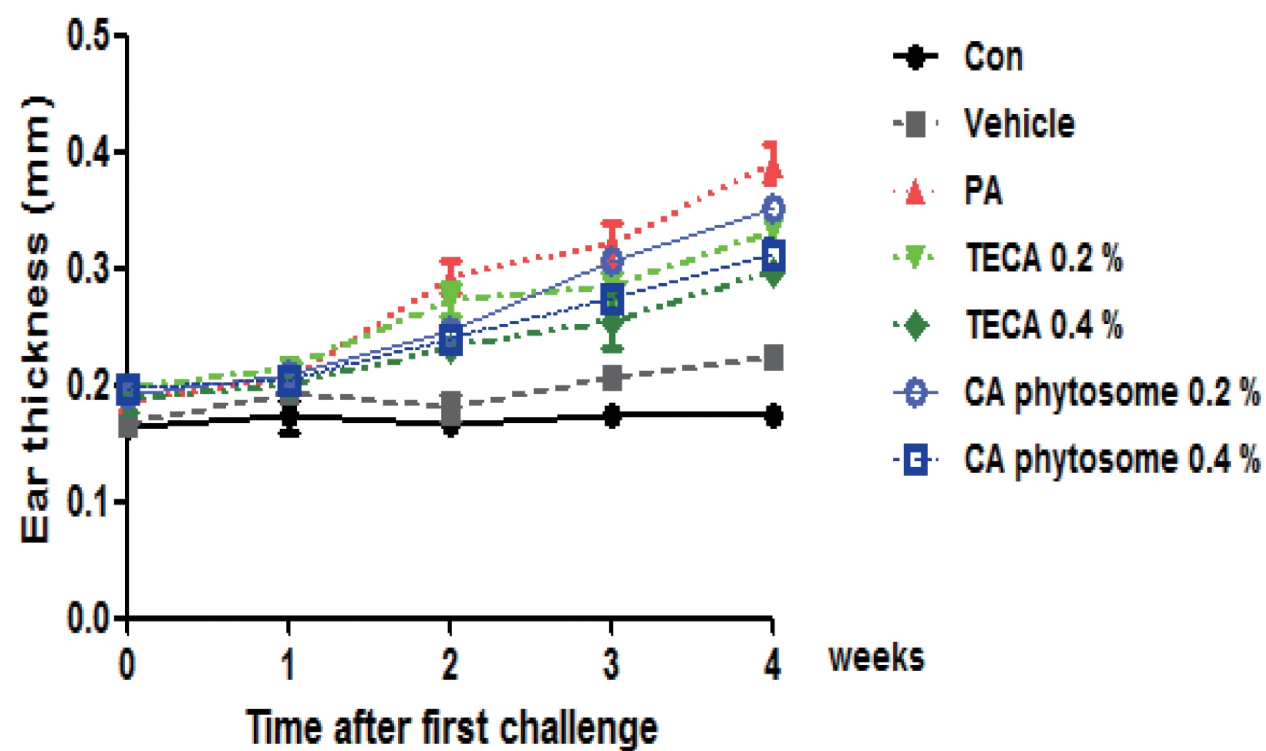
References

- Ali, S.O., Darwish, H.A., Ismail, N.A., 2016. Curcumin, Silybin Phytosome((R)) and alpha-R-Lipoic Acid Mitigate Chronic Hepatitis in Rat by Inhibiting Oxidative Stress and Inflammatory Cytokines Production. *Basic Clin. Pharmacol. Toxicol.* 118, 369–380.
- Angelico, R., Ceglie, A., Sacco, P., Colafemmina, G., Ripoli, M., Mangia, A., 2014. Phytoliposomes as nanoshuttles for water-insoluble silybin-phospholipid complex. *Int. J. Pharm.* 471, 173–181.
- Appendino, G., Belcaro, G., Cornelli, U., Luzzi, R., Togni, S., Dugall, M., Cesarone, M.R., Feragalli, B., Ippolito, E., Errichi, B.M., Pellegrini, L., Ledda, A., Ricci, A., Bavera, P., Hosoi, M., Stuard, S., Corsi, M., Errichi, S., Gizzi, G., 2011. Potential role of curcumin phytosome (Meriva) in controlling the evolution of diabetic microangiopathy. A pilot study. *Panminerva Med* 53, 43–49.
- Barnes, P.J., Karin, M., 1997. Nuclear factor-kappaB: a pivotal transcription factor in chronic inflammatory diseases. *N. Engl. J. Med.* 336, 1066–1071.
- Brandt, E.B., Sivaprasad, U., 2011. Th2 Cytokines and Atopic Dermatitis. *J. Clin. Cell. Immunol.* 2 pii: 110.
- Carini, M., Stefani, R., Aldini, G., Ozioli, M., Facino, R.M., 2001. Procyanidins from Vitis vinifera seeds inhibit the respiratory burst of activated human neutrophils and lysosomal enzyme release. *Planta Med* 67, 714–717.
- Chen, L., Martinez, O., Overbergh, L., Mathieu, C., Prabhakar, B.S., Chan, L.S., 2004. Early up-regulation of Th2 cytokines and late surge of Th1 cytokines in an atopic dermatitis model. *Clin. Exp. Immunol.* 138, 375–387.
- Choi, E.J., Debnath, T., Tang, Y., Ryu, Y.B., Moon, S.H., Kim, E.K., 2016. Topical application of Moringa oleifera leaf extract ameliorates experimentally induced atopic dermatitis by the regulation of Th1/Th2/Th17 balance. *Biomed. Pharmacother.* 84, 870–877.
- Cohen, D.E., Heidary, N., 2004. Treatment of irritant and allergic contact dermatitis. *Dermatol. Ther.* 17, 334–340.
- Comoglio, A., Tomasi, A., Malandrino, S., Poli, G., Albano, E., 1995. Scavenging effect of silipide, a new silybin-phospholipid complex, on ethanol-derived free radicals. *Biochem. Pharmacol.* 50, 1313–1316.
- Damle, M., Mallya, R., 2016. Development and Evaluation of a Novel Delivery System Containing Phytospholipid Complex for Skin Aging. *AAPS PharmSciTech* 17, 607–617.
- Di Pierro, F., Menghi, A.B., Barreca, A., Lucarelli, M., Calandrelli, A., 2009. Greenselect Phytosome as an adjunct to a low-calorie diet for treatment of obesity: a clinical trial. *Altern. Med. Rev.* 14, 154–160.
- Di Pierro, F., Settembre, R., 2013. Safety and efficacy of an add-on therapy with curcumin phytosome and piperine and/or lipoic acid in subjects with a diagnosis of peripheral neuropathy treated with dexibuprofen. *J. Pain Res.* 6, 497–503.
- Dokmeci, E., Herrick, C.A., 2008. The immune system and atopic dermatitis. *Semin. Cutan. Med. Surg.* 27, 138–143.
- El-Gazayerly, O.N., Makhlof, A.I., Soelm, A.M., Mohmoud, M.A., 2014. Antioxidant and hepatoprotective effects of silymarin phytosomes compared to milk thistle extract in CCl4 induced hepatotoxicity in rats. *J. Microencapsul.* 31, 23–30.
- Gianni, L.M., Sulli, M.M., 2001. Topical tacrolimus in the treatment of atopic dermatitis. *Ann. Pharmacother.* 35, 943–946.
- Gupta, N.K., Dixit, V.K., 2011. Bioavailability enhancement of curcumin by complexation with phosphatidyl choline. *J. Pharm. Sci.* 100, 1987–1995.
- Harada, D., Takada, C., Tsukumo, Y., Takaba, K., Manabe, H., 2005. Analyses of a mouse model of the dermatitis caused by 2,4,6-trinitro-1-chlorobenzene (TNCl)-repeated application. *J. Dermatol. Sci.* 37, 159–167.
- Hashim, P., Sidek, H., Helan, M.H., Sabery, A., Palanisamy, U.D., Ilham, M., 2011. Triterpene composition and bioactivities of *Centella asiatica*. *Molecules* 16, 1310–1322.
- Hashimoto, Y., Takaoka, A., Sugimoto, M., Honma, Y., Sakurai, T., Futaki, N., Arai, I., 2011. Itch-associated scratching contributes to the development of dermatitis and hyperimmunoglobulinemia E in NC/Nga mice. *Exp. Dermatol.* 20, 820–825.
- Homey, B., Steinhoff, M., Ruzicka, T., Leung, D., 2006. Cytokines and chemokines orchestrate atopic skin inflammation. *J. Allergy Clin. Immunol.* 118, 178–189.
- Hulbert, A.J., Else, P.L., 2005. Membranes and the setting of energy demand. *J. Exp. Biol.* 208, 1593–1599.
- Jeong, H.J., Koo, H.N., Na, H.J., Kim, M.S., Hong, S.H., Eom, J.W., Kim, K.S., Shin, T.Y., Kim, H.M., 2002. Inhibition of TNF-alpha and IL-6 production by Aucubin through blockade of NF-kappaB activation RBL-2H3 mast cells. *Cytokine* 18, 252–259.
- Klewicka, E., Cukrowska, B., Libudzisz, Z., Slizewska, K., Motyl, I., 2011. Changes in gut microbiota in children with atopic dermatitis administered the bacteria *Lactobacillus casei* DN-114001. *Pol. J. Microbiol.* 60, 329–333.
- Kumar, N., Rai, A., Reddy, N.D., Raj, P.V., Jain, P., Deshpande, P., Mathew, G., Kutty, N.G., Udupa, N., Rao, C.M., 2014. Silymarin liposomes improves oral bioavailability of silybin besides targeting hepatocytes, and immune cells. *Pharmacol. Rep.* 66,

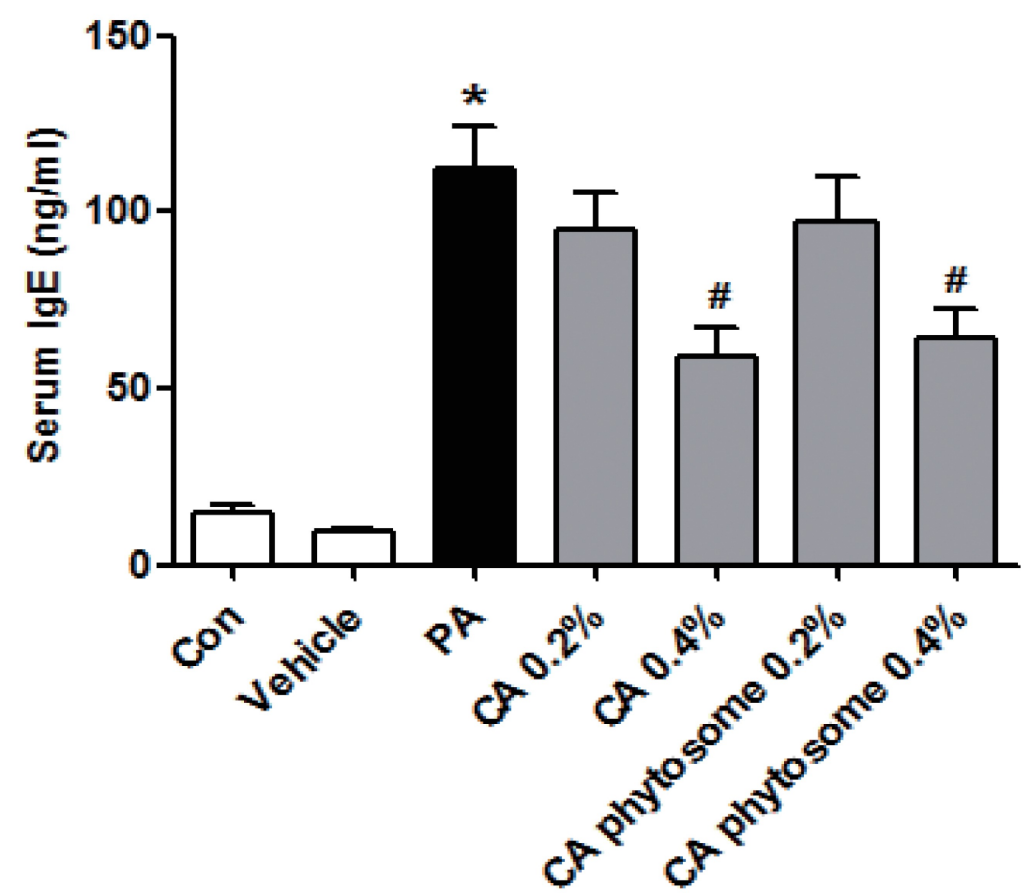
- 788–798.
- Kuramoto, T., Yokoe, M., Tanaka, D., Yuri, A., Nishitani, A., Higuchi, Y., Yoshimi, K., Tanaka, M., Kuwamura, M., Hiai, H., 2015. Atopic dermatitis-like skin lesions with IgE hyperproduction and pruritus in KFRS4/Kyo rats. *J. Dermatol. Sci.* 80, 116–123.
- Kwak, M., Kim, J., Hwang, I., Lee, Y., An, B., Hong, J., Lee, S., Hwang, D., 2013. Quantitative evaluation of therapeutic effect of *Liriope platyphylla* on phthalic anhydride-induced atopic dermatitis in IL-4/Luc/CNS-1 Tg mice. *J. Ethnopharmacol.* 148, 880–889.
- Leung, D.Y., 2000. Atopic dermatitis: new insights and opportunities for therapeutic intervention. *J. Allergy Clin. Immunol.* 105, 860–876.
- Leung, D.Y., Bieber, T., 2003. Atopic dermatitis. *Lancet* 361, 151–160.
- Li, Y., Yang, D.J., Chen, S.L., Chen, S.B., Chan, A.S., 2008. Process parameters and morphology in puerarin, phospholipids and their complex microparticles generation by supercritical antisolvent precipitation. *Int. J. Pharm.* 359, 35–45.
- Maffei Facino, R., Carini, M., Aldini, G., Calloni, M.T., Bombardelli, E., Morazzoni, P., 1998. Sparing effect of procyanidins from *Vitis vinifera* on vitamin E: in vitro studies. *Planta Med* 64, 343–347.
- Marquardt, D.L., Walker, L.L., 2000. Dependence of mast cell IgE-mediated cytokine production on nuclear factor-kappaB activity. *J. Allergy Clin. Immunol.* 105, 500–505.
- Matsumoto, M., Yamada, T., Yoshinaga, S.K., Boone, T., Horan, T., Fujita, S., Li, Y., Mitani, T., 2002. Essential role of NF-kappa B-inducing kinase in T cell activation through the TCR/CD3 pathway. *J. Immunol.* 169, 1151–1158.
- Nedoszytko, B., Sokolowska-Wojdylo, M., Ruckemann-Dziurdzinska, K., Roszkiewicz, J., Nowicki, R.J., 2014. Chemokines and cytokines network in the pathogenesis of the inflammatory skin diseases: atopic dermatitis, psoriasis and skin mastocytosis. *Postepy Dermatol. Alergol.* 31, 84–91.
- Neis, M.M., Peters, B., Dreuw, A., Wenzel, J., Bieber, T., Mauch, C., Krieg, T., Stanzel, S., Heinrich, P.C., Merk, H.F., Bosio, A., Baron, J.M., Hermanns, H.M., 2006. Enhanced expression levels of IL-31 correlate with IL-4 and IL-13 in atopic and allergic contact dermatitis. *J. Allergy Clin. Immunol.* 118, 930–937.
- Nhiem, N.X., Tai, B.H., Quang, T.H., Van Kiem, P., Van Minh, C., Nam, N.H., Kim, J., Im, L., Lee, Y., Kim, Y.H., 2011. A new ursane-type triterpenoid glycoside from *Centella asiatica* leaves modulates the production of nitric oxide and secretion of TNF- α in activated RAW 264.7 cells. *Bioorg. Med. Chem. Lett.* 21, 1777–1781.
- Pacor, M., Di Lorenzo, G., Martinelli, N., Mansueto, P., Rini, G., Corrocher, R., 2004. Comparing tacrolimus ointment and oral cyclosporine in adult patients affected by atopic dermatitis: a randomized study. *Clinical & Experimental Allergy* 34, 639–645.
- Park, J., Kim, M., Jeong, G., Yoon, J., 2015. Xanthii fructus extract inhibits TNF- α /IFN- γ -induced Th2-chemokines production via blockade of NF- κ B, STAT1 and p38-MAPK activation in human epidermal keratinocytes. *J. Ethnopharmacol.* 171, 85–93.
- Park, S., Lee, Y., Lee, K.H., Kim, T., 2013. Effect of eriodictyol on the development of atopic dermatitis-like lesions in ICR mice. *Biological and Pharmaceutical Bulletin* 36, 1375–1379.
- Pastore, S., Lulli, D., Potapovich, A.I., Fidanza, P., Kostyuk, V.A., Dellambra, E., De Luca, C., Maurelli, R., Korkina, L.G., 2011. Differential modulation of stress-inflammation responses by plant polyphenols in cultured normal human keratinocytes and immortalized HaCaT cells. *J. Dermatol. Sci.* 63, 104–114.
- Peng, Q., Gong, T., Zuo, J., Liu, J., Zhao, D., Zhang, Z., 2008. Enhanced the oral bioavailability of salvianolic acid B by phospholipid complex loaded nanoparticles. *Die Pharmazie-An International Journal of Pharmaceutical Sciences* 63, 661–666.
- Schakel, K., Dobel, T., Bosselmann, I., 2014. Future treatment options for atopic dermatitis - small molecules and beyond. *J. Dermatol. Sci.* 73, 91–100.
- Semalty, A., Semalty, M., Rawat, M.S.M., Franceschi, F., 2010. Supramolecular phospholipids-polyphenolics interactions: The PHYTOSOME® strategy to improve the bioavailability of phytochemicals. *Fitoterapia* 81, 306–314.
- Seo, W.Y., Youn, G.S., Choi, S.Y., Park, J., 2015. Butein, a tetrahydrochalcone, suppresses pro-inflammatory responses in HaCaT keratinocytes. *BMB Rep* 48, 495–500.
- Sikarwar, M.S., Sharma, S., Jain, A.K., Parial, S., 2008. Preparation, characterization and evaluation of marsupin-phospholipid complex. *AAPS PharmSciTech* 9, 129–137.
- Singh, D., SM Rawat, M., Semalty, A., Semalty, M., 2012. Rutin-phospholipid complex: an innovative technique in novel drug delivery system-NDDS. *Current drug delivery* 9, 305–314.
- Sung, J., Lee, H., Kim, J., Go, J., Seo, E., Yun, W., Kim, D., Son, H., Lee, C., Lee, H., 2016. Therapeutic effect of ethyl acetate extract from *Asparagus cochinchinensis* on phthalic anhydride-induced skin inflammation. *Laboratory animal research* 32, 34–45.
- Suzuki, H., Makino, Y., Nagata, M., Furuta, J., Enomoto, H., Hirota, T., Tamari, M., Noguchi, E., 2016. A rare variant in CYP27A1 and its association with atopic dermatitis with high serum total IgE. *Allergy* 71, 1486–1489.
- Tan, H.Y., Zhang, A.L., Chen, D., Xue, C.C., Lenon, G.B., 2013. Chinese herbal medicine for atopic dermatitis: a systematic review. *J. Am. Acad. Dermatol.* 69, 295–304.
- Tanaka, A., Konno, M., Muto, S., Kambe, N., Morii, E., Nakahata, T., Itai, A., Matsuda, H., 2005. A novel NF-kappaB inhibitor, IMD-0354, suppresses neoplastic proliferation of human mast cells with constitutively activated c-kit receptors. *Blood* 105, 2324–2331.
- Tokura, Y., 2010. Extrinsic and intrinsic types of atopic dermatitis. *J. Dermatol. Sci.* 58, 1–7.
- Wullaert, A., Bonnet, M.C., Pasparakis, M., 2011. NF- κ B in the regulation of epithelial homeostasis and inflammation. *Cell Res* 21, 146–158.
- Yamashita, H., Tanaka, H., Inagaki, N., 2013. Treatment of the chronic itch of atopic dermatitis using standard drugs and kampo medicines. *Biological and Pharmaceutical Bulletin* 36, 1253–1257.
- Zhang, Y., Wang, A., Xu, L., Shen, N., Zhu, J., Tu, C., 2016. Characteristics of peripheral blood CD4 CD25 regulatory T cells and related cytokines in severe atopic dermatitis. *European Journal of Dermatology* 26, 240–246.

Supplementary Fig. 1

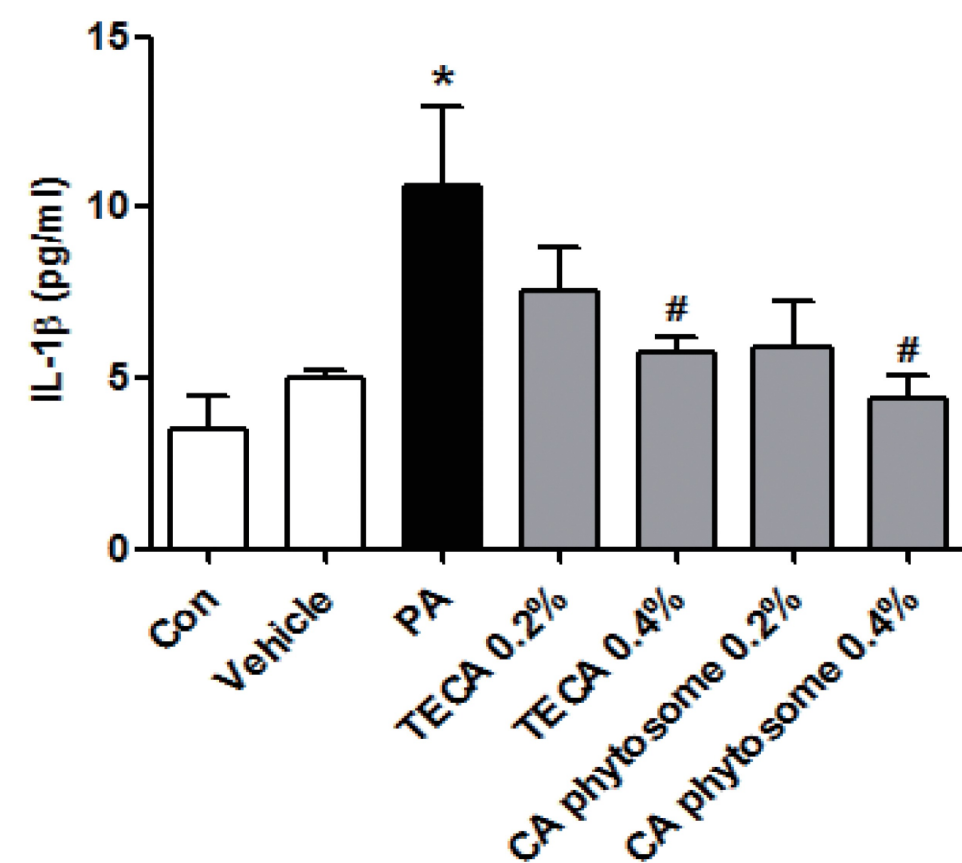
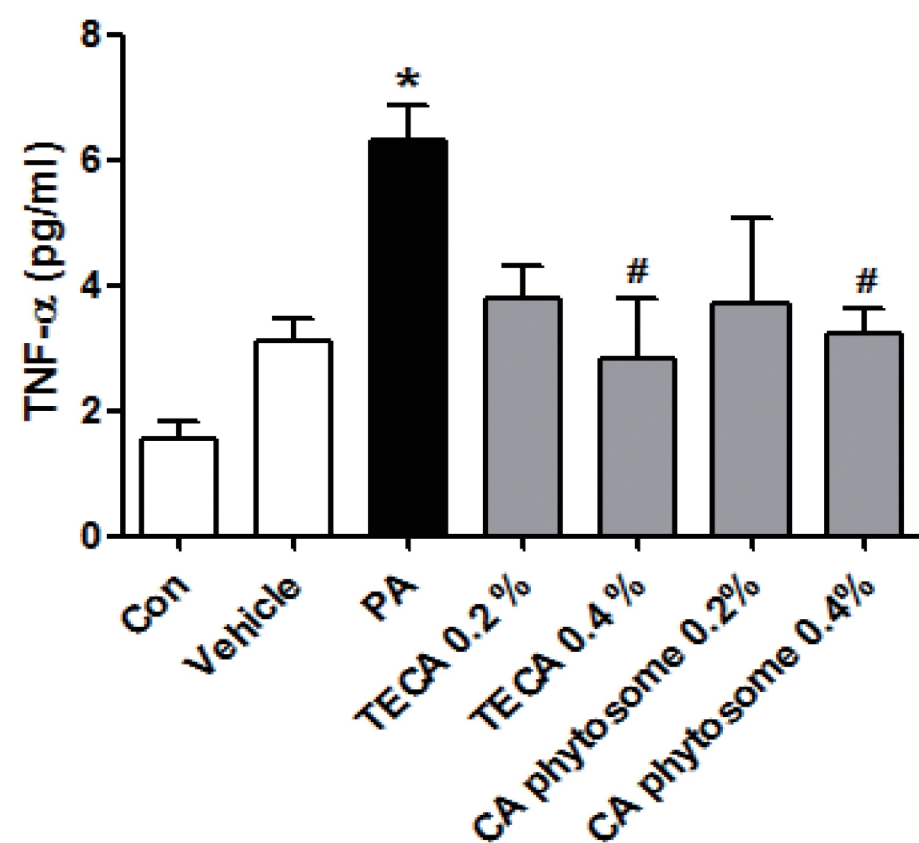
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B

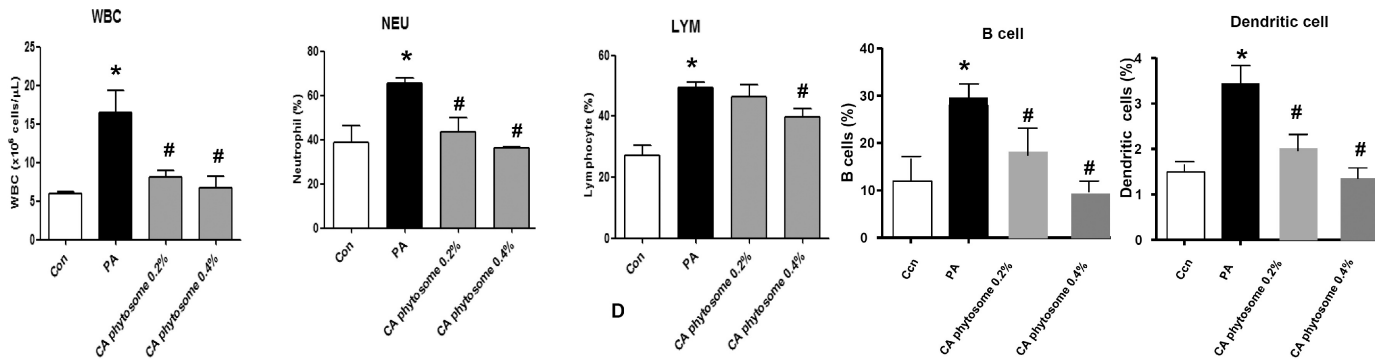


C



Supplementary Fig. 2

A



B

